

# Boswellic Acids Stimulate Arachidonic Acid Release and 12-Lipoxygenase Activity in Human Platelets Independent of $\text{Ca}^{2+}$ and Differentially Interact with Platelet-Type 12-Lipoxygenase

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Received March 22, 2006; accepted June 20, 2006

## 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. Using 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  $\beta$ -boswellic acid and 3-O-acetyl-11-keto-boswellic acid (AKBA) markedly enhanced the release of arachidonic acid via cytosolic phospholipase  $\text{A}_2$  (cPLA<sub>2</sub>), whereas for generation of 12-hydro(pero)xyeicosatetraenoic acid [12-H(P)ETE], AKBA was less potent than  $\beta$ -boswellic acid and was without effect at higher concentrations ( $\geq 30 \mu\text{M}$ ). In contrast to thrombin,  $\beta$ -boswellic acid-induced release of arachidonic acid and formation of 12-H(P)ETE was more rapid and occurred in the absence of  $\text{Ca}^{2+}$ . The  $\text{Ca}^{2+}$ -independent re-

lease of arachidonic acid and 12-H(P)ETE production elicited by  $\beta$ -boswellic acid was not affected by pharmacological inhibitors of signaling molecules relevant for agonist-induced arachidonic acid liberation and metabolism. It is noteworthy that in cell-free assays,  $\beta$ -boswellic acid increased p12-LO catalysis approximately 2-fold in the absence but not in the presence of  $\text{Ca}^{2+}$ , whereas AKBA inhibited p12-LO activity. No direct modulatory effects of boswellic acids on cPLA<sub>2</sub> activity in cell-free assays were evident. Therefore, immobilized KBA (linked to Sepharose beads) selectively precipitated p12-LO from platelet lysates but failed to bind cPLA<sub>2</sub>. 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  $\text{Ca}^{2+}$ -independent routes, and we identified p12-LO as a selective molecular target of boswellic acids.

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 antitumorigenic potential of boswellic acids based on experimental cellular and animal models (Safayhi et al., 1992; Winking

et al., 2000; Syrovets et al., 2005a,b; Anthoni et al., 2006; Poeckel et al., 2006). 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 $\kappa$ B kinases (Safayhi et al., 1995, 1997; Syrovets et al., 2000, 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 re-

This study was supported by the Deutsche Forschungsgemeinschaft (WE 2260/4-1 and GRK 757).

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.  
doi:10.1124/mol.106.024836.

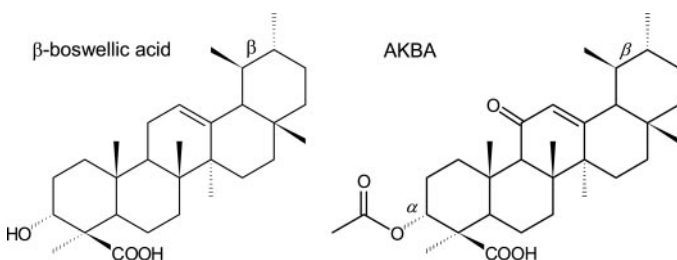
**ABBREVIATIONS:** cPLA<sub>2</sub>, cytosolic phospholipase  $\text{A}_2$ ; A $\beta$ -BA, 3-O-acetyl-boswellic acid; AKBA, 3-O-acetyl-11-keto-boswellic acid; CDC, cinnamyl-3,4-dihydroxy- $\alpha$ -cyanocinnamate; 12-H(P)ETE, 12-hydro(pero)xyeicosatetraenoic acid; KBA, 11-keto-boswellic acid; MAFP, methyl-arachidonyl-fluorophosphonate; MAPK, mitogen-activated protein kinase; p12-LO, platelet-type 12-lipoxygenase; PG buffer, phosphate-buffered saline and glucose; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol-4,5-bisphosphate; PMNL, polymorphonuclear leukocytes; SDS-b, 2 $\times$  SDS-polyacrylamide gel electrophoresis sample loading buffer; PAGE, polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; AM, acetoxymethyl ester; Seph, Sepharose beads without ligand; Seph-KBA, Sepharose beads linked with 11-keto-boswellic acid; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; SU6656, 2-oxo-3-(4,5,6,7-tetrahydro-1*H*-indol-2-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; A23187, calcimycin; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; PP3, 4-amino-7-phenylpyrazolo[3,4-*d*]pyrimidine.

garded as the most important pharmacological action of boswellic acids accounting for their anti-inflammatory properties (Safayhi et al., 1995, 1997).

Many cell types are able to release arachidonic acid from phospholipids within cellular membranes by the action of specific phospholipases A<sub>2</sub> (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 cytochrome P450 family. The 85-kDa cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>) has been accounted as a 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<sup>2+</sup> 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 (PIP2) (Balsinde et al., 2000) or ceramide(1-phosphate) (Huwiler et al., 2001; Pettus et al., 2004; Subramanian et al., 2005) via specific binding-site(s) may promote cPLA<sub>2</sub> catalysis.

Exposure of leukocytes or platelets to boswellic acids differentially affects signaling pathways and functional responses including Ca<sup>2+</sup> mobilization, MAPK activation, formation of reactive oxygen species, release of arachidonic acid, and stimulation of 5-lipoxygenase product formation. Thus, stimulating properties (Safayhi et al., 2000; Altmann et al., 2002, 2004; Poeckel et al., 2005) and inhibitory effects (Safayhi et al., 1992, 1995; Werz et al., 1998; Poeckel et al., 2006) 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, IC<sub>50</sub> values in the range of 1.5  $\mu$ M (Safayhi et al., 1995) up to 50  $\mu$ M (Werz et al., 1997, 1998) were determined, but also 5-lipoxygenase stimulatory effects in this concentration range were described previously (Safayhi et al., 2000; Altmann et al., 2004).

We observed recently 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.



**Fig. 1.** Chemical structures of  $\beta$ -boswellic acid and AKBA. AKBA lacking the 3-*O*-acetyl group yields KBA; 3-*O*-acetylation of  $\beta$ -boswellic acid results in A $\beta$ -BA.

## Materials and Methods

**Materials.** Boswellic acids were synthesized and prepared as described previously (Jauch and Bergmann, 2003). Antibodies against human p12-LO were kindly provided by Dr. Colin D. Funk (Queen's University, Kingston, ON, Canada). SB203580, PP2, PP3, SU6656, methyl-arachidonyl-fluorophosphonate (MAFP), bromoenol lactone, the cPLA<sub>2</sub> inhibitor, and U0126 were from Calbiochem (Bad Soden, Germany); BAPTA/AM and Fura-2/AM were from Alexis (Grünberg, Germany); wortmannin was from Biotrend (Köln, Germany); cinnamyl-3,4-dihydroxy- $\alpha$ -cyanocinnamate (CDC) was from BIOMOL Research Laboratories (Plymouth Meeting, PA); EAH-Sepharose 4B was from GE Healthcare (Freiburg, Germany); and 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 previously (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 CaCl<sub>2</sub>. For incubations with solubilized compounds, ethanol or DMSO was used as vehicle, never exceeding 1% (v/v). For the measurement of [<sup>3</sup>H]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 750g.

**Determination of Release of <sup>3</sup>H-Labeled Arachidonic Acid from Intact Platelets.** Platelet rich plasma was labeled with 19.2 nM [<sup>3</sup>H]arachidonic acid (1  $\mu$ Ci/ml; specific activity, 200 Ci/mmol) for 2 h at 37°C in the presence of 100  $\mu$ M aspirin to avoid clotting. Then, cells were washed twice with PBS, pH 5.9, plus 1 mM MgCl<sub>2</sub>, 11.5 mM NaHCO<sub>3</sub>, 1 g/l glucose, and 1 mg/ml fatty acid-free bovine serum albumin and finally resuspended in PG buffer (10<sup>8</sup>/ml). Preparation of cells at pH 5.9 is believed to minimize temperature-induced activation. Platelets were incubated at 37°C with 1 mM EDTA plus 30  $\mu$ M BAPTA/AM for 15 min or incubated with CaCl<sub>2</sub> (1 mM) for 2.5 min before stimulation with the indicated agents. After the indicated times, incubations were put on ice for 10 min, followed by centrifugation (5000g, 15 min). Aliquots (300  $\mu$ l) of the supernatants were measured (Wallac MicroBeta TriLux; PerkinElmer Life and Analytical Sciences, Boston, MA) to detect the amounts of <sup>3</sup>H-labeled arachidonic acid released into the medium.

**Determination of 12-Lipoxygenase Formation.** To determine p12-LO product formation in intact cells, freshly isolated platelets (10<sup>8</sup>/ml PG buffer) were supplemented with either 1 mM CaCl<sub>2</sub>, 1 mM EDTA, or 1 mM EDTA plus 30  $\mu$ M BAPTA/AM. Platelets were preincubated with the indicated agents for 15 min at 37°C. After the 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 high-performance liquid chromatography as described previously (Albert et al., 2002). 12-HETE and 12-H(P)ETE elute as one major peak, and integration of this peak represents p12-LO product formation, expressed as nanograms of metabolites per 10<sup>8</sup> cells.

For the determination of p12-LO product formation in broken cell preparations, platelets (10<sup>8</sup>/ml PG buffer plus 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride) were sonicated (3  $\times$  10 s) and centrifuged (100,000g/70 min/4°C). To the resulting 100,000g supernatant, boswellic acids were added, and samples were prewarmed at 37°C for 30 s. CaCl<sub>2</sub> (2 mM) was added as indicated, and p12-LO product formation was started by the addition of arachidonic acid (10  $\mu$ M). 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 (N. Kather, L. Tausch, D. Poeckel, O. Werz, E. Herdtweck, and J. Jauch, unpublished data). In brief, KBA was treated with glutaric anhydride to form the half-ester glutaroyl-KBA and analyzed by <sup>1</sup>H and <sup>13</sup>C NMR and by mass spectrometry. This substance was ready for immobilization at EAH

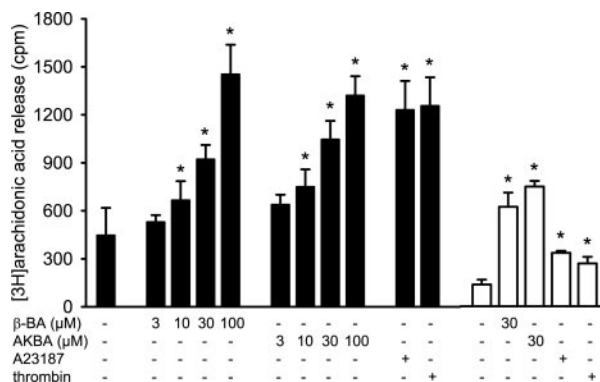
Sephacrose 4B by standard amide coupling procedures. The carboxylic acid of the KBA core was unlikely to react under standard conditions because of steric crowding. The success of the coupling reaction was determined by two methods: 1) glutaroyl-KBA was used in defined excess (2  $\mu\text{mol}$  of glutaroyl-KBA per 1  $\mu\text{mol}$   $\text{NH}_2$  groups of the EAH Sepharose 4B), and after the coupling reaction, the hypothetical excess of glutaroyl-KBA (1  $\mu\text{mol}$ ) could be indeed recovered; and 2) treatment of glutaroyl-KBA with KOH in isopropanol under reflux for approximately 3 h, cleaved the ester bond, and gave KBA, which was then analyzed by thin-layer chromatography.

For protein fishing experiments,  $10^9$  platelets were lysed in 1 ml of lysis buffer (50 mM HEPES, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 2  $\mu\text{g}/\text{ml}$  leupeptin, and 120  $\mu\text{g}/\text{ml}$  soybean trypsin inhibitor). After sonication ( $3 \times 8$  s) and centrifugation for 10 min at 12,000g, 50  $\mu\text{l}$  of the Sepharose slurries [50% (v/v)] was added to supernatants and incubated at 4°C overnight under continuous rotation. The Sepharose beads were washed three times with binding buffer (HEPES, pH 7.4, 200 mM NaCl, and 1 mM EDTA), and precipitated proteins were finally separated and denatured by the addition of 2 $\times$  SDS-polyacrylamide gel electrophoresis (PAGE) sample loading buffer [SDS-b; 20 mM Tris/HCl, pH 8, 2 mM EDTA, 5% SDS (w/v), and 10%  $\beta$ -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 previously (Poeckel et al., 2005). Proteins were visualized by Western blotting (Poeckel et al., 2005) or Coomassie staining, respectively.

**Statistics.** Statistical evaluation of the data was performed by one-way analyses of variance for independent or correlated samples followed by Tukey honestly significantly different 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  $\text{Ca}^{2+}$ .** In the presence of extracellular  $\text{Ca}^{2+}$  (1 mM),  $\beta$ -boswellic acid and its 11-keto counterpart AKBA (Fig. 1) concentration-dependently increased the liberation of arachidonic acid with significant effects at 10  $\mu\text{M}$  each (Fig. 2). No marked differences in the potencies between  $\beta$ -boswellic acid and AKBA were obvious, and the efficacy of boswellic acids was comparable with thrombin (2 U/ml) or  $\text{Ca}^{2+}$ -ionophore A23187 (5  $\mu\text{M}$ ) (Fig. 2). When cells were



**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  $\mu\text{M}$  BAPTA/AM for 15 min (□) or incubated with  $\text{CaCl}_2$  (1 mM) for 2.5 min (■) and then stimulated with the indicated concentrations of  $\beta$ -boswellic acid ( $\beta$ -BA) or AKBA, thrombin (2 U/ml), or ionophore A23187 (5  $\mu\text{M}$ ). [ $^3\text{H}$ ]Arachidonic acid released into the medium was measured after 5 min in the presence of  $\text{Ca}^{2+}$  (■) or after 15 min in the absence of  $\text{Ca}^{2+}$  (□). Data are given as counts per minute; mean  $\pm$  S.E.,  $n = 5$ ,  $p < 0.05$  (\*).

depleted from intracellular (chelation with BAPTA/AM) and extracellular (chelation with EDTA)  $\text{Ca}^{2+}$ , boswellic acids still exhibited a strong stimulatory effect on arachidonic acid release with similar efficacies for  $\beta$ -boswellic acid and AKBA (Fig. 2). Although the absolute levels of arachidonic acid released into the medium in response to  $\beta$ -boswellic acid or AKBA (30  $\mu\text{M}$  each) were higher in the presence of  $\text{Ca}^{2+}$ , the relative increases in the absence of  $\text{Ca}^{2+}$  were more pronounced (4.6- to 5.4-fold), as when  $\text{Ca}^{2+}$  was present (2.1- and 2.4-fold), which apparently is due to reduced basal arachidonic acid levels in unstimulated cells in which  $\text{Ca}^{2+}$  has been depleted. However, the release of arachidonic acid evoked by boswellic acids in the absence of  $\text{Ca}^{2+}$  was much slower compared with conditions in which  $\text{Ca}^{2+}$  was present. Note that in  $\text{Ca}^{2+}$ -depleted cells, thrombin and A23187 were much less active compared with boswellic acids, but still, an approximately 2-fold stimulation over untreated cells was evident (Fig. 2). This effect of A23187 is surprising and not readily explainable, but it possibly could be caused by  $\text{Ca}^{2+}$ -independent, unspecific actions on phospholipid membranes. Taken together, boswellic acids are capable of substantially releasing arachidonic acid from intact platelets also in the absence of  $\text{Ca}^{2+}$ .

**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 reverse-phase high-performance liquid chromatography, representing a sensitive readout for the evaluation of platelet arachidonic acid metabolism. Washed platelets were incubated with vehicle (DMSO),  $\beta$ -boswellic acid and AKBA (30  $\mu\text{M}$  each), thrombin (2 U/ml), or exogenous arachidonic acid (10  $\mu\text{M}$ , positive control), either in  $\text{Ca}^{2+}$ -containing medium or under  $\text{Ca}^{2+}$ -free conditions (pretreatment with BAPTA/AM plus EDTA). As shown in Fig. 3A,  $\beta$ -boswellic acid strongly stimulated the formation of 12-H(P)ETE to a level comparable with that of thrombin. AKBA exerted a much weaker effect than  $\beta$ -boswellic acid. In  $\text{Ca}^{2+}$ -depleted cells, stimulation with thrombin is virtually ineffective, whereas  $\beta$ -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 ( $\beta$ -boswellic acid and  $\alpha$ -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  $\text{Ca}^{2+}$ -depleted cells (data not shown). Selective inhibitors of cPLA<sub>2</sub> (cPLA<sub>2</sub> $\alpha$  inhibitor, 1  $\mu\text{M}$ ; MAFP, 10  $\mu\text{M}$ ) and p12-LO (CDC, 10  $\mu\text{M}$ ) strongly suppressed 12-H(P)ETE formation under all experimental conditions, whereas an inhibitor of the  $\text{Ca}^{2+}$ -independent PLA<sub>2</sub> (bromoenol lactone, 5  $\mu\text{M}$ ) caused no suppression (Fig. 3C). In conclusion, both  $\beta$ -boswellic acid and AKBA induce the release of arachidonic acid equally well, but only  $\beta$ -boswellic acid and not AKBA potentially stimulates 12-H(P)ETE formation, which in part is  $\text{Ca}^{2+}$ -independent.

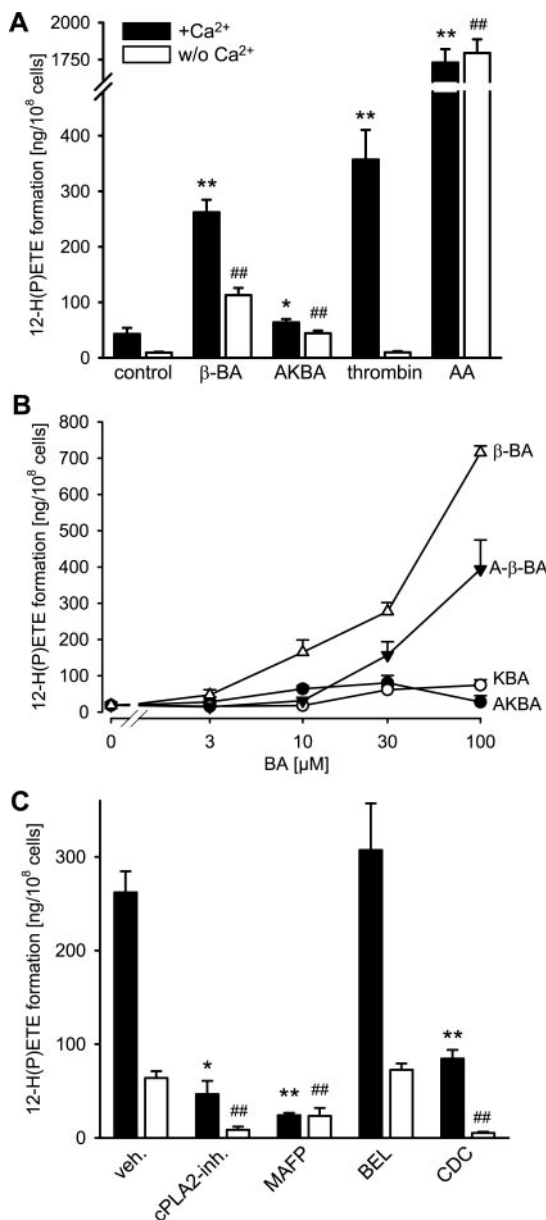
**Kinetic Analysis of 12-H(P)ETE Formation.** The kinetics of 12-H(P)ETE formation in platelets was studied. The time necessary for half-maximal 12-H(P)ETE synthesis ( $t_{\text{max}1/2}$ ) was determined by regression analysis using a



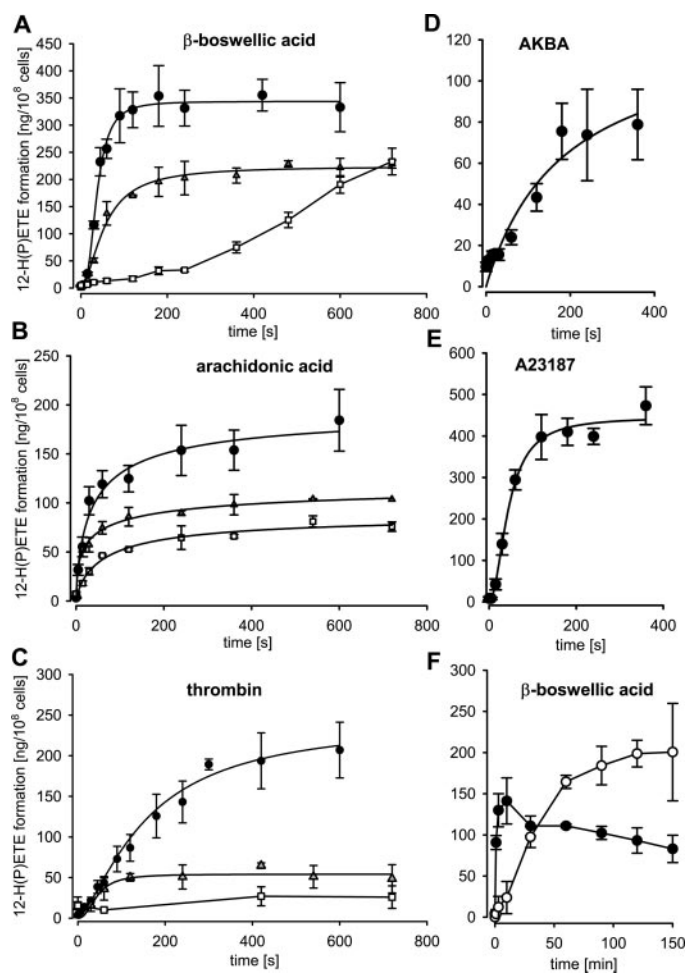
three-parameter Hill equation:  $f(t) = a \times t^b / (c^b + t^b)$ , where  $t$  is time,  $f(t)$  is the amount of 12-H(P)ETE per milliliter, and  $a$ ,  $b$ , and  $c$  are fitting constants. In the presence of  $\text{Ca}^{2+}$ ,  $\beta$ -boswellic acid (30  $\mu\text{M}$ ) induced a rapid 12-H(P)ETE 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. It is noteworthy that 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  $\geq 100$  s (Fig. 4D).

Lack of extracellular  $\text{Ca}^{2+}$  (1 mM EDTA) did not strongly alter the kinetic progression of 12-H(P)ETE formation induced by  $\beta$ -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 intracellular  $\text{Ca}^{2+}$  was removed by BAPTA/AM,  $\beta$ -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  $\text{Ca}^{2+}$ -containing buffer gradually decreases after approximately 60 min, it continuously increases up to a plateau after 150 min in  $\text{Ca}^{2+}$ -depleted cells (Fig. 4F). When  $\text{Ca}^{2+}$ -depleted cells were stimulated with thrombin, no detectable increase in 12-H(P)ETE formation was observed (Fig. 4C). Together,  $\beta$ -boswellic acid induces arachidonic acid liberation/12-H(P)ETE formation in



**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  $\text{CaCl}_2$  or 1 mM EDTA plus 30  $\mu\text{M}$  BAPTA/AM as indicated.  $\beta$ -Boswellic acid ( $\beta$ -BA, 30  $\mu\text{M}$ ), AKBA (30  $\mu\text{M}$ ), thrombin (2 U/ml), arachidonic acid (AA, 10  $\mu\text{M}$ ), or vehicle (DMSO) was added and 12-H(P)ETE formation was determined. B, concentration-response experiments for 12-H(P)ETE formation. Platelets were treated with  $\beta$ -boswellic acid ( $\beta$ -BA), A- $\beta$ -BA, KBA, or AKBA at the indicated concentrations and 12-H(P)ETE formation was determined. C, effects of p12-LO and PLA<sub>2</sub> inhibitors. Platelets were supplemented with either 1 mM  $\text{CaCl}_2$  (■) or 1 mM EDTA plus 30  $\mu\text{M}$  BAPTA/AM (□) and preincubated with CDC (10  $\mu\text{M}$ ), MAFP (10  $\mu\text{M}$ ), cPLA<sub>2</sub> inhibitor (1  $\mu\text{M}$ ), and bromoenol lactone (BEL, 5  $\mu\text{M}$ ). After 10 min,  $\beta$ -boswellic acid ( $\beta$ -BA, 30  $\mu\text{M}$ ) was added, and 12-H(P)ETE formation was determined. Data are given as mean  $\pm$  S.E.,  $n = 3$  to 5. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .



**Fig. 4.** Kinetics of 12-H(P)ETE formation in intact platelets. Platelets ( $10^9$ ) were resuspended in 10 ml of PG buffer containing either 1 mM  $\text{CaCl}_2$  (●), 1 mM EDTA (triangles), or 1 mM EDTA plus 30  $\mu\text{M}$  BAPTA/AM (open symbols). Cells were stimulated with either  $\beta$ -boswellic acid [30  $\mu\text{M}$  (A), and 10  $\mu\text{M}$  (F)], 2  $\mu\text{M}$  arachidonic acid (B), 1 U/ml thrombin (C), 30  $\mu\text{M}$  AKBA (D), or 5  $\mu\text{M}$  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  $\pm$  S.E.,  $n = 3$  to 5.

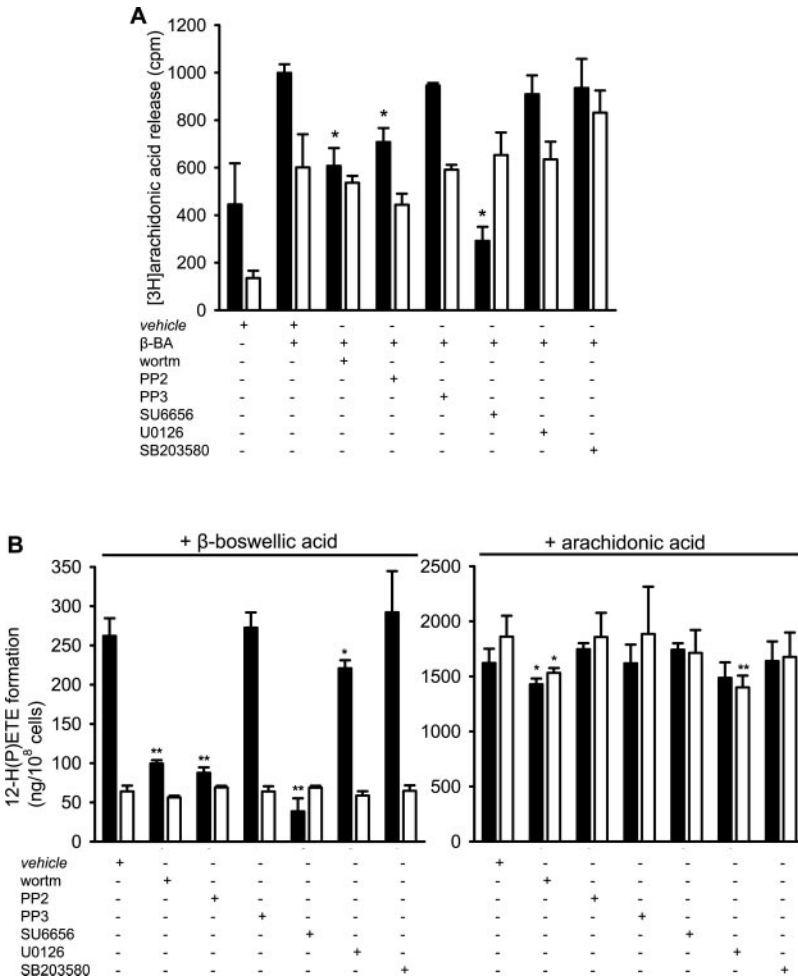
platelets by a rapid  $\text{Ca}^{2+}$ -mediated pathway and by a  $\text{Ca}^{2+}$ -independent route(s).

**Pharmacological Dissection of Signaling Pathways Activated by  $\beta$ -Boswellic Acid.** The signaling pathways underlying the  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ -independent mechanisms of arachidonic acid liberation and generation of 12-H(P)ETE were investigated using a pharmacological inhibitor approach. In the presence of  $\text{Ca}^{2+}$ , increased arachidonic acid liberation due to  $\beta$ -boswellic acid was suppressed by the PI3K inhibitor wortmannin (Fig. 5A). In addition, the Src family kinase inhibitors SU6656 and PP2 (but not its inactive variant PP3) reduced the effects of  $\beta$ -boswellic acid. In contrast, inhibitors of mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (U0126) and p38 MAPK (SB203580) failed in this respect. Note that in  $\text{Ca}^{2+}$ -depleted cells, no significant suppression of  $\beta$ -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  $\text{Ca}^{2+}$ , the  $\beta$ -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. In addition, in the absence of  $\text{Ca}^{2+}$ ,  $\beta$ -boswellic acid-evoked p12-LO product formation was not sensitive to any of these inhibitors (Fig. 5B). Moreover, in control experiments, in which 12-H(P)ETE was elicited by the addition of exogenous arachidonic acid to circumvent the supply of endogenous

substrate, no or only minor effects of the inhibitors were observed, regardless of the presence of  $\text{Ca}^{2+}$  (Fig. 5B). Thus, the inhibitory effects of wortmannin, PP2, and SU6656 on  $\beta$ -boswellic acid-induced 12-H(P)ETE generation seem to primarily affect release of arachidonic acid rather than p12-LO activity. In conclusion,  $\beta$ -boswellic acid-evoked arachidonic acid release/12-H(P)ETE formation in the presence of  $\text{Ca}^{2+}$  seemingly involves PI3K and Src family kinases, whereas in  $\text{Ca}^{2+}$ -depleted cells, none of these signaling molecules apparently contribute.

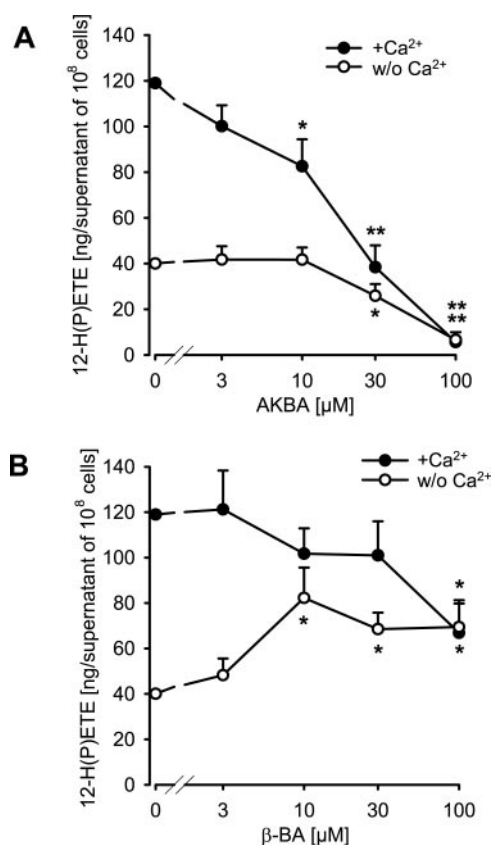
**Effects of Boswellic Acids on cPLA<sub>2</sub> and p12-LO Activity in Cell-Free Assays.** To test the stimulation of cPLA<sub>2</sub> by boswellic acids in vitro, we determined the effects of  $\beta$ -boswellic acid or AKBA on arachidonic acid release from platelet membrane lipids in the absence (inclusion of 1 mM EDTA) and in the presence of 2 mM  $\text{Ca}^{2+}$ . Arachidonic acid release was increased by  $\text{Ca}^{2+}$  by approximately 2.4-fold, and was suppressed by the cPLA<sub>2</sub> inhibitor, ensuring that cPLA<sub>2</sub> 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  $\mu\text{M}$  boswellic acid (data not shown), regardless of the presence of  $\text{Ca}^{2+}$ , implying that boswellic acids do not stimulate cPLA<sub>2</sub> activity in vitro. In addition, there was no increased association of cPLA<sub>2</sub> with platelet membranes after either incubation of platelet homogenates with boswellic acids or exposure of intact platelets to boswellic acids (assessed by Western



**Fig. 5.** Effects of relevant pharmacological inhibitors on  $\beta$ -boswellic acid-induced arachidonic acid release and 12-H(P)ETE formation. A, arachidonic acid release. Platelets were labeled with [ $^3\text{H}$ ]arachidonic acid as described in the legend to Fig. 2. After washing, cells were either left untreated (■) or 1 mM EDTA plus 30  $\mu\text{M}$  BAPTA/AM (□) was added. Then, cells were preincubated with 200 nM wortmannin (wortm), 3  $\mu\text{M}$  PP2 or PP3, 5  $\mu\text{M}$  SU6656, 3  $\mu\text{M}$  U0126, 10  $\mu\text{M}$  SB203580, or vehicle (DMSO, negative/positive) as indicated for 15 min at 37°C.  $\text{CaCl}_2$  (1 mM) was added to the cells as indicated, and after 2.5 min, cells were stimulated with 30  $\mu\text{M}$   $\beta$ -boswellic acid ( $\beta$ -BA). [ $^3\text{H}$ ]Arachidonic acid released into the medium was measured after 5 min (■) or 15 min (□), respectively. B, 12-H(P)ETE formation. Platelets were resuspended in 1 ml of PG buffer plus 1 mM  $\text{CaCl}_2$  (■) or in PG buffer containing 1 mM EDTA plus 30  $\mu\text{M}$  BAPTA/AM (□) and preincubated with 200 nM wortmannin (wortm), 3  $\mu\text{M}$  PP2 or PP3, 5  $\mu\text{M}$  SU6656, 3  $\mu\text{M}$  U0126, 10  $\mu\text{M}$  SB203580, or vehicle (DMSO) as indicated. Then, 30  $\mu\text{M}$   $\beta$ -boswellic acid ( $\beta$ -BA, left) or 10  $\mu\text{M}$  arachidonic acid (AA, right) was added and 12-H(P)ETE formation was determined. Data are expressed as a percentage of control (100%, vehicle), and values are given as mean  $\pm$  S.E.,  $n = 3$  to 4. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

blotting; data not shown), independent of the presence of  $\text{Ca}^{2+}$ , suggesting that boswellic acids do not promote binding of cPLA<sub>2</sub> to membrane phospholipids *in vitro*.

The effects of boswellic acids on p12-LO activity in the platelet 100,000g supernatant were investigated. Platelet 100,000g supernatant was incubated with AKBA plus 2  $\mu\text{M}$  arachidonic acid in the presence of either 1 mM EDTA or 1 mM  $\text{Ca}^{2+}$ . 12-H(P)ETE formation was approximately 3-fold higher in the presence of  $\text{Ca}^{2+}$  (Fig. 6, A and B). AKBA caused a concentration-dependent inhibition of p12-LO activity (Fig. 6A). In the presence of  $\text{Ca}^{2+}$ , the  $\text{IC}_{50}$  value was approximately 15  $\mu\text{M}$ , whereas without  $\text{Ca}^{2+}$ , the  $\text{IC}_{50}$  value was approximately 50  $\mu\text{M}$ . In contrast to AKBA, 12-H(P)ETE formation was differentially modulated by  $\beta$ -boswellic acid. Thus, only a weak inhibition of p12-LO activity by  $\beta$ -boswellic acid ( $\text{IC}_{50} > 100 \mu\text{M}$ ) was detectable in the presence of  $\text{Ca}^{2+}$  (Fig. 6B). However, in the absence of  $\text{Ca}^{2+}$ ,  $\beta$ -boswellic acid elevated 12-H(P)ETE up to approximately 2-fold at a threshold concentration of 10  $\mu\text{M}$  (Fig. 6B), which was sensitive to the p12-LO inhibitor CDC (data not shown). Together,  $\beta$ -boswellic acid stimulates the catalysis of crude p12-LO in the absence of  $\text{Ca}^{2+}$ , whereas AKBA generally suppresses the catalytic activity of p12-LO, and no direct modulation of cPLA<sub>2</sub> in cell-free assays is apparent for either boswellic acid.

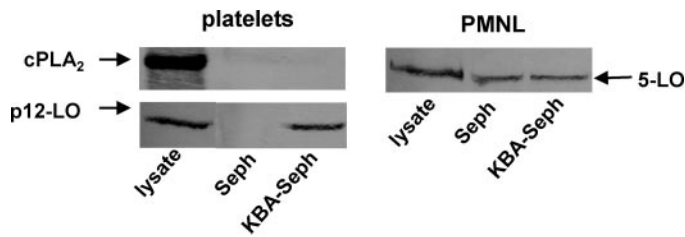


**Fig. 6.** Effects of boswellic acids on the activity of p12-LO in cell-free assays. Platelets were sonicated, and a 100,000g supernatant was prepared. AKBA (A) or  $\beta$ -boswellic acid ( $\beta$ -BA) (B) were added to the 100,000g supernatant at the indicated concentrations, and the synthesis of 12-H(P)ETE was started by the addition of arachidonic acid (2  $\mu\text{M}$ ) with or without 2 mM  $\text{CaCl}_2$ , as indicated. 12-H(P)ETE was determined by high-performance liquid chromatography. Data are given as mean  $\pm$  S.E.,  $n = 3$  to 5; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

**Interaction of Boswellic Acids with cPLA<sub>2</sub> and p12-LO.** To assess the direct interaction of boswellic acids with cPLA<sub>2</sub> 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-Sep). EAH-Sepharose beads without ligand (Seph) were used as control, and platelet 12,000g supernatants served as protein source. Coomassie-staining of gels after SDS-PAGE or Ponceau S staining of membranes after blotting ensured comparable unspecific protein-binding by Seph and KBA-Sep (data not shown). As shown in Fig. 7, no cPLA<sub>2</sub> protein was detectable (by Western-blotting analysis) in precipitates using Seph or KBA-Sep. cPLA<sub>2</sub> was abundantly present in the corresponding platelet lysates and clearly detectable. However, substantial amounts of p12-LO were present in KBA-Sep pull-downs but not in precipitates using Seph as negative control. Because 5-lipoxygenase was postulated as an AKBA-binding protein (Sailer et al., 1998), we attempted to confirm 5-lipoxygenase binding by our protein fishing strategy using 12,000g supernatants of PMNL as a source for 5-lipoxygenase. Both Seph and KBA-Sep 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 cPLA<sub>2</sub>, connected to subsequent conversion by cyclooxygenase-1 and p12-LO, depending on the strength of the stimuli and the nature of the signaling molecules involved (Hamberg and Samuelsson, 1974; Holmsen, 1994; Coffey et al., 2004).  $\text{Ca}^{2+}$  is a determinant for these processes because it stimulates cellular activation and catalysis of both cPLA<sub>2</sub> (Gijon and Leslie, 1999; Leslie, 2004) and p12-LO (Baba et al., 1989). Besides  $\text{Ca}^{2+}$ , 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 cPLA<sub>2</sub>. 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  $\text{Ca}^{2+}$  is known as a (moderate) stimulatory cofactor (Baba et al., 1989). It is assumed that the capacity of platelets to form 12-H(P)ETE is essentially



**Fig. 7.** AKBA selectively binds p12-LO; 12,000g supernatants of platelet lysates (for precipitation of cPLA<sub>2</sub> and p12-LO) or of PMNL lysates (for 5-lipoxygenase) were incubated overnight at 4°C with either KBA-Sep 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 cPLA<sub>2</sub>, 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.



linked to the supply of arachidonic acid. Because boswellic acids induce massive mobilization of  $\text{Ca}^{2+}$  and activate MAPK in platelets (Poeckel et al., 2005), it was reasonable that boswellic acids as a result may elicit the release of arachidonic acid and concomitantly 12-H(P)ETE synthesis.

$\beta$ -Boswellic acid and AKBA evoked arachidonic acid release with comparable potencies, similar to those of the strong platelet agonists thrombin or ionophore that act by recruiting cPLA<sub>2</sub> via phosphorylation and/or elevation of  $[\text{Ca}^{2+}]_i$  (Borsch-Haubold et al., 1995; Kramer et al., 1996). The liberation of arachidonic acid was rapid and sensitive to selective inhibitors of the  $\text{Ca}^{2+}$ -dependent cPLA<sub>2</sub>, suggesting that in analogy to thrombin and ionophore, cPLA<sub>2</sub> is the responsible PLA<sub>2</sub> isoform. However, in contrast to thrombin and ionophore, boswellic acids may induce cPLA<sub>2</sub> activation, at least in part, independent of  $\text{Ca}^{2+}$ . Note that bromoenol lactone did not compromise arachidonic acid release in the absence of  $\text{Ca}^{2+}$ , which excludes the  $\text{Ca}^{2+}$ -independent PLA<sub>2</sub> (Hazen et al., 1991) as a responsible enzyme. In addition, the determination of 12-H(P)ETE shows that boswellic acids but not thrombin partially act in a  $\text{Ca}^{2+}$ -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 favors an additional  $\text{Ca}^{2+}$ -independent cPLA<sub>2</sub>/p12-LO activation pathway. Thus, 12-H(P)ETE formation induced by  $\beta$ -boswellic acid was much more rapid than by thrombin, although increases in  $[\text{Ca}^{2+}]_i$  by  $\beta$ -boswellic acid are delayed compared with thrombin (Poeckel et al., 2005). Therefore, it is unlikely that the rapid and robust 12-H(P)ETE synthesis induced by  $\beta$ -boswellic acid is mediated solely by the elevation of  $[\text{Ca}^{2+}]_i$ .

The  $\text{Ca}^{2+}$  dependence of cPLA<sub>2</sub> in platelets is well established, but alternate signaling routes such as phosphorylation by MAPKs contribute (Borsch-Haubold et al., 1995, 1999; Kramer et al., 1996). In fact, boswellic acids activate MAPK in platelets (Poeckel et al., 2005); however, MAPK inhibitors failed to suppress  $\beta$ -boswellic acid-induced arachidonic acid release and 12-H(P)ETE synthesis. Nevertheless, our inhibitor approach indicates that PI3K and Src family kinases may be integrated in  $\beta$ -boswellic acid-evoked responses, at least under conditions in which  $\text{Ca}^{2+}$  is present. Because Src family kinases and PI3K are also involved in  $\beta$ -boswellic acid-induced  $\text{Ca}^{2+}$  mobilization (Poeckel et al., 2005), the suppressive effects of the respective inhibitors are likely to be due to inhibition of  $\text{Ca}^{2+}$  mobilization rather than uncoupling  $\text{Ca}^{2+}$ -independent signals to cPLA<sub>2</sub>. This is supported by the fact that the inhibitors completely failed to suppress  $\beta$ -boswellic acid-induced responses in the absence of  $\text{Ca}^{2+}$ . 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. PI3K 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).

Apart from  $\text{Ca}^{2+}$  and phosphorylation, cPLA<sub>2</sub> 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 seemed possible that also boswellic acids could activate cPLA<sub>2</sub> by direct interactions.

However, cPLA<sub>2</sub> failed to bind KBA-Seph, and boswellic acids did not stimulate cPLA<sub>2</sub> activity in cell-free assays, excluding such interrelations. We conclude that, collectively, boswellic acids activate cPLA<sub>2</sub> independent of  $\text{Ca}^{2+}$  and phosphorylation by a yet-unrecognized mechanism.

Boswellic acids were initially identified as inhibitors of 5-lipoxygenase (Safayhi et al., 1992, 1995) that may interfere with a regulatory arachidonic acid-binding site in a  $\text{Ca}^{2+}$ -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  $\text{IC}_{50}$  value (15  $\mu\text{M}$ ) significantly lower than the values determined for 5-lipoxygenase under comparable assay conditions (50  $\mu\text{M}$ ) (Werz et al., 1997, 1998). It is interesting that 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 12,000g supernatants of PMNL lysates were approximately the same, implying unspecific binding of 5-lipoxygenase to KBA-Seph.

Direct suppression of p12-LO activity by AKBA may explain why despite the induction of marked arachidonic acid release in intact platelets, no subsequent conversion to 12-H(P)ETE was evident, whereas  $\beta$ -boswellic acid (or A $\beta$ -BA) concentration-dependently induced 12-H(P)ETE formation. In agreement with others (Baba et al., 1989),  $\text{Ca}^{2+}$  increased p12-LO activity in platelet 100,000g supernatants approximately 3-fold, and  $\beta$ -boswellic acid mimicked this effect because it stimulated p12-LO activity without  $\text{Ca}^{2+}$ . In contrast, in the presence of  $\text{Ca}^{2+}$ ,  $\beta$ -boswellic acid did not further stimulate p12-LO. The 11-keto moiety seems to determine 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, 2006).

The conclusions from our results deviate from the long-established view of boswellic acids as negative modulatory agents of the arachidonic acid cascade, because we demonstrate strong induction of arachidonic acid release and formation of 12-H(P)ETE by boswellic acids in platelets. In addition, we suggest p12-LO as a definite target of boswellic acids with superior susceptibility compared with 5-lipoxygenase. The question of the pharmacological consequence resulting from the divergent effects of  $\beta$ -boswellic acid and AKBA on 12-H(P)ETE biosynthesis in vivo remains to be answered. After oral intake of  $4 \times 786$  mg of *B. serrata* extracts (containing approximately 3.7% AKBA, 10.5% A $\beta$ -BA, 6.1% KBA, and 18.2%  $\beta$ -boswellic acid) per day, the plasma levels of AKBA (0.1  $\mu\text{M}$ ) (Buchele and Simmet, 2003) are far lower than the concentrations required to efficiently suppress p12-LO ( $\text{IC}_{50} = 15$   $\mu\text{M}$ ). On the other hand,  $\beta$ -boswellic acid reached plasma levels (10.1  $\mu\text{M}$ ), virtually sufficient to induce 12-H(P)ETE formation. In our in vitro assays, relevant amounts (approximately 5  $\mu\text{g}/\text{ml}$ ) of *B. serrata* extracts, containing diverse boswellic acids, strongly induced arachidonic acid release and 12-H(P)ETE synthesis (data not shown). 12-H(P)ETE may act as a chemoattractant for leukocytes (Goetzl, 1980), mediate angiogenesis and tumor metastasis (Honn et al., 1994), possess inhibitory neuromodulatory effects (Piomelli et al., 1987), and be 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 *B. serrata*, it also remains a future challenge to fully elucidate the  $\text{Ca}^{2+}$ /phosphorylation-independent signaling routes, leading to cPLA<sub>2</sub> activation and increased release of arachidonic acid by boswellic acids.

## Acknowledgments

We thank Sven George for expert technical assistance.

## References

- Albert D, Zundorf I, Dingermann T, Muller WE, Steinhilber D, and Werz O (2002) Hyperforin is a dual inhibitor of cyclooxygenase-1 and 5-lipoxygenase. *Biochem Pharmacol* **64**:1767–1775.
- Altmann A, Fischer L, Schubert-Zsilavecz M, Steinhilber D, and Werz O (2002) Boswellic acids activate p42 (MAPK) and p38 MAPK and stimulate  $\text{Ca}^{2+}$  mobilization. *Biochem Biophys Res Commun* **290**:185–190.
- Altmann A, Poeckel D, Fischer L, Schubert-Zsilavecz M, Steinhilber D, and Werz O (2004) Coupling of boswellic acid-induced  $\text{Ca}^{2+}$  mobilisation and MAPK activation to lipid metabolism and peroxide formation in human leucocytes. *Br J Pharmacol* **141**:223–232.
- Anthoni C, Laukoetter MG, Rijcken E, Vowinkel T, Mennigen R, Muller S, Senninger N, Russell J, Jauch J, Bergmann J, et al. (2006) Mechanisms underlying the anti-inflammatory actions of boswellic acid derivatives in experimental colitis. *Am J Physiol* **290**:G1131–G1137.
- Baba A, Sakuma S, Okamoto H, Inoue T, and Iwata H (1989) Calcium induces membrane translocation of 12-lipoxygenase in rat platelets. *J Biol Chem* **264**:15790–15795.
- Balsinde J, Balboa MA, Li WH, Llopis J, and Dennis EA (2000) Cellular regulation of cytosolic group IV phospholipase A2 by phosphatidylinositol bisphosphate levels. *J Immunol* **164**:5398–5402.
- Borsch-Haubold AG, Ghomashchi F, Pasquet S, Goedert M, Cohen P, Gelb MH, and Watson SP (1999) Phosphorylation of cytosolic phospholipase A2 in platelets is mediated by multiple stress-activated protein kinase pathways. *Eur J Biochem* **265**:195–203.
- Borsch-Haubold AG, Kramer RM, and Watson SP (1995) Cytosolic phospholipase A2 is phosphorylated in collagen- and thrombin-stimulated human platelets independent of protein kinase C and mitogen-activated protein kinase. *J Biol Chem* **270**:25885–25892.
- Bryant RW, Simon TC, and Bailey JM (1982) Role of glutathione peroxidase and hexose monophosphate shunt in the platelet lipoxygenase pathway. *J Biol Chem* **257**:14937–14942.
- Buchele B and Simmet T (2003) Analysis of 12 different pentacyclic triterpenic acids from frankincense in human plasma by high-performance liquid chromatography and photodiode array detection. *J Chromatogr B Analyt Technol Biomed Life Sci* **795**:355–362.
- Coffey MJ, Jarvis GE, Gibbins JM, Coles B, Barrett NE, Wylie OR, and O'Donnell VB (2004) Platelet 12-lipoxygenase activation via glycoprotein VI: involvement of multiple signaling pathways in agonist control of H(P)ETE synthesis. *Circ Res* **94**:1598–1605.
- Gijon MA and Leslie CC (1999) Regulation of arachidonic acid release and cytosolic phospholipase A2 activation. *J Leukoc Biol* **65**:330–336.
- Goetzl EJ (1980) Mediators of immediate hypersensitivity derived from arachidonic acid. *N Engl J Med* **303**:822–825.
- Gonzalez-Nunez D, Claria J, Rivera F, and Poch E (2001) Increased levels of 12(S)-HETE in patients with essential hypertension. *Hypertension* **37**:334–338.
- Hamberg M and Samuelsson B (1974) Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. *Proc Natl Acad Sci USA* **71**:3400–3404.
- Hazen SL, Zupan LA, Weiss RH, Getman DP, and Gross RW (1991) Suicide inhibition of canine myocardial cytosolic calcium-independent phospholipase A2. Mechanism-based discrimination between calcium-dependent and -independent phospholipases A2. *J Biol Chem* **266**:7227–7232.
- Holmsen H (1994) Significance of testing platelet functions in vitro. *Eur J Clin Invest* **24**:3–8.
- Honn KV, Tang DG, Gao X, Butovich IA, Liu B, Timar J, and Hagmann W (1994) 12-lipoxygenases and 12(S)-HETE: role in cancer metastasis. *Cancer Metastasis Rev* **13**:365–396.
- Huwyler A, Johansen B, Skarstad A, and Pfeilschifter J (2001) Ceramide binds to the CaLB domain of cytosolic phospholipase A2 and facilitates its membrane docking and arachidonic acid release. *FASEB J* **15**:7–9.
- Jauch J and Bergmann J (2003) An efficient method for the large-scale preparation of 3-O-acetyl-11-oxo-beta-boswellic acid and other boswellic acids. *Eur J Org Chem* **4752**–4756.
- Kramer RM, Roberts EF, Um SL, Borsch-Haubold AG, Watson SP, Fisher MJ, and Jakubowski JA (1996) p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A2 (cPLA2) in thrombin-stimulated platelets. Evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by cPLA2. *J Biol Chem* **271**:27723–27729.
- Leslie CC (2004) Regulation of the specific release of arachidonic acid by cytosolic phospholipase A2. *Prostaglandins Leukot Essent Fatty Acids* **70**:373–376.
- Pettus BJ, Bielawska A, Subramanian P, Wijesinghe DS, Maceyka M, Leslie CC, Evans JH, Freiberg J, Roddy P, Hannun YA, et al. (2004) Ceramide 1-phosphate is a direct activator of cytosolic phospholipase A2. *J Biol Chem* **279**:11320–11326.
- Piomelli D, Volterra A, Dale N, Siegelbaum SA, Kandel ER, Schwartz JH, and Belardetti F (1987) Lipoxygenase metabolites of arachidonic acid as second messengers for presynaptic inhibition of Aplysia sensory cells. *Nature (Lond)* **328**:38–43.
- Poeckel D, Tausch L, Altmann A, Feisst C, Klinkhardt U, Graff J, Harder S, and Werz O (2005) Induction of central signalling pathways and select functional effects in human platelets by beta-boswellic acid. *Br J Pharmacol* **146**:514–524.
- Poeckel D, Tausch L, George S, Jauch J, and Werz O (2006) 3-O-Acetyl-11-keto-boswellic acid decreases basal intracellular  $\text{Ca}^{2+}$  levels and inhibits agonist-induced  $\text{Ca}^{2+}$  mobilization and mitogen-activated protein kinase activation in human monocytic cells. *J Pharmacol Exp Ther* **316**:224–232.
- Safayhi H, Boden SE, Schweizer S, and Ammon HP (2000) Concentration-dependent potentiating and inhibitory effects of Boswellia extracts on 5-lipoxygenase product formation in stimulated PMNL. *Planta Med* **66**:110–113.
- Safayhi H, Mack T, Sabieraj J, Anazodo MI, Subramanian LR, and Ammon HP (1992) Boswellic acids: novel, specific, nonredox inhibitors of 5-lipoxygenase. *J Pharmacol Exp Ther* **261**:1143–1146.
- Safayhi H, Rall B, Sailer ER, and Ammon HP (1997) Inhibition by boswellic acids of human leukocyte elastase. *J Pharmacol Exp Ther* **281**:460–463.
- Safayhi H, Sailer ER, and Ammon HP (1995) Mechanism of 5-lipoxygenase inhibition by acetyl-11-keto-beta-boswellic acid. *Mol Pharmacol* **47**:1212–1216.
- Sailer ER, Schweizer S, Boden SE, Ammon HP, and Safayhi H (1998) Characterization of an acetyl-11-keto-beta-boswellic acid and arachidonate-binding regulatory site of 5-lipoxygenase using photoaffinity labeling. *Eur J Biochem* **256**:364–368.
- Six DA and Dennis EA (2000) The expanding superfamily of phospholipase A<sub>2</sub> enzymes: classification and characterization. *Biochim Biophys Acta* **1488**:1–19.
- Subramanian P, Stahelin RV, Szulc Z, Bielawska A, Cho W, and Chalfant CE (2005) Ceramide 1-phosphate acts as a positive allosteric activator of group IVA cytosolic phospholipase A2α and enhances the interaction of the enzyme with phosphatidylcholine. *J Biol Chem* **280**:17601–17607.
- Syrovetz T, Buchele B, Gedig E, Slupsky JR, and Simmet T (2000) Acetyl-boswellic acids are novel catalytic inhibitors of human topoisomerases I and IIα. *Mol Pharmacol* **58**:71–81.
- Syrovetz T, Buchele B, Krauss C, Laumonnier Y, and Simmet T (2005a) Acetyl-boswellic acids inhibit lipopolysaccharide-mediated TNF-α induction in monocytes by direct interaction with IκB kinases. *J Immunol* **174**:498–506.
- Syrovetz T, Gschwend JE, Buchele B, Laumonnier Y, Zugmaier W, Genze F, and Simmet T (2005b) Inhibition of IκB kinase activity by acetyl-boswellic acids promotes apoptosis in androgen-independent PC-3 prostate cancer cells in vitro and in vivo. *J Biol Chem* **280**:6170–6180.
- Werz O, Schneider N, Brungs M, Sailer ER, Safayhi H, Ammon HPT, and Steinhilber D (1997) A test system for leukotriene synthesis inhibitors based on the in-vitro differentiation of the human leukemic cell lines HL-60 and Mono Mac 6. *Naunyn-Schmiedeberg's Arch Pharmacol* **356**:441–445.
- Werz O, Szellas D, Henseler M, and Steinhilber D (1998) Nonredox 5-lipoxygenase inhibitors require glutathione peroxidase for efficient inhibition of 5-lipoxygenase activity. *Mol Pharmacol* **54**:445–451.
- Winking M, Sarikaya S, Rahmani A, Jodicke A, and Boker DK (2000) Boswellic acids inhibit glioma growth: a new treatment option? *J Neurooncol* **46**:97–103.
- Yoshimoto T and Takahashi Y (2002) Arachidonate 12-lipoxygenases. *Prostaglandins Other Lipid Mediat* **68–69**:245–262.

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