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Boswellic acids stimulate arachidonic acid release and 12-lipoxygenase activity in human platelets independent of Ca^{2+} and differentially interact with platelet-type 12-lipoxygenase¹

Daniel Poeckel, Lars Tausch, Nicole Kather, Johann Jauch, and Oliver Werz

Department of Pharmaceutical Analytics, Institute of Pharmacy, Eberhard-Karls-University Tübingen, Auf der Morgenstelle 8, D-72076 Tübingen, Germany (DP, OW).

Institute of Pharmaceutical Chemistry, University of Frankfurt, Max-von-Laue Str.9, D-60439 Frankfurt, Germany (LT).

Institute of Organic Chemistry, University of Saarland, Im Stadtwald C4.2, D-66123 Saarbrücken, Germany (NK, JJ).

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Correspondence to: Prof. Dr. Oliver Werz, Department of Pharmaceutical Analytics, Institute of Pharmacy, Eberhard-Karls-University Tübingen, Auf der Morgenstelle 8, D-72076 Tübingen, Germany. Phone: +49-7071-2978793; Fax: +49-7071-294565. e-mail: oliver.werz@uni-tuebingen.de

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Abbreviations used: A β -BA, 3-*O*-acetyl-boswellic acid; AKBA, 3-*O*-acetyl-11-keto-boswellic acid; CDC, cinnamyl-3,4-dihydroxy- α -cyanocinnamate; cPLA₂, cytosolic phospholipase A₂; 12-H(P)ETE, 12-hydro(pero)xyicosatetraenoic acid; KBA, 11-keto-boswellic acid; MAFP, methyl-arachidonyl-fluorophosphonate; MAPK, mitogen-activated protein kinase; p12-LO, platelet-type 12-lipoxygenase; PG buffer, PBS plus 1 mg/ml glucose; PGC buffer, PBS containing 1 mg/ml glucose and 1 mM CaCl₂; PI 3-K, phosphatidylinositol 3-kinase; PIP₂, phosphatidylinositol-4,5-bisphosphat; PMNL, polymorphonuclear leukocytes; SDS-b, 2 \times sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer.

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Abstract

Boswellic acids inhibit the transformation of arachidonic acid to leukotrienes via 5-lipoxygenase but can also enhance the liberation of arachidonic acid in human leukocytes and platelets. Utilizing human platelets, we explored the molecular mechanisms underlying the boswellic acid-induced release of arachidonic acid and the subsequent metabolism by platelet-type 12-lipoxygenase (p12-LO). Both, β -boswellic acid as well as 3-*O*-acetyl-11-keto-boswellic acid (AKBA) markedly enhanced the release of arachidonic acid via cytosolic phospholipase (PLA)₂, whereas for generation of 12-hydro(pero)xyeicosatetraenoic acid (12-H(P)ETE), AKBA was less potent than β -boswellic acid and was without effect at higher concentrations ($\geq 30 \mu\text{M}$). In contrast to thrombin, β -boswellic acid-induced release of arachidonic acid and formation of 12-H(P)ETE was more rapid and occurred also in the absence of Ca²⁺. The Ca²⁺-independent release of arachidonic acid and 12-H(P)ETE production elicited by β -boswellic acid was not affected by pharmacological inhibitors of signaling molecules relevant for agonist-induced arachidonic acid liberation and metabolism. Notably, in cell-free assays, β -boswellic acid increased p12-LO catalysis about two-fold in the absence, but not in the presence of Ca²⁺, whereas AKBA inhibited p12-LO activity. No direct modulatory effects of boswellic acids on cPLA₂ activity in cell-free assays were evident. Accordingly, immobilized KBA (linked to sepharose beads) selectively precipitated p12-LO from platelet lysates but failed to bind cPLA₂. Taken together, we show that boswellic acids induce the release of arachidonic acid and the synthesis of 12-H(P)ETE in human platelets by unique, Ca²⁺-independent routes, and we identified p12-LO as a selective molecular target of boswellic acids.

Introduction

The pentacyclic triterpenes boswellic acids (**fig. 1**) are regarded as the active pharmacological principles of ethanolic extracts of *Boswellia serrata*, and there is accumulating evidence for an anti-inflammatory and anti-tumorigenic potential of boswellic acids based on experimental cellular and animal models (Anthoni et al., 2006; Poeckel et al., 2006; Safayhi et al., 1992; Syrovets et al., 2005a; Syrovets et al., 2005b; Winking et al., 2000). Attempts to identify the responsible molecular mechanisms and/or receptors revealed a number of proteins that may be targeted by boswellic acids including 5-lipoxygenase, human leukocyte elastase, topoisomerases and I κ B kinases (Safayhi et al., 1997; Safayhi et al., 1995; Syrovets et al., 2000; Syrovets et al., 2005b). Interaction with these targets may indeed provide a molecular basis for the pharmacological effects observed in animals and human subjects. In particular suppression of leukotriene biosynthesis from arachidonic acid by inhibition of 5-lipoxygenase is generally regarded as the most important pharmacological action of boswellic acids accounting for their anti-inflammatory properties (Safayhi et al., 1997; Safayhi et al., 1995).

Many cell types are able to release arachidonic acid from phospholipids within cellular membranes by the action of specific phospholipases (PL) A_2 (Six and Dennis, 2000). Arachidonic acid is an important precursor for a number of highly bioactive metabolites formed by various oxygenases including cyclooxygenases, lipoxygenases, and monooxygenases of the CYP family. The 85-kDa cytosolic PLA $_2$ (cPLA $_2$) has been accounted as responsible enzyme providing free arachidonic acid as substrate for cyclooxygenases and lipoxygenases in leukocytes and platelets (Leslie, 2004). This soluble enzyme is distributed within the cytosol of resting cells and associates with membranes upon elevation of intracellular Ca $^{2+}$ and/or serine phosphorylations by members of the mitogen-activated protein kinase (MAPK) family (Gijon and Leslie, 1999), occurring in response to a number of agonists. In addition, binding to phosphatidylinositol-4,5-bisphosphate (PIP $_2$) (Balsinde et al.,

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2000) or ceramide(1-phosphate) (Huwiler et al., 2001; Pettus et al., 2004; Subramanian et al., 2005) via specific binding-site(s) may promote cPLA₂ catalysis.

Exposure of leukocytes or platelets to boswellic acids differentially affects signaling pathways and functional responses including Ca²⁺ mobilization, MAPK activation, formation of reactive oxygen species, release of arachidonic acid and stimulation of 5-lipoxygenase product formation. Thus, stimulating properties (Altmann et al., 2002; Altmann et al., 2004; Poeckel et al., 2005; Safayhi et al., 2000) as well as inhibitory effects (Poeckel et al., 2006; Safayhi et al., 1992; Safayhi et al., 1995; Werz et al., 1998) of boswellic acids have been reported for these functions, depending on the cell type and the respective experimental settings. For example, for inhibition of 5-lipoxygenase by AKBA, IC₅₀ values in the range of 1.5 μM (Safayhi et al., 1995) up to 50 μM (Werz et al., 1997; Werz et al., 1998) were determined, but also 5-lipoxygenase stimulatory effects in this concentration range were described (Altmann et al., 2004; Safayhi et al., 2000).

We recently observed that boswellic acids are capable of elevating the release of arachidonic acid in human isolated polymorphonuclear leukocytes (PMNL) (Altmann et al., 2004) and platelets (Poeckel et al., 2005). Platelets do not express 5-lipoxygenase, but contain the closely related p12-LO that converts arachidonic acid to 12-hydro(pero)xyeicosatetraenoic acid (12-H(P)ETE) (Yoshimoto and Takahashi, 2002). Here we characterized the liberation of arachidonic acid by boswellic acids and the subsequent conversion by p12-LO, and we investigated the underlying molecular mechanisms.

Materials and Methods

Materials

Boswellic acids were synthesized and prepared as described (Jauch and Bergmann, 2003). Antibodies against human p12-LO were kindly provided by Dr Colin D. Funk, Kingston, Canada. SB203580, PP2, PP3, SU6656, methyl-arachidonyl-fluorophosphonate (MAFP), bromoenol lactone, the cPLA₂α inhibitor, and U0126, Calbiochem (Bad Soden, Germany); BAPTA/AM and Fura-2/AM, Alexis (Grünberg, Germany); wortmannin, Biotrend (Köln, Germany); cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC), Biomol (Plymouth Meeting, PA); EAH-Sepharose 4B, GE Healthcare Bio-Sciences (Freiburg, Germany); all other chemicals were obtained from Sigma (Deisenhofen, Germany).

Cells

Platelets were freshly isolated from human venous blood of healthy adult donors (St. Markus Hospital, Frankfurt, Germany) as described (PoECKel et al., 2005). Washed platelets were finally resuspended in PBS pH 7.4 and 1 mg/ml glucose (PG buffer) or in PBS pH 7.4 and 1 mg/ml glucose plus 1 mM CaCl₂ (PGC buffer). For incubations with solubilized compounds, ethanol or DMSO was used as vehicle, never exceeding 1 % (vol/vol). For the measurement of [³H]arachidonic acid release, platelet rich plasma was prepared from freshly drawn blood (in 3.13% citrate) from healthy adult donors by centrifugation for 10 min at 750 × g.

Determination of release of [³H]-labeled arachidonic acid from intact platelets

Platelet rich plasma was labelled with 19.2 nM [³H]arachidonic acid (1 μCi/ml, specific activity 200 Ci/mmol) for 2 hours at 37°C in the presence of 100 μM aspirin to avoid clotting. Then, cells were washed twice with PBS pH 5.9 plus 1 mM MgCl₂, 11.5 mM NaHCO₃, 1 g/l glucose, and 1 mg/ml fatty acid-free BSA, and finally resuspended in PG buffer (10⁸/ml). Preparation of cells at pH 5.9 is thought to minimize temperature-induced activation. Platelets

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were incubated at 37°C with 1 mM EDTA plus 30 µM BAPTA/AM for 15 min or incubated with CaCl₂ (1 mM) for 2.5 min prior stimulation with the indicated agents. After the indicated times, incubations were put on ice for 10 min, followed by centrifugation (5,000 × g, 15 min). Aliquots (300 µl) of the supernatants were measured (Micro Beta Trilux, Perkin Elmer) to detect the amounts of [³H]-labeled arachidonic acid released into the medium.

Determination of 12-lipoxygenase formation

To determine p12-LO product formation in intact cells, freshly isolated platelets (10⁸/ml PG buffer) were supplemented with either 1 mM CaCl₂, 1 mM EDTA, or 1 mM EDTA plus 30 µM BAPTA/AM. Platelets were preincubated with the indicated agents for 15 min at 37°C. After addition of stimuli and further incubation at 37°C for the times indicated, p12-LO products (12(S)-hydro(pero)xy-6-trans-8,11,14-cis-eicosatetraenoic acid (12-H(P)ETE) were extracted and then analyzed by HPLC as described (Albert et al., 2002). 12-HETE and 12-HPETE elute as one major peak, integration of this peak represents p12-LO product formation, expressed as ng metabolites per 10⁸ cells.

For determination of p12-LO product formation in broken cell preparations, platelets (10⁸/ml PG buffer plus 1 mM EDTA and 1 mM PMSF) were sonicated (3 × 10 sec) and centrifuged (100,000×g/70 min/4°C). To the resulting 100,000×g supernatant, boswellic acids were added and samples were pre-warmed at 37°C for 30 sec. CaCl₂ (2 mM) was added as indicated and p12-LO product formation was started by addition of arachidonic acid (10 µM). After 10 min at 37°C, the formation of 12-H(P)ETE was determined as described for intact cells.

Immobilization of boswellic acids and protein pull-down assays

For immobilization of KBA at EAH Sepharose 4B beads, the free 3-OH group of KBA was used (manuscript in preparation: Kather, N., Tausch, L., Poeckel, D., Werz, O., Herdtweck, E.

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and Jauch, J. (2006)). In brief, KBA was treated with glutaric anhydride to form the half-ester glutaroyl-KBA, and analyzed by ^1H - and ^{13}C -NMR as well as by mass spectrometry. This substance was ready for immobilization at EAH Sepharose 4B by standard amide coupling procedures. The carboxylic acid of the KBA-core was unlikely to react under standard conditions due to steric crowding. The success of the coupling reaction was determined by two methods: a) glutaroyl-KBA was used in defined excess (2 μmol of glutaroyl-KBA per 1 μmol NH_2 -groups of the EAH Sepharose 4B). After the coupling reaction, the hypothetical excess of glutaroyl-KBA (1 μmol) could be indeed recovered. b) Treatment of glutaroyl-KBA with KOH in *iso*-propanol under reflux for ca. 3 h cleaved the ester bond and gave KBA, analyzed by thin layer chromatography.

For protein fishing experiments, 10^9 platelets were lysed in 1 ml lysis buffer (50 mM HEPES pH 7.4, 200 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM PMSF, 2 $\mu\text{g}/\text{ml}$ leupeptin, 120 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor). After sonication (3×8 s) and centrifugation for 10 min at $12,000\times g$, 50 μl of the sepharose slurries (50 %, vol/vol) were added to supernatants and incubated at 4°C over night under continuous rotation. The sepharose beads were washed 3 times with binding buffer (HEPES pH 7.4, 200 mM NaCl, 1 mM EDTA) and precipitated proteins were finally separated and denatured by addition of 2 \times sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer (SDS-b; 20 mM Tris/HCl, pH 8, 2 mM EDTA, 5% SDS (w/v), 10% β -mercaptoethanol). After boiling (95°C , 6 min), sepharose beads were removed by centrifugation and proteins in the supernatant were analyzed by SDS-PAGE as described (Poeckel et al., 2005). Proteins were visualized by Western-blotting (Poeckel et al., 2005) or Coomassie staining, respectively.

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Statistics

Statistical evaluation of the data was performed by one-way ANOVAs for independent or correlated samples followed by Tukey HSD post-hoc tests. Where appropriate, Student's *t* test for paired and correlated samples was applied. A *p* value of <0.05 (*) or <0.01 (**, ##) was considered significant.

Results

Boswellic acids induce arachidonic acid release independent of Ca^{2+}

In the presence of extracellular Ca^{2+} (1 mM), β -boswellic acid and its 11-keto counterpart AKBA (**fig. 1**) concentration-dependently increased the liberation of arachidonic acid with significant effects at 10 μM , each (**fig. 2**). No marked differences in the potencies between β -boswellic acid and AKBA were obvious and the efficacy of boswellic acids was comparable to thrombin (2 U/ml) or Ca^{2+} -ionophore A23187 (5 μM) (**fig. 2**). Intriguingly, when cells were depleted from intracellular (chelation with BAPTA/AM) and extracellular (chelation with EDTA) Ca^{2+} , boswellic acids still exhibited a strong stimulatory effect on arachidonic acid release with similar efficacies for β -boswellic acid and AKBA (**fig. 2**). Although the absolute levels of arachidonic acid released into the medium in response to β -boswellic acid or AKBA (30 μM , each) were higher in the presence of Ca^{2+} , the relative increases in the absence of Ca^{2+} were more pronounced (4.6- to 5.4-fold), as when Ca^{2+} was present (2.1- and 2.4-fold), which apparently is due to reduced basal arachidonic acid levels in unstimulated cells where Ca^{2+} has been depleted. However, the release of arachidonic acid evoked by boswellic acids in the absence of Ca^{2+} was much slower as compared to conditions where Ca^{2+} was present. Note that in Ca^{2+} -depleted cells, thrombin and A23187 were much less active as compared to boswellic acids, but still an about two-fold stimulation over untreated cells was evident (**fig. 2**). This effect of A23187 is surprising and not readily explainable, but possibly could be caused by Ca^{2+} -independent, unspecific actions on phospholipid membranes. Taken together, boswellic acids are capable to substantially release arachidonic acid from intact platelets, also in the absence of Ca^{2+} .

Effect of boswellic acids on 12-H(P)ETE formation

12-H(P)ETE is a major metabolite of arachidonic acid in platelets produced by p12-LO (Hamberg and Samuelsson, 1974) that can be easily monitored by RP-HPLC representing a

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sensitive read out for evaluation of platelet arachidonic acid metabolism. Washed platelets were incubated with vehicle (DMSO), β -boswellic acid and AKBA (30 μ M, each), thrombin (2 U/ml), or exogenous arachidonic acid (10 μ M, positive control), either in Ca^{2+} -containing medium or under Ca^{2+} -free conditions (pre-treatment with BAPTA/AM plus EDTA). As shown in **fig. 3A**, β -boswellic acid strongly stimulated the formation of 12-H(P)ETE to a comparable level as thrombin. AKBA exerted a much weaker effect than β -boswellic acid. In Ca^{2+} -depleted cells, stimulation with thrombin is virtually ineffective, whereas β -boswellic acid clearly stimulated 12-H(P)ETE formation, and a minor stimulation was also seen for AKBA (**fig. 3A**). As can be seen from **fig. 3B**, boswellic acids lacking the 11-keto group (β -boswellic acid and A β -BA) caused a concentration-dependent increase in 12-H(P)ETE formation, whereas boswellic acids containing the 11-keto moiety (KBA and AKBA) were hardly effective, and for AKBA the formation of 12-H(P)ETE was even lower at higher concentrations. Thus, the 11-keto group seemingly hampers the formation of 12-H(P)ETE. A similar pattern was found in Ca^{2+} -depleted cells (not shown). Selective inhibitors of cPLA₂ (cPLA₂ α inhibitor, 1 μ M; MAFP, 10 μ M) and p12-LO (CDC, 10 μ M) strongly suppressed 12-H(P)ETE formation under all experimental conditions, whereas an inhibitor of the Ca^{2+} -independent iPLA₂ (bromo-enol lactone, 5 μ M) caused no suppression (**fig. 3C**). In conclusion, both β -boswellic acid and AKBA induce the release of arachidonic acid equally well, but only β -boswellic acid but not AKBA potently stimulates 12-H(P)ETE formation, which in part is Ca^{2+} -independent.

Kinetic analysis of 12-H(P)ETE formation

The kinetics of 12-H(P)ETE formation in platelets was studied. The time necessary for half maximal 12-H(P)ETE synthesis ($t_{\text{max}1/2}$) was determined by regression analysis using a 3-parameter Hill equation ($f(t) = a \times t^b / (c^b + t^b)$). In the presence of Ca^{2+} , β -boswellic acid (30

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μM) induced a rapid 12-H(P)ETE generation ($t_{\text{max}1/2} = 37$ s) entering a plateau phase after 3 min (**fig. 4A**). A similarly rapid 12-H(P)ETE production was recorded when arachidonic acid ($2 \mu\text{M}$, $t_{\text{max}1/2} = 41$ s, **fig. 4B**) or ionophore A23187 ($2.5 \mu\text{M}$, $t_{\text{max}1/2} = 44$ s, **fig. 4E**), were added to platelets. Importantly, the kinetic profile of thrombin was different and was considerably delayed ($t_{\text{max}1/2} = 157$ s, **fig. 4C**). AKBA ($30 \mu\text{M}$) gave a less consistent kinetic profile with a $t_{\text{max}1/2}$ of ≥ 100 s (**fig. 4D**).

Lack of extracellular Ca^{2+} (1 mM EDTA) did not strongly alter the kinetic progression of 12-H(P)ETE formation induced by β -boswellic acid ($t_{\text{max}1/2} = 28$ s, **fig. 4A**) or by exogenously added arachidonic acid ($t_{\text{max}1/2} = 33$ s, **fig. 4B**). However, when also intracellular Ca^{2+} was removed by BAPTA/AM, β -boswellic acid-, but not arachidonic acid-induced 12-H(P)ETE formation was remarkably delayed but continuously increased. Long-term kinetic recordings show that whereas the 12-H(P)ETE level in Ca^{2+} -containing buffer gradually decreases after approx. 60 min, it continuously increases up to a plateau after 150 min in Ca^{2+} -depleted cells (**fig. 4F**). When Ca^{2+} -depleted cells were stimulated with thrombin, no detectable increase in 12-H(P)ETE formation was observed (**fig. 4C**). Together, β -boswellic acid induces arachidonic acid liberation/12-H(P)ETE formation in platelets by a rapid Ca^{2+} -mediated pathway as well as by a Ca^{2+} -independent route(s).

Pharmacological dissection of signalling pathways activated by β -boswellic acid

The signalling pathways underlying the Ca^{2+} -dependent and Ca^{2+} -independent mechanisms of arachidonic acid liberation and generation of 12-H(P)ETE were investigated using a pharmacological inhibitor approach. In the presence of Ca^{2+} , increased arachidonic acid liberation due to β -boswellic acid was suppressed by the PI 3-K inhibitor wortmannin (**fig. 5A**). Also, the Src family kinase inhibitors SU6656 and PP2 (but not its inactive variant PP3) reduced the effects of β -boswellic acid. In contrast, inhibitors of MEK/ERK (U0126) and p38

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MAPK (SB203580) failed in this respect. Note that in Ca^{2+} -depleted cells, no significant suppression of β -boswellic acid-evoked arachidonic acid release by any of the above inhibitors was observed (**fig. 5A**).

In analogy to arachidonic acid release, in the presence of Ca^{2+} , the β -boswellic acid-evoked generation of 12-H(P)ETE (**fig. 5B**) was blocked by wortmannin, SU6656 and PP2 (but not by PP3), whereas U0126 or SB203580 were hardly active. Also, in the absence of Ca^{2+} , β -boswellic acid-evoked p12-LO product formation was not sensitive to any of these inhibitors (**fig. 5B**). Moreover, in control experiments, where 12-H(P)ETE was elicited by addition of exogenous arachidonic acid in order to circumvent supply of endogenous substrate, no or only minor effects of the inhibitors were observed, regardless of the presence of Ca^{2+} (**fig. 5B**). Thus, the inhibitory effects of wortmannin, PP2 and SU6656 on β -boswellic acid-induced 12-H(P)ETE generation appear to primarily affect release of arachidonic acid, rather than p12-LO activity. In conclusion, β -boswellic acid-evoked arachidonic acid release/12-H(P)ETE formation in the presence of Ca^{2+} seemingly involves PI 3-K and Src family kinases, whereas in Ca^{2+} -depleted cells none of these signalling molecules apparently contribute.

Effects of boswellic acids on cPLA₂ and p12-LO activity in cell-free assays

To test stimulation of cPLA₂ by boswellic acids *in vitro*, we determined the effects of β -boswellic acid or AKBA on arachidonic acid release from platelet membrane lipids in the absence (inclusion of 1 mM EDTA) as well as in the presence of 2 mM Ca^{2+} . Arachidonic acid release was increased by Ca^{2+} by about 2.4-fold, and was suppressed by the cPLA₂ α inhibitor, assuring that cPLA₂ is the arachidonic acid-releasing enzyme in this assay. No significant and concentration-dependent modulation of the arachidonic acid release was observed by 1 to 100 μM boswellic acid (not shown), regardless of the presence of Ca^{2+} , implying that boswellic acids do not stimulate cPLA₂ activity *in vitro*. Also, there was no

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increased association of cPLA₂ with platelet membranes after either incubation of platelet homogenates with boswellic acids or after exposure of intact platelets to boswellic acids (assessed by Western-blotting, not shown), independent of the presence of Ca²⁺, suggesting that boswellic acids do not promote binding of cPLA₂ to membrane phospholipids *in vitro*. The effects of boswellic acids on p12-LO activity in the platelet 100,000×g supernatant were investigated. Platelet 100,000×g supernatant was incubated with AKBA plus 2 μM arachidonic acid in the presence of either 1 mM EDTA or 1 mM Ca²⁺. 12-H(P)ETE formation was about 3-fold higher in the presence of Ca²⁺ (**fig. 6A, B**). AKBA caused a concentration-dependent inhibition of p12-LO activity (**fig. 6A**). In the presence of Ca²⁺, the IC₅₀ was about 15 μM, whereas without Ca²⁺ the IC₅₀ was approx. 50 μM. In contrast to AKBA, 12-H(P)ETE formation was differentially modulated by β-boswellic acid. Thus, only a weak inhibition of p12-LO activity by β-boswellic acid (IC₅₀ > 100 μM) was detectable in the presence of Ca²⁺ (**fig. 6B**). However, in the absence of Ca²⁺, β-boswellic acid elevated 12-H(P)ETE up to approx. 2-fold at a threshold concentration of 10 μM (**fig. 6B**), which was sensitive to the p12-LO inhibitor CDC (not shown). Together, β-boswellic acid stimulates the catalysis of crude p12-LO in the absence of Ca²⁺, whereas AKBA generally suppresses the catalytic activity of p12-LO, and no direct modulation of cPLA₂ in cell-free assays is apparent for either boswellic acid.

Interaction of boswellic acids with cPLA₂ and p12-LO

To assess direct interaction of boswellic acids with cPLA₂ or p12-LO, a protein fishing assay was performed using KBA as bait that was covalently linked to EAH Sepharose 4B beads via a glutaric acid linker (KBA-Seph). EAH-Sepharose beads without ligand (Seph) were used as control, platelet 12,000×g supernatants served as protein source. Coomassie-staining of gels after SDS-PAGE or Ponceau S staining of membranes after blotting assured comparable

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unspecific protein-binding by Seph and KBA-Seph (not shown). As shown in **fig. 7**, no cPLA₂ protein was detectable (by Western-blotting analysis) in precipitates using Seph or KBA-Seph. cPLA₂ was abundantly present in the corresponding platelet lysates and clearly detectable. However, substantial amounts of p12-LO were present in KBA-Seph pull-downs, but not in precipitates using Seph as negative control. Since 5-lipoxygenase was postulated as AKBA-binding protein (Sailer et al., 1998), we attempted to confirm 5-lipoxygenase binding by our protein fishing strategy using 12,000×g supernatants of PMNL as source for 5-lipoxygenase. Both Seph and KBA-Seph moderately bound 5-lipoxygenase, without significant quantitative differences (**fig. 7**). In summary, p12-LO could be selectively precipitated by KBA immobilized to sepharose beads.

Discussion

Activation of platelets by adequate stimuli may lead to substantial release of arachidonic acid by cPLA₂, connected to subsequent conversion by cyclooxygenase-1 and p12-LO, depending on the strength of the stimuli and the nature of the signalling molecules involved (Coffey et al., 2004; Hamberg and Samuelsson, 1974; Holmsen, 1994). Ca²⁺ is a determinant for these processes, as it stimulates cellular activation and catalysis of both cPLA₂ (Gijon and Leslie, 1999; Leslie, 2004) and p12-LO (Baba et al., 1989). Besides Ca²⁺, serine phosphorylations by MAPK (Borsch-Haubold et al., 1999) and interaction with PIP2 (Balsinde et al., 2000) or sphingolipids (Huwiler et al., 2001; Pettus et al., 2004; Subramanian et al., 2005) activate cPLA₂. In contrast, for p12-LO, there is only limited information regarding cellular activation (Coffey et al., 2004), and except the redox-tone (Bryant et al., 1982), which is of general importance for lipoxygenase activation, only Ca²⁺ is known as (moderate) stimulatory cofactor (Baba et al., 1989). It is assumed that the capacity of platelets to form 12-H(P)ETE is essentially linked to the supply of arachidonic acid. Since boswellic acids induce massive mobilization of Ca²⁺ and activate MAPK in platelets (Poeckel et al., 2005), it was reasonable that boswellic acids as a result may elicit release of arachidonic acid and concomitantly also 12-H(P)ETE synthesis.

β-Boswellic acid and AKBA evoked arachidonic acid release with comparable potencies, similar as the strong platelet agonists thrombin or ionophore that act by recruiting cPLA₂ via phosphorylation and/or elevation of [Ca²⁺]_i (Borsch-Haubold et al., 1995; Kramer et al., 1996). The liberation of arachidonic acid was rapid and sensitive to selective inhibitors of the Ca²⁺-dependent cPLA₂, suggesting that in analogy to thrombin and ionophore, cPLA₂ is the responsible PLA₂ isoform. However, in contrast to thrombin and ionophore, boswellic acids may induce cPLA₂ activation, at least in part, independent of Ca²⁺. Note that bromoenol lactone did not compromise arachidonic acid release in the absence of Ca²⁺, which excludes the Ca²⁺-independent iPLA₂ (Hazen et al., 1991) as responsible enzyme. Also, determination

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of 12-H(P)ETE shows that boswellic acids but not thrombin partially act in a Ca^{2+} -independent manner. AKBA failed to substantially induce 12-H(P)ETE synthesis, probably related to its inhibitory action on p12-LO (discussed below). Moreover, our kinetic analysis of cellular 12-H(P)ETE production favours an additional, Ca^{2+} -independent cPLA₂/p12-LO activation pathway. Thus, 12-H(P)ETE formation induced by β -boswellic acid was much more rapid than by thrombin, although increases in $[\text{Ca}^{2+}]_i$ by β -boswellic acid are delayed as compared to thrombin (Poeckel et al., 2005). Therefore, it is unlikely that the rapid and robust 12-H(P)ETE synthesis induced by β -boswellic acid is mediated solely by elevation of $[\text{Ca}^{2+}]_i$. The Ca^{2+} -dependency of cPLA₂ in platelets is well established, but alternate signaling routes such as phosphorylation by MAPKs contribute (Borsch-Haubold et al., 1999; Borsch-Haubold et al., 1995; Kramer et al., 1996). In fact, boswellic acids activate MAPK in platelets (Poeckel et al., 2005), however, MAPK inhibitors failed to suppress β -boswellic acid-induced arachidonic acid release and 12-H(P)ETE synthesis. Nevertheless, our inhibitor approach indicates that PI 3-K and Src family kinases may be integrated in β -boswellic acid-evoked responses, at least under conditions where Ca^{2+} is present. Since Src family kinases and PI 3-K are also involved in β -boswellic acid-induced Ca^{2+} mobilisation (Poeckel et al., 2005), the suppressive effects of the respective inhibitors are likely to be due to inhibition of Ca^{2+} mobilisation, rather than uncoupling Ca^{2+} -independent signals to cPLA₂. This is supported by the fact that the inhibitors completely failed to suppress β -boswellic acid-induced responses in the absence of Ca^{2+} . Moreover, no inhibition of 12-H(P)ETE formation was evident after stimulation with exogenous arachidonic acid, implying that arachidonic acid release rather than p12-LO activation is primarily affected by the inhibitors. PI 3-K and Src family kinases have also been implicated in the formation of 12-H(P)ETE from endogenous arachidonic acid in platelets stimulated by collagen and collagen-related peptide (Coffey et al., 2004).

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Apart from Ca^{2+} and phosphorylation, cPLA₂ is activated by direct interaction with PIP2 or ceramide and ceramide 1-phosphate (Huwiler et al., 2001; Pettus et al., 2004; Subramanian et al., 2005), and it appeared possible that also boswellic acids could activate cPLA₂ by direct interactions. However, cPLA₂ failed to bind KBA-Seph, and boswellic acids did not stimulate cPLA₂ activity in cell-free assays, excluding such interrelations. Collectively, we conclude that boswellic acids activate cPLA₂ independent of Ca^{2+} and phosphorylation by a yet unrecognized mechanism.

Initially, boswellic acids were identified as inhibitors of 5-lipoxygenase (Safayhi et al., 1992; Safayhi et al., 1995) that may interfere with a regulatory arachidonic acid-binding site in a Ca^{2+} -dependent manner (Sailer et al., 1998). Among the boswellic acids, AKBA is the most potent 5-lipoxygenase inhibitor with high selectivity for 5-lipoxygenase whereas inhibition of p12-LO in intact platelets was excluded (Safayhi et al., 1992). We found that AKBA inhibits p12-LO in cell-free assays with an IC₅₀ value (15 μM), significantly below the values determined for 5-lipoxygenase under comparable assay conditions (50 μM) (Werz et al., 1997; Werz et al., 1998). Of interest, p12-LO bound to KBA-Seph, but was absent in pull-downs using Seph, implying a rather selective interaction between KBA-Seph and p12-LO. Note that the amounts of 5-lipoxygenase in KBA-Seph and Seph precipitates from 12,000 \times g supernatants of PMNL lysates were about the same, implying unspecific binding of 5-lipoxygenase to KBA-Seph.

Direct suppression of p12-LO activity by AKBA may explain why despite induction of marked arachidonic acid release in intact platelets, no subsequent conversion to 12-H(P)ETE was evident, whereas β -boswellic acid (or A β -BA) concentration-dependently induced 12-H(P)ETE formation. In agreement with others (Baba et al., 1989), Ca^{2+} increased p12-LO activity in platelet 100,000 \times g supernatant about 3-fold, and β -boswellic acid mimicked this effect as it stimulated p12-LO activity without Ca^{2+} . In contrast, in the presence of Ca^{2+} , β -boswellic acid did not further stimulate p12-LO. Apparently, the 11-keto moiety determines

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the quality of p12-LO modulation by boswellic acids, and contrasting effects of boswellic acids depending on the 11-keto moiety were observed before also in other experimental settings (Altmann et al., 2004; Poeckel et al., 2005; Poeckel et al., 2006).

The conclusions from our results deviate from the long established view of boswellic acids as negative modulatory agents of the arachidonic acid cascade, as we demonstrate strong induction of arachidonic acid release and formation of 12-H(P)ETE by boswellic acids in platelets. Also, we suggest p12-LO as a definite target of boswellic acids with superior susceptibility as compared to 5-lipoxygenase. The question of the pharmacological consequence resulting from the divergent effects of β -boswellic acid and AKBA on 12-H(P)ETE biosynthesis *in vivo* remains to be answered. After oral intake of 4×786 mg *Boswellia serrata* extracts (containing about 3.7 % AKBA, 10.5 % A β -BA, 6.1 % KBA and 18.2 % β -boswellic acid) per day, the plasma levels of AKBA (0.1 μ M) (Buchele and Simmet, 2003) are far below the concentrations required to efficiently suppress p12-LO ($IC_{50} = 15$ μ M). On the other hand, β -boswellic acid reached plasma levels (10.1 μ M), virtually sufficient to induce 12-H(P)ETE formation. In our *in vitro* assays, relevant amounts (approx. 5 μ g/ml) of *Boswellia serrata* extracts, containing diverse boswellic acids, strongly induced arachidonic acid release as well as 12-H(P)ETE synthesis (not shown). 12-H(P)ETE may act as chemoattractant for leukocytes (Goetzl, 1980), mediates angiogenesis and tumour metastasis (Honn et al., 1994), possesses inhibitory neuromodulatory effects (Piomelli et al., 1987) and is involved in cardiovascular diseases (Gonzalez-Nunez et al., 2001), which should be taken into account when administering boswellic acid-containing medicine. Besides the dissection of the influences of boswellic acids on 12-H(P)ETE as mediator in (patho-)physiology applied as complex composed extracts of *Boswellia serrata*, it also remains a future challenge to fully elucidate the Ca^{2+} /phosphorylation-independent signalling routes leading to cPLA₂ activation and increased release of arachidonic acid by boswellic acids.

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¹Footnotes

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Figure legends

Fig. 1 Chemical structures of β -boswellic acid and AKBA

AKBA lacking the 3-*O*-acetyl group yields KBA; 3-*O*-acetylation of β -boswellic acid results in A β -BA.

Fig. 2 Boswellic acids elevate the liberation of arachidonic acid in platelets

Platelets (10^8) were incubated at 37°C with 1 mM EDTA plus 30 μ M BAPTA/AM for 15 min (white bars) or incubated with CaCl₂ (1 mM) for 2.5 min (black bars), and then stimulated with the indicated concentrations of β -boswellic acid (β -BA) or AKBA, thrombin (2 U/ml), or ionophore A23187 (5 μ M). [³H]arachidonic acid released into the medium was measured after 5 min in the presence of Ca²⁺ (black bars) or after 15 min in the absence of Ca²⁺ (white bars). Data are given as cpm, mean + S.E., n = 5, p < 0.05 (*).

Fig. 3 Boswellic acids stimulate the formation of p12-LO in intact platelets

(A) 12-H(P)ETE formation. Platelets were supplemented with either 1 mM CaCl₂ or 1 mM EDTA plus 30 μ M BAPTA/AM as indicated. β -Boswellic acid (β -BA, 30 μ M), AKBA (30 μ M) thrombin (2 U/ml), arachidonic acid (AA, 10 μ M) or vehicle (DMSO) were added and 12-H(P)ETE formation was determined. (B) Concentration-response experiments for 12-H(P)ETE formation. Platelets were treated with β -boswellic acid (β -BA), A β -BA, KBA or AKBA at the indicated concentrations and 12-H(P)ETE formation was determined. (C) Effects of p12-LO and PLA₂ inhibitors. Platelets were supplemented with either 1 mM CaCl₂ (black bars) or 1 mM EDTA plus 30 μ M BAPTA/AM (white bars) and preincubated with CDC (10 μ M), MAFP (10 μ M), cPLA₂ α inhibitor (1 μ M), and bromoenol lactone (BEL, 5 μ M). After 10 min, β -boswellic acid (β -BA, 30 μ M) was added and 12-H(P)ETE formation was determined. Data are given as mean + S.E., n = 3-5. * p < 0.05; **, ## p < 0.01.

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Fig. 4 Kinetics of 12-H(P)ETE formation in intact platelets. Platelets (10^9) were resuspended in 10 ml PG buffer containing either 1 mM CaCl_2 (filled circles), 1 mM EDTA (triangles), or 1 mM EDTA plus 30 μM BAPTA/AM (open symbols). Cells were stimulated with either β -boswellic acid (30 μM **A**, and 10 μM **F**), 2 μM arachidonic acid (**B**), 1 U/ml thrombin (**C**), 30 μM AKBA (**D**), or 5 μM ionophore (**E**). Aliquots of 1 ml corresponding to 10^8 cells were mixed with 1 ml of ice-cold methanol after the indicated times, and 12-H(P)ETE formation was determined. Data are given as mean + S.E., $n = 3-5$.

Fig. 5 Effects of relevant pharmacological inhibitors on β -boswellic acid-induced arachidonic acid release and 12-H(P)ETE formation.

(**A**) Arachidonic acid release. Platelets were labelled with [^3H]arachidonic acid as described in the legend of fig. 2. After washing, cells were either left untreated (black bars), or 1 mM EDTA plus 30 μM BAPTA/AM (white bars) was added. Then, cells were preincubated with 200 nM wortmannin (wort), 3 μM PP2 or PP3, 5 μM SU6656, 3 μM U-0126, 10 μM SB203580, or vehicle (DMSO, negative/positive) as indicated for 15 min at 37°C. CaCl_2 (1 mM) was added to the cells as indicated and after 2.5 min, cells were stimulated with 30 μM β -boswellic acid (β -BA). [^3H]arachidonic acid released into the medium was measured after 5 min (black bars) or 15 min (white bars), respectively. (**B**) 12-H(P)ETE formation. Platelets were resuspended in 1 ml PG buffer plus 1 mM CaCl_2 (black bars) or in PG buffer containing 1 mM EDTA plus 30 μM BAPTA/AM (white bars) and preincubated with 200 nM wortmannin (wort), 3 μM PP2 or PP3, 5 μM SU6656, 3 μM U-0126, 10 μM SB203580 or vehicle (DMSO) as indicated. Then, 30 μM β -boswellic acid (β -BA, left panel) or 2 μM arachidonic acid (AA, right panel) was added and 12-H(P)ETE formation was determined. Data are expressed as percentage of control (100 %, vehicle) and values are given as mean + S.E., $n = 3-4$. * $p < 0.05$; ** $p < 0.01$.

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Fig. 6 Effects of boswellic acids on the activity of p12-LO in cell free assays

Platelets were sonicated and a 100,000×g supernatant was prepared. AKBA (**A**) or β-boswellic acid (β-BA) (**B**) were added to the 100,000×g supernatant at the indicated concentrations, and the synthesis of 12-H(P)ETE was started by addition of arachidonic acid (2 μM) with or without 2 mM CaCl₂, as indicated. 12-H(P)ETE was determined by HPLC. Data are given as mean + S.E., n = 3-5, **p* < 0.05; ***p* < 0.01.

Fig. 7 AKBA selectively binds p12-LO

12,000×g supernatants of platelet lysates (for precipitation of cPLA₂ and p12-LO) or of PMNL lysates (for 5-lipoxygenase), were incubated over night at 4°C with either KBA-Seph or with crude Seph. Precipitates were intensively washed, solubilized by addition of SDS-b and separated by SDS-PAGE. Proteins were visualized by Western-blotting using specific antibodies against cPLA₂, p12-LO or 5-lipoxygenase (5-LO). Aliquots of the corresponding lysates were used as positive controls. Similar results were obtained in three additional experiments.

Fig. 1

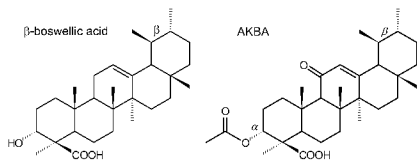


Fig. 2

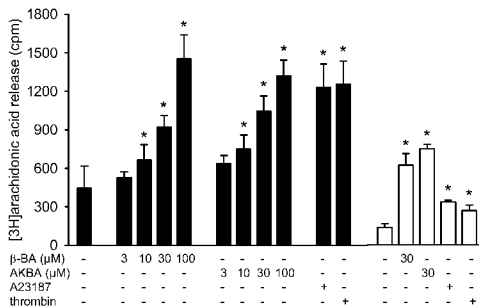


Fig. 3

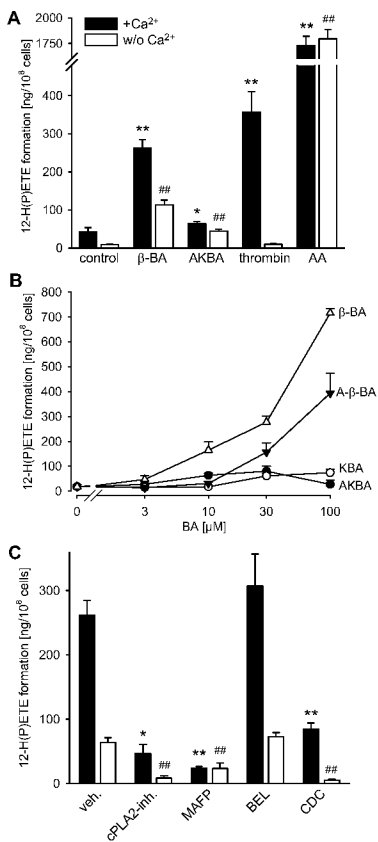


Fig. 4

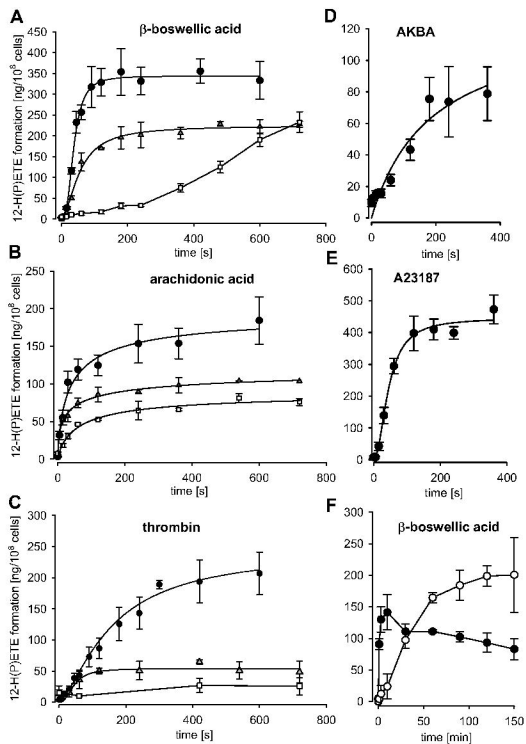


Fig. 5

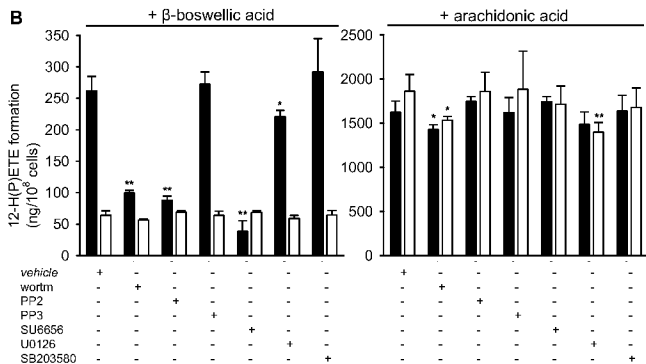
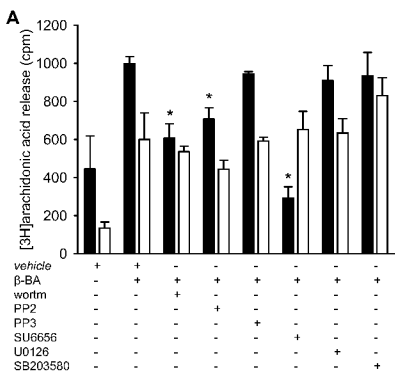


Fig. 6

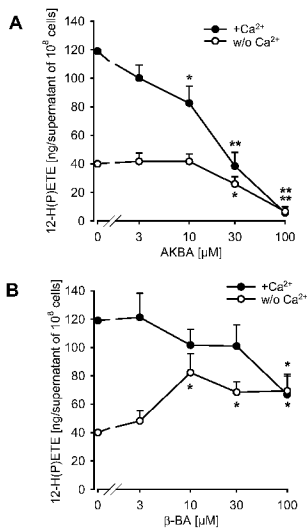


Fig. 7

