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Ginger Phenylpropanoids Inhibit IL-1β and Prostanoid Secretion and Disrupt Arachidonate-Phospholipid Remodeling by Targeting Phospholipases A2

Andreas Nievergelt,* Janine Marazzi,* Roland Schoop,† Karl-Heinz Altmann,‡ and Jürg Gertsch*

The rhizome of ginger (Zingiber officinale) is employed in Asian traditional medicine to treat mild forms of rheumatoid arthritis and fever. We have profiled ginger constituents for robust effects on proinflammatory signaling and cytokine expression in a validated assay using human whole blood. Independent of the stimulus used (LPS, PMA, anti-CD28 Ab, anti-CD3 Ab, and thapsigargin), ginger constituents potently and specifically inhibited IL-1β expression in monocytes/macrophages. Both the calcium-independent phospholipase A2 (iPLA2)-triggered maturation and the cytosolic phospholipase A2 (cPLA2)-dependent secretion of IL-1β from isolated human monocytes were inhibited. In a fluorescence-coupled PLA2 assay, most major ginger phenylpropanoids directly inhibited i/cPLA2 from U937 macrophages, but not hog pancreas secretory phospholipase A2. The effects of the ginger constituents were additive and the potency comparable to the mechanism-based inhibitor bromoeno lactone for iPLA2 and methyl arachidonyl fluorophosphonate for cPLA2, with 10-gingerol/-shogaol being most effective. Furthermore, a ginger extract (2 μg/ml) and 10-shogaol (2 μM) potently inhibited the release of PGE2 and thromboxane B2 (>50%) and partially also leukotriene B4 in LPS-stimulated macrophages. Intriguingly, the total cellular arachidonic acid was increased 2- to 3-fold in U937 cells under all experimental conditions. Our data show that the concurrent inhibition of iPLA2 and prostanoid production causes an accumulation of free intracellular arachidonic acid by disrupting the phospholipid deacylation-reacylation cycle. The inhibition of i/cPLA2, the resulting attenuation of IL-1β secretion, and the simultaneous inhibition of prostanoid production by common ginger phenylpropanoids uncover a new anti-inflammatory molecular mechanism of dietary ginger that may be exploited therapeutically. The Journal of Immunology, 2011, 187: 000–000.

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Abbreviations used in this article: AA, arachidonic acid; BEL, bromoeno lactone; CBA, cytometric bead array; COX, cyclooxygenase; cPLA2, cytosolic phospholipase A2; DBA, 2,4′-dibromoacetophenone; iPLA2, calcium-independent phospholipase A2; MAFP, methyl arachidonoyl fluorophosphonate; NMR, nuclear magnetic resonance; PAK, palmitoyl-6-O-acetate potassium salt; PEA, palmitoylethanolamide; PLA2, phospholipase A2; RT, room temperature; sPLA2, secretory PLA2; TXB2, thromboxane B2.

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phospholipids, thus liberating lysophospholipids and free fatty acids, which are precursors for a variety of different mediators of physiological and pathological processes (20, 21). PL\(\alpha\) iso-enzymes are not only involved in the generation of free arachidonic acid (AA), which is metabolized by cyclo- and lipoxygenases to eicosanoids (22), but also in the incorporation of free AA into membranes and redistribution into specific compartment (remodeling) (23, 24). Besides regulatory functions in lipid catabolism/metabolism, PL\(\alpha\) enzymes are critically involved in signal transduction, phospholipid and cell membrane bilayer remodeling (25), store-operated cation channels and Ca\(^{2+}\) release-activated Ca\(^{2+}\) channels (26), Fas-induced apoptosis (27), cellular proliferation and differentiation (28, 29), glucocorticoid and insulin secretion (30), and, most important for this study, they play a central role in acute (18) and chronic inflammation (31).

Both iPL\(\alpha\) and ePL\(\alpha\) are crucial for IL-1\(\beta\) maturation and secretion (membrane translocation and fusion of storage vesicles) (32, 33). IL-1\(\beta\) is mainly secreted by monocytes/macrophages and dendritic cells, but also fibroblasts; it is involved in a variety of inflammatory processes, such as costimulus for T cell activation; and is one of the key players in rheumatoid arthritis and inflammatory bowel disease (34, 35). IL-1\(\beta\) further induces COX-2 expression in endothelial cells and is involved in edema formation, where it synergizes with PGE\(_2\) (36). IL-1\(\beta\) is also a major mediator of fever induction (34, 37). In this study, we provide evidence that the inhibition of i/cPL\(\alpha\) by phenylpropanoids is directly linked to the global inhibition of IL-1\(\beta\) secretion by ginger extracts in vitro. Moreover, ginger phenylpropanoids induce dramatic changes in arachidonate-phospholipid remodeling, due to their simultaneous inhibition of iPL\(\alpha\)\(_3\) and prostanoid synthesis. This apparently robust mechanism of action could provide a rational basis for the reported antipyretic and anti-inflammatory (i.e., immunomodulatory) effects of ginger preparations.

**Materials and Methods**

**Chemicals and reagents**

Ethanol 99\%, KOH, NaCl, D- (+)-glucose, EDTA, LPS from *Escherichia coli*, ATP, PMA from Euphorbiaceae, BSA fraction V (BSA), and 2,4’-dibromoacetophenone (DBA) were obtained from Fluka Chemie AG (Buchs, Switzerland). DMEM, HEPES, MOPS, chloroform-d, monobromobiane, cyclosporin A, aspirin, diclofenac sodium salt, cetirizine, curcumin, parthenolide, fMLP, and pamitoylthio-2-deoxy-sn-glycero-3-phosphorylcholine were purchased from Sigma-Aldrich. All enzymes were from Euphorbiaceae, BSA fraction V (BSA), and 2,4’-dibromoacetophenone (DBA) were obtained from Fluka Chemie AG (Buchs, Switzerland). DMEM, HEPES, MOPS, chloroform-d, monobromobiane, cyclosporin A, aspirin, diclofenac sodium salt, cetirizine, curcumin, parthenolide, fMLP, and pamitoylthio-2-deoxy-sn-glycero-3-phosphorylcholine were purchased from Sigma-Aldrich.

**Whole blood, assay conditions, and purification of immune cells**

Human venous whole blood was drawn from healthy volunteers with the BD vacutainer system. Isolation of PBMCs was conducted in accordance with the guidelines of the World Medical Association’s Declaration of Helsinki and approved by ETH Zurich. PBMCs were isolated with Opti-Prep (Axis-Shield, Oslo, Norway) density gradient flotation and CD14\(^+\) monocytes were enriched by adhesion to culture flasks (FACS analysis was used to check for purity). Whole blood was immediately pipetted in a 96-well plate at 200 \(\mu\)l/well. Ethanol extracts, pure compounds, or ethanol as vehicle control were added at different concentrations with a maximal concentration of 0.5% ethanol and incubated for 30 min at 37\(^\circ\)C. Where indicated, stimuli (LPS [312 ng/ml]) or a combination of PMA (15 ng/ml) and thapsigargin (1 \(\mu\)M), anti-CD3 Ab, or anti-CD28 Ab [each 1 \(\mu\)g/ml] were added, and blood was incubated for another 18 h at 37\(^\circ\)C. For cytokine measurements, the plates were centrifuged, and an appropriate amount (usually 50 \(\mu\)l) of the supernatant serum was removed for immediate analysis. Isolation of PBMCs and T lymphocytes from peripheral whole blood was performed, as previously reported (39).

**Cell cultures.** U937 cells (CRL-1593.2) (40) obtained from American Type Culture Collection and isolated human monocytes/macrophages were cultivated in RPMI 1640 medium containing penicillin and streptomycin (100 \(\mu\)g/ml), 2 mM glutamate (all from Life Technologies Inviron, Basel, Switzerland), amphotericin B (2 \(\mu\)g/ml; Sigma-Aldrich, Steinheim, Germany), and 10% FCS (heat inactivated for 30 min at 56\(^\circ\)C) at 37\(^\circ\)C, 5% CO\(_2\), and humidified atmosphere. In the experiments measuring AA and eicosanoids, the cells were washed and incubated in serum-free medium (see below).

**Cytokine measurements** 

Cytokine quantification in whole blood was done with BD human inflammatory cytokine kit (BD kit 551811) for IL-8, IL-1\(\beta\), IL-6, IL-10, TNF-\(\alpha\), IL-12p70, and the BD CBA human allay mediators kit (BD kit 558022) for IL-3, IL-4, IL-5, IL-7, IL-10, and GM-CSF, or with isolated monocytes using the BD human IL-1\(\beta\) Flex Set (538279), according to the manufacturer’s manuals, measured with a FACScan flow cytometer equipped with an argon laser, and evaluated with the CBA software V1.4 (all by BD Biosciences).

**ELISA measurements of thromboxane B\(_2\) and leukotriene B\(_4\)** 

The thromboxane B\(_2\) (TXB\(_2\)) ELISA colorimetric kit was from Cayman Chemicals (10004023), limit of detection 35 pg/ml, and leukotriene B\(_4\) (LTB\(_4\)) ELISA colorimetric kit (EHLT4B) was from Thermo Scientific (Pierce Protein Research Products), limit of detection 20 pg/ml. ELISA measurements were carried out as specified by the manufacturer’s instructions. A total of 2 \(\times\) 10\(^5\) U937 macrophages was stimulated with LPS (1 \(\mu\)g/ml) to measure TXB\(_2\) and 10 \(\times\) 10\(^5\) primary CD14\(^+\) monocytes/macrophages to measure LTB\(_4\). Cells were preincubated with DMSO (vehicle) or test compounds for 30 min prior to stimulation with LPS (1 \(\mu\)g/ml) and for full LTB\(_4\) release costimulation with fMLP (1 \(\mu\)M). LTB\(_4\) could not be measured from stimulated U937 macrophages.

**Western blots**

Gels for Western blots were run on denaturing NuPage Novex 4–12% Bis-Tris precast gels and corresponding MOPS buffer from Invitrogen, duskov, Basel, Switzerland), amphotericin B (2 \(\mu\)g/ml; Sigma-Aldrich, Steinheim, Germany), and 10% FCS (heat inactivated for 30 min at 56\(^\circ\)C) at 37\(^\circ\)C, 5% CO\(_2\), and humidified atmosphere. In the experiments measuring AA and eicosanoids, the cells were washed and incubated in serum-free medium (see below).

**Gels for Western blots were run on denaturing NuPage Novex 4–12% Bis-Tris precast gels and corresponding MOPS buffer from Invitrogen, according to the manufacturer’s recommendations, using 10 \(\mu\)l samples dissolved in LDS blower. Blotting was done on nitrocellulose membranes in a NuPage blotting chamber, according to the instruction manual.

**Staining** 

Staining was done according to the ECL manual from PerkinElmer using TBST (pH 7.6 at used temperatures) and nitrocellulose membranes blocked with TBST plus 5% defatted milk powder. Ab labeling was done with TBST, 1% defatted milk powder and primary Ab (anti-IL-1\(\beta\); Abnova MaxPab and Sigma-Aldrich) were used at 2 \(\mu\)l/ml; and secondary Ab (anti-mouse IgG [goat] HRP labeled by GE Healthcare) at 1/8 \(\mu\)l/ml. Chemiluminescence detection was done with ECL Plus Western blotting detect. (Amersham Biosciences by GE Healthcare). For IL-1\(\beta\) detection with Western blot analysis, isolated monocytes, in 96-well plates at a density of 2 \(\times\) 10\(^5\) cells/ml, and 100 \(\mu\)l/well, were stimulated in fresh RPMI 1640 with 100 ng/ml LPS for 4 h. Medium was changed after LPS priming to a gluconate basal salt solution described earlier (35), omitting glucose. Further processing was in analogy to the CBA (BD Biosciences) protocol, but lipopolisates were directly dissolved in 10 \(\mu\l\) LBS sample buffer for gel electrophoreses.

**Ehudiium bromide uptake to measure PZK-mediated ion fluxes**

Ehudiium\(^+\) uptake was measured in isolated CD14\(^+\) human monocytes (according to 41), but without measuring additional cell markers. Primary
human cells were used for this assay because they gave a better signal-to-noise ratio than U937 cells.

Measurement of IL-1β maturation and secretion

Cytometric bead arrays. For IL-1β detection with CBA, the isolated monocytes, in 96-well plates at a density of 2 × 10⁶ cells/ml and 100 µl well, were stimulated in fresh RPMI 1640 for 4 h under different priming conditions (vehicle, 100 ng/ml LPS and/or 10 µg/ml gentamic acid). Cells were spun down for 5 min at 200 × g, and medium was replaced to be processed as described below. With 100 µl fresh one containing different stimuli (vehicle, 1 mM ATP and/or 10 µg/ml gentamic acid), then incubated for 30 min and centrifuged for 5 min at 200 × g, and supernatant was removed. Medium samples and cells were shock frozen and lyophilized, to be stored at −80°C until measurement. Lyophilisates were reconstituted in 10 µl hypotone buffer (10 mM HEPES, 0.3 mM EGTA, 0.1% Triton X-100, adjusted with KOH to pH 7.4 at RT), and IL-1β was quantified with IL-1β BD FlexSet.

Establishment of PLA2 assay

Phospholipase preparation. U-937 cells (10⁶ cells/ml) were kept in culture medium, according to the recommendations by American Type Culture Collection. Two to three hours before metabolic processing, cells were stimulated with 35 ng/ml PMA, according to published protocols (42, 43). Two times 2.8 × 10⁶ U-937 cells were lysed in a hypotone lysis buffer (340 mM sucrose, 10 mM HEPES, 1 mM EDTA, 1 mM DTT, pH adjusted with KOH to 7.4 at 4°C) and centrifuged at 1000 × g for 30 min at 4°C. The pellets were kept as whole membrane preparations because cPLA2 does colocalize with cell membranes. Supernatants were ultrafiltrated with a 50-kDa molecular mass cut-off, resulting in lysate concentrates of 0.625–1.5 mg protein/ml for the U-937 (quantified with the reducing agent and detergent-compatible protein assay by Bio-Rad).

Substrate synthesis and isolation procedure. Palmitoyl-6-o-acidic ascorbic acid was synthesized (according to 44). Reaction conditions were as follows: 35°C was the reaction temperature; 8.8 ml 95% sulphuric acid, 2.29 g palmitic acid, and 1.76 g acetic acid; 100% ultrasonic power output with stirring rate of 150 rpm was confirmed by electron spray ionization mass spectrometry and two-dimensional nuclear magnetic resonance (NMR). Palmitoyl-6-o-acidic acid sodium salt (PAA) was generated before use by mixing appropriate solutions of palmitoyl-6-o-acidic acid in chloroform with KOH in methanol to result in a 100 mM mixture of 95:5 chloroform to methanol. Crude phosphatidylcholine was isolated from commercial grade lecithin (Hänseler AG) by quantitative de-oiling with acetone, enrichment of phosphatidylcholine by quantitative extraction with boiling ethanol, and finally, precipitation of impurities at 0°C. Crude phosphatidylcholine was isolated from medium, according to the recommendations by American Type Culture Collection. Establishing of PLA2 assay

Fluorescence detection and HPLC. Solutions were mixed at room temperature (RT), heated to 40°C (giving a clear solution), and again incubated at RT under gentle shaking. The enzymatic reaction was stopped after 3 h (≥50% hydrolysis) with 16.6 µl ice-cold MeOH. A total of 0.2 µl DT (100 mM aq. sol., for reduction of dithio-lyso-phosphatidylcholine), 0.2 µl CsClEDTA (100 mM aqueous solution, to chelate free calcium, which otherwise would interfere with the reaction), and 2 µl Cs₂CO₃ (200 mM aq. sol., to achieve a pH >8) was added and incubated for 1 h at RT. Then 20 µl monobromobimane (10 mM stock in MeOH) was added and incubated for 1 h at RT. The reaction was stopped and stabilized by acidification to 1% trichloroacetic acid (2.2 M aqueous solution, to achieve a pH <3) and centrifuged at 500 × g for 5 min. The supernatant could be stored with no detectable decomposition for 3 d at −18°C until HPLC measurement. Analytical HPLC was performed on an Elite LaChrome device (VWR International, Hitachi) equipped with a fluorescence detector using a Nucleodur Sphären column (4 µm particle size, 4.6 × 100-mm diameter). A Macherey-Nagel column (5 µm particle size, 9 × 250-mm diameter) was used for preparative HPLC. Detection was in the wavelength range of 200 to 350 nm (LiChrosolv; Merck) from 30:70 to 10:90 was run for analysis, and UV detection at λₑm 385 nm and λₑm 485 nm was carried out.

The enzyme activity was calculated from the area under the curve in absolute values (nmol product total) and (nmol product/min/mg protein).

TLC. TLC was done with a solvent mixture (according to 47). For detection of phosphate-containing lipids, a molybdenum blue spray solution and charring were used.

Phospho-MAPKs

MAPK phosphorylation in leukocytes was quantified using BD FlexSets, according to the manufacturer’s recommendations, but with 5 times smaller volumes.

Isolated human lymphocytes were adjusted to 4 × 10⁶ cells/ml and aliquoted at 50 µl into 96-well plates. The 50 µl diluted stimuli were added as 2X stock and incubated at 37°C for the indicated time. Conditions for p38 phosphorylation were 10 µl/ml anti-CD3 Ab and 2 mM PMA for 20 min; for ERK1/2 phosphorylation, 10 nM PMA for 20 min; and for JNK1/2 phosphorylation, 10 µl/ml anti-CD3 Ab and 10 nM PMA for 60 min. Subsequently, samples were cooled on ice and centrifuged at 300 × g for 5 min at 4°C. The pellets were lysed with 5 µl diluted BD denaturation buffer containing phosphatase inhibitors (4 mM sodium potassium tartrate, 2 mM imidazole, 1.15 mM sodium molybdate, 1 mM sodium fluoride, and 1 mM apyrase). A 15-min gradient of 0.25% NH₄H₂O₄ in water-acetonitril (LiChrosolv; Merck) from 30:70 to 10:90 was run for analysis, and UV detection at λₑm 385 nm and λₑm 485 nm was carried out.

For in process controls for palmitoyl-6-o-acidic acid the scooting was used, and sample mixture was analyzed on a zetasizer, giving 3.6 nm by number (area of 99.3%), and 27.9 nm by volume (area of 99.7%) and 6.25 nm by number 3.25. Estimated by density were 30% water with 0.1% formic acid and 70% acetonitrile; 20 min volumes.

Quantification of lipids (AA, PGE2, palmitoylethanolamide) by gas chromatography/mass spectrometry

Initial fraction containing 0.625 µl BrdU stock solution and 2 µl anti-CD3 Ab per ml or 50 µl RPMI 1640 medium containing vehicle control or test compounds prior to incubation for 5 d.

Proliferation was determined with the BD FITC BrdU Flow Kit by FACS, according to the recommendations by the manufacturer (48).

A clear correlation between the proliferation rate and residual CD14 monocytes could be observed and is in agreement with earlier reports (49, 50).

Characterization of compounds

Nuclear magnetic resonance. Palmitoyl-6-o-acetate was verified by two-dimensional NMR done on a Bruker 400 UltraShield and Bruker TOPSPIN 1.3 software.

Mass spectrometry. For in process controls for palmitoyl-6-o-acetate synthesis and phospholipase assay establishment, mass spectrometry (Waters Alliance HT with separation module 2795 coupled to a dual λ absorption detector 2487 and MassLynx V4.0 software) was used. Solvent was 30% water with 0.1% formic acid and 70% acetonitrile; 20 µl sample solution was injected. The mass range measured was m/z 100–500 and/or m/z 200–600, electrospray ionization (cone voltage): ±20 eV, ±40 eV, and ±80 eV; capillary voltage 3.0 kV; RF lens 0.2 V; desolvation temperature was set at 120°C and desolvation temperature at 250°C. N₂ was used as carrier gas at a flow rate of 600 l/h for desolvation and for cone flow at 40 l/h.

Quantification of lipids (AA, PGE₂, palmitoylolethanolamide) by gas chromatography/mass spectrometry

Quantification was performed by a gas chromatography/mass spectrometry method. A total of 5 × 10⁶ U-937 cells was cultured in complete RPMI 1640 medium and differentiated with 2 mM PMA for 48 h and washed with PBS, and the medium was replaced with FBS-free medium. Cells were
incubated with test compounds (DMSO, 2 μM 10-shogaol, 2 μg/ml ginger Hot Flavor extract, 50 and 100 μM aspirin, 0.5 μM methyl arachidonoyl fluorophosphonate [MAFP]) and incubated for 30 min. Then LPS (1 μg/ml) was added, and the cells were incubated for another 4 h. Cells were scraped off and separated from the supernatant by centrifugation for 5 min at 1000 rpm in a Heraeus Biofuge fresco (rotor 3325).

Lipids were extracted from the supernatants and the cell fractions as follows: 1 ml ethanol was added to internal standards (vide infra) and mixed with 9 ml supernatant. The pH was brought to 3 with hydrochloric acid and the samples added to C18 Sep-Pak cartridge (Waters) (preactivated with 3 ml methanol and equilibrated with 3 ml 10% ethanol). Cartridges were washed with 10% ethanol and eluted with 3 ml acetonitrile/ethyl acetate (1:1). Supernatant samples were evaporated to dryness.

Cells were extracted similar to the lipid extraction described by Folch et al. (51). In short, cell pellets were sonicated for 5 min at 4°C ice-cold chloroform (1 ml containing the internal standards), methanol (0.5 ml), and PBS (0.25 ml), and then centrifuged for 5 min at 800 × g. The organic phase was dried in a glass vial and dried under N2 and reconstituted in 1 ml ethanol by vortexing at RT (<5 min), diluted with 9 ml water, and extracted by solid-phase extraction, as described for the supernatant.

Internal standards were as follows: PGE2-d4, 4 ng/μl (100 ng/sample); AA-d8, 4 ng/μl (100 ng/sample); and palmitoylethanolamide-d5, 1 ng/μl (25 ng/sample).

Derivatization of the hydroxyl group of palmitoylethanolamide (PEA) was performed with the silylating agent dimethylisopropylsilyl imidazole. As described by Obata et al. (52), the molecular stability is increased and large fragments could be detected. Derivatization of AA was achieved by esterification with pentafluorobenzylbromide and N,N-diisopropylethylamine (53). The hydroxyl groups of PGE2 were readily silylated with dimethylisopropylsilyl imidazole and the carboxylic acid as for AA with pentafluorobenzylbromide. The remaining ketone was transformed into an O-methyl oxime by methoxiyamine hydrochloride in pyridine (52).

The samples were analyzed by GC/electron ionization mass spectrometry using an Agilent 6890N GC equipped with a 30 m HP-5MS column and a 5975C MS with triple-axis detector. As carrier, gas helium was used at a constant flow rate of 1.5 ml/min with splitless injection at an inlet temperature of 250°C. Optimal separation of the three analytes was achieved with the following oven program: initial temperature, 150°C for 1 min, followed by an increase to 280°C at 8°C/min, with a final time of 20 min. Specific ions were used for selected ion monitoring (54).

Quantification of TXB2 and LTB4

TXB2 and LTB4 were quantified from cell culture supernatants using commercially available ELISA kits.

Results

Setup of whole blood assay and immunopharmacological profiling of ginger extract

A convenient high-content in vitro assay was established to profile the effects of traditional anti-inflammatory multicomponent agents (i.e., botanical drugs) with yet unknown molecular mechanisms of action. It was our aim to employ an assay that was potentially less susceptible to false positives (e.g., effects only detected in tumor cell lines, but not primary cells) and more closely associated with physiological parameters like blood plasma components. We used human venous whole blood, which was immediately transferred to 96-well plates (200-μl aliquots) and incubated with discrete stimuli over 18 h. As the primary readout, we measured differential Th1/Th2 cytokine expression (Supplemental Fig. 1). To induce patterns of differential cytokine expression, stimuli engaging distinct receptors and signaling pathways were applied. These stimuli included bacterial LPS acting at CD14/TLR4/MyD88 (55); PMA that activates protein kinase C (56) and nonspecifically amplifies cellular signals; anti-CD28 and anti-CD3 Abs acting at TCR subtypes (57, 58); and thapsigargin that nonspecifically triggers a cytosolic calcium increase in all cells (59).

As shown in Supplemental Fig. 1, different stimuli that target distinct leukocyte populations triggered significantly distinctive patterns of cytokine expression. The data show mean values from independent experiments performed with blood from different female and male donors (n = 18) and clearly demonstrate that the cytokine network is widely conserved between different individuals, thus providing a robust high content readout. As anticipated,
LPS stimulation induced mainly Th1 monokines with strong TNF-α, IL-1β, IL-6, IL-8, and IL-10 expressions (>10,000 pg/ml), but weak IL-12 expression (Supplemental Fig. 1). Under these conditions, the T cell cytokines IL-3, IL-4, IL-5, IL-7, and GM-CSF were not induced. In contrast, PMA/anti-CD3 Ab stimulation led to a robust Th2 cell response with similar IL-8, IL-12, and TNF-α expressions; less pronounced IL-1β, IL-6, and IL-10 expressions; but significantly stronger IL-3, IL-4, IL-5, IL-7, and GM-CSF expressions.

To validate our assay, we applied specific inhibitors of key inflammatory processes or signal transduction events. Clinically and experimentally used inhibitors were dexamethasone, cyclosporin, diclofenac, cetirizine, parthenolide, the MAPK kinase inhibitors SB203580 (p38 MAPK inhibitor), SB202190 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), U0126 (MEK inhibitor), and PD98059 (selective MEK1 inhibitor) (Fig. 1). As expected, dexamethasone potently inhibited the expression of proinflammatory cytokines independent of the stimulus used. Intriguingly, in this assay, only dexamethasone strongly inhibited LPS-stimulated TNF-α expression. The MEK1/2 inhibitor U0126 and the p38 inhibitor SB203580 weakly inhibited TNF-α. Distinct MEK and p38 inhibitors like U0126 and PD98059, as well as the broad-spectrum enzyme inhibitor 2,4-dibromoacetophenone (4-bromophenacyl bromide or DBA), significantly inhibited LPS-stimulated IL-1β expression, but with a high interassay variability. Based on our assumption that certain ginger constituents may be able to modulate proinflammatory cytokine expression (vide supra), the commercial food-grade ginger Hot Flavor extract (50 µg/ml) was analyzed in this assay. Additional anti-inflammatory medicinal plant extracts, like Harpagophytum procumbens and Salix spp., were tested in the same setup. The ginger extract potentely inhibited IL-1β expression with little interassay variability, whereas the H. procumbens and Salix extracts (both 50 µg/ml) and curcumin (25 µg/ml) were largely ineffective (Fig. 1). Moreover, the ginger extract differentially modulated the expression of several cytokines, depending on the stimulus used (vide infra). All inhibitors of kinases (SB203580, SB202190, SP600125, U0126) and NF-κB (parthenolid and curcumin), as well as DBA and cetirizine, potently inhibited the PMA/anti-CD3 Ab-stimulated GM-CSF (Fig. 1), which may be explained by the fact that this factor is least strongly induced (<1500 pg/ml). As anticipated, cyclosporine potently inhibited the expression of CD3/CD28-induced factors, as exemplified by GM-CSF and TNF-α (Fig. 1), but also other calcium-dependent cytokines (IL-3, IL-4, IL-5, IL-7) (data not shown). The COX-2 inhibitor diclofenac and the H1-antagonist cetirizine were largely ineffective, whereas the broad-spectrum enzyme inhibitor DBA significantly inhibited most cytokines (Fig. 1). As blood from different donors was employed, we concluded that the inhibitory effects observed were robust and meaningful.

Ginger extract globally inhibits IL-1β expression in human whole blood and in primary monocytes

Intriguingly, the ginger extract (50 µg/ml) potently inhibited IL-1β expression (≥35%) in all experiments in which whole blood was stimulated, irrespective of the stimulus applied (Fig. 2). In contrast, the other cytokines were not or only partially inhibited (Figs. 1, 2), also reflecting the lack of general cytotoxicity of ginger extracts under these assay conditions (data not shown). Based on this finding, we concluded that ginger constituents could selectively and globally interfere with the IL-1β expression machinery by concrete, yet unknown mechanisms (Supplemental Fig. 2). Because the stimuli employed induced distinctly different signal

![FIGURE 2](http://www.jimmunol.org/Downloadedfrom)  
**FIGURE 2.** Global inhibition of IL-1β expression by ginger Hot Flavor extract in differentially stimulated whole blood. A total of 50 µg/ml extract was incubated together with different stimuli (z-axis) for 18 h, and cytokine expression was determined by CBA. Arrow indicates that IL-1β is robustly inhibited by 30–50%, independent of the stimulus applied, whereas TNF-α, IL-6, and IL-10 are only partially inhibited. IL-8 expression was not modulated by ginger. Data are the mean values from blood of at least three different donors, each measured in triplicates. The SD was <20%.
transduction events, we excluded the possibility that upstream events, such as, for example, inhibition of MAPKs or transcription factors, could be responsible for this effect.

The inhibition of IL-1β expression by ginger extract is mediated by phenylpropanoids that inhibit i/cPLA₂, but not sPLA₂.

Both the purinoreceptor P2X₇ and PLA₂ enzymes have been shown to be crucial for efficient IL-1β expression (maturation and secretion) from monocytes/macrophages (60). To explore the effects of ginger on IL-1β, experiments in which LPS stimulation was coactivated by ATP were performed in U937 cells, a human monocyte/macrophage cell line, to differentiate between IL-1β maturation and secretion. U937 cells that are stimulated by LPS empty the IL-1β stores (by yet unknown mechanisms), leading only to partial IL-1β section (Fig. 3A) (61). When the cells are costimulated by ATP (which is elevated under inflammatory conditions and present in whole blood), the purinoceptor P2X₇, a ligand-gated ion channel, is activated, leading to activation of caspase-1 that cleaves pro–IL-1β into the mature IL-1β. In parallel, this causes an increase in [Ca²⁺], which triggers the release of the IL-1β storage vesicles. As shown in Fig. 3A, the ginger extract significantly and specifically inhibited the ATP/LPS-stimulated IL-1β secretion from U937 cells, thus indicating potential effects on P2X₇, PLA₂ enzymes, and/or caspase-1. The level of ethidium bromide uptake after ATP stimulation can be regarded as equivalent to general cation influx through large P2X₇ receptor adjacent pores (41). The assay was performed in isolated human monocytes, as they showed a better signal-to-noise ratio than U937 cells. In this assay, P2X₇ activity was not inhibited by the ginger extract (Fig. 3B).

Western blot and cytometric bead analyses showed that the ginger extract inhibits maturation and release of IL-1β from activated monocytes by ~60% when the ginger extract was added before ATP (Fig. 3C). Under nonstimulated conditions, the isolated human monocytes (2 × 10⁵) secrete ~4 pg mature IL-1β within 4 h in culture and ~60 pg/ml when incubated (primed) with LPS. The constitutive secretion was statistically unchanged by 10 μg/ml ginger extract (Fig. 3A). A total of 1 mM ATP did not modulate cytokine secretion in nonprimed cells. However, stimulating LPS-primed cells with ATP caused an increase in cytokine secretion (180 pg/ml) (Fig. 3A). This stimulated secretion was significantly reduced by >50% when ginger extract (10 μg/ml) was added prior to the LPS priming and by ~40% when added after LPS and prior to ATP. These levels were significantly different between LPS plus ATP but insignificant when compared with LPS alone, ruling out an effect on caspase-1. A differential effect on intra- and extracellular pro- and mature IL-1β species was clearly visible in Western blots (Fig. 3C) even though the
inhibition of maturation and secretion was less pronounced than in the assay conditions used for quantitative CBA (Fig. 3A). In isolated human monocytes, the iPLA2-dependent IL-1β maturation and the cPLA2-dependent IL-1β secretion were reduced to ∼60% (at 10 μg/ml ginger extract), whereas transcription/translation and constitutive maturation/secretion were unaffected (Fig. 3C). Therefore, we concluded that the major ginger constituents (Fig. 4) could be potential inhibitors of PLA2 enzymes (Supplemental Fig. 2).

To measure i/cPLA2 enzyme activities, a suitable assay was established using mixed micelles and a fluorescence-coupled assay (Fig. 5A, 5B) (for experimental details, see Materials and Methods). The major lipophilic ginger constituents (Fig. 4) were purchased or isolated, as previously reported (17), and tested at 10 μM. As shown in Fig. 5C, the ginger extract and its main ginger constituents inhibited both iPLA2 and cPLA2 enzyme activities. Overall, the phenylpropanoids more strongly inhibited iPLA2. The inhibition of ∼50% was in the same range as the inhibition by the irreversible PLA2 inhibitors MAFP and BEL. The relatively low efficacy of the latter two may be due to the preincubation scheme used (62, 63) or more likely residual calcium- and phospholipase-independent hydrolytic activities. Nevertheless, the assay employed was robust enough to detect i/cPLA2 inhibitors and to compare potencies of individual compounds. The inhibitor concentration was as low as 0.1 μg/ml of the total detergent molecules, a possible inhibition by surface dilution kinetics could be excluded (64). The ginger extract and 10-shogaol concentration-dependently inhibited both iPLA2 and cPLA2 with similar potencies as BEL and MAFP (Fig. 5D). However, the inhibition of iPLA2 was clearly stronger at lower concentrations than the inhibition of cPLA2; in particular with the ginger extract (EC50 values 0.7 μg/ml versus 3 μg/ml). For iPLA2, the eight homologs were less active than the controls at equimolar concentrations (10 μM), but 6-shogaol and 10-gingerol were somewhat more potent, with an overall inhibition of ∼65% (Fig. 5C). Although 6-,8-gingerols did not inhibit cPLA2 and 10-shogaol was the most potent inhibitor, structure-activity relationships were not apparent.

To assess the specificity of this effect, we also measured sPLA2. Porcine pancreatic sPLA2 activity was tested using isolated phosphatidylcholine, and an established TLC detection method (65, 66) was not inhibited by ginger constituents up to 20 μM, but strongly inhibited by the nonspecific enzyme inhibitor DBA (data not shown).

Weak effects of ginger extract on T cell proliferation and MAPKs

iPLA2 inhibition has been shown to contribute to the proliferation of lymphocytes, Jurkat T cells (28), and monocytes (29). As shown in Fig. 6, the proliferation of anti-CD3 Ab-stimulated human lymphocytes was weakly, but significantly inhibited by the ginger CO2 extract (10 μg/ml) and 10-shogaol (>5 μM), but not by a totum extract containing a higher essential oil content. To address the effect of ginger constituents on MAPKs previously reported with cancer cells (54), we also measured the effects of gingerols and shogaols on ERK, JNK, and p38 in primary human T cells. PMA/anti-CD3 Ab-stimulated lymphocytes were used to determine the modulation of MAPKs by measuring kinase phosphorylation states using a commercial CBA assay (from BD Biosciences). As shown in Fig. 6, experiments using primary human T cells did not show significant modulations. Phosphorylation of p38 was even increased by ginger constituents. Only 10-shogaol significantly inhibited JNK phosphorylation by ∼35%, but the whole extract showed a trend toward activation of this kinase, and thus, no conclusive picture.

Inhibition of prostanoid secretion and modulation of arachidonate-phospholipid remodeling by ginger phenylpropanoids

Based on the finding that ginger phenylpropanoids (Fig. 4) inhibit the PLA2 enzymes in the mixed micelles assay (Fig. 5), we next assessed the effects of ginger extract and 10-shogaol on free AA levels and PGE2 release directly in differentiated U937 macrophages (differentiated with 5 nM PMA for 48 h). A quantitative gas chromatography/mass spectrometry analysis was employed to

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**FIGURE 6.** A. Proliferation of anti-CD3 Ab-stimulated human CD4+ T lymphocytes, as determined by BrdU incorporation. The ginger Hot Flavor extract (10 μg/ml) and 10-shogaol (5–10 μM) show weak, but significantly reduced proliferation, whereas essential ginger oil, a ginger totum extract, and the other phenylpropanoids had no effect. Shown are the mean values ± SEM of triplicates performed with lymphocytes of at least three different donors. B–D, MAPK phosphorylation in isolated human CD4+ lymphocytes stimulated with PMA/anti-CD3 Ab together with the ginger Hot Flavor extract (10 μg/ml) (2) and four of its main constituents (10 μM). Phosphorylation of kinases was measured using selective Abs and CBA (BD Biosciences). Shown are the mean values ± SEM of triplicates performed with lymphocytes from at least three different donors. *p < 0.05.
determine both AA and PGE2 levels in cell supernatants (cell medium) and in the cellular fractions (see Materials and Methods). Whereas free AA could be detected in both supernatant (5.4 nmol/l × 10^7 cells) and in the thoroughly washed cellular fraction (0.4 nmol/l × 10^7 cells) of undifferentiated U937 cells, PGE2 was only found in the supernatant of U937 macrophages. U937 macrophages constitutively released PGE2 (0.1 nmol/l × 10^7 cells) even without LPS stimulation (Fig. 7A). Upon stimulation by LPS, the PGE2 levels stably increased ~3-fold. As shown in Fig. 7B, the positive control acetyl salicylic acid (aspirin) at concentrations at which COX-1/2 is fully inhibited in vitro (>10 μM) inhibited PGE2 release from LPS-stimulated U937 cells. The PLA2 and nonspecific hydrolase inhibitor MAFP (0.5 μM) and 10-shogaol (2 μM) inhibited the release of PGE2 and the prostanoid metabolite TXB2 by >50% (Figs. 7A, 7B, 8). In nonstimulated U937 macrophages, 10-shogaol inhibited constitutive PGE2 expression by >50% (Fig. 7A). Noteworthily, even concentrations of ginger extract as low as 2 μg/ml showed a significant inhibition of PGE2 and TXB2 release, whereas LTb4 was only weakly inhibited. As expected, BEL, which more specifically inhibits iPLA2, had no effect on PGE2, TXB2, or LTb4 production, whereas MAFP also inhibited LTb4 (Figs. 7, 8). Moreover, PEA, which is also constitutively released by U937 macrophages (0.1 nmol/l × 10^7 cells), was not modulated by ginger extract or 10-shogaol (Fig. 7F, 7G). LPS stimulation did not increase PEA secretion. Somewhat unexpectedly, ginger extract and 10-shogaol significantly increased

**FIGURE 7.** Effects of ginger extract and 10-shogaol on PEG2, AA, and PEA levels in LPS-stimulated U937 macrophages (PMA differentiated). A and B, PGE2 levels in supernatant. C and D, Free AA levels in supernatant. E, Free AA levels in cells. F, PEA levels in supernatant. G, PEA levels in cells. Supernatant levels of AA, PGE2, and PEA and the intracellular level of AA and PEA were measured by GC/MC. Ginger Hot Flavor extract (ginger, 2 μg/ml), 10-shogaol (2 μM), MAFP (0.5 μM), and acetyl salicylic acid (aspirin, 50 and 100 μM) were used as controls. Indicated are the relative mean values ± SD of three independent experiments compared with untreated controls (one sample t test). *p < 0.05, **p < 0.01, ***p < 0.001.

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iPLA2 and COX, we speculated that this double effect may lead to rather decrease free AA levels. Because the ginger extract and 10-shogaol inhibit both iPLA2 and COX, we predicted that this double effect may lead to a dramatic and unexpected increase of free AA similar to what was observed with ginger phenylpropanoids. We concluded that the inhibition of iPLA2 leads to a dramatic increase of free AA. This was corroborated in an experiment in which BEL and aspirin were coincubated, and in this combination also led to significant increase of free AA levels (Fig. 7). Free fatty acids are first bound to CoA and then incorporated into lipids by either de novo synthesis of triglycerides (Kennedy pathway) or remodeling of phospholipids (Lands cycle) (23, 24) (Supplemental Fig. 3). In the case of AA, the latter involves incorporation of arachidonate into phosphatidylcholine by a deacylation/reacylation reaction, followed by a phospholipid class switch via CoA-independent transacylation (73, 74). This is the main pathway for AA incorporation in most cell types (75). In both of these processes, phospholipases, and especially iPLA2, play major regulatory roles by providing free fatty acid acceptor molecules (e.g., lyso-phosphatidylcholine) (25). In contrast, immune cells generate free AA mainly from phospholipids by cPLA2 IVA, the only PLA2 with a preference for AA (46, 76–79), and only under certain conditions by sPLA2 IIA, V, and X and iPLA2 VIA (74). Because we observed a significant increase of free cellular AA by ginger phenylpropanoids in U937 macrophages (Fig. 7E), the iPLA2 inhibition is likely to be predominant in cells, and sPLA2 enzymes are not affected. The sPLA2 enzymes may be the cause of the free AA observed in our assays. The reason that MAFP at concentrations around the IC50 of reported i/cPLA2 inhibition (80, 81) only weakly inhibits free AA in the supernatant is not clear, but may be due to the combined i/cPLA2 isomerase inhibition and a resulting indirect reduction of extracellular sPLA2 activity. Moreover, a fraction of the AA that is released by PLA2 activation will be rapidly reincorporated into phospholipid, whereas the remainder will be lost by conversion to eicosanoids or other products, or by β-oxidation (82, 83). Unesterified AA in plasma rapidly replaces the amount lost, and this replacement is proportional to PLA2 activation (84, 85). In our experiments, BEL alone inhibited free AA, but increased free AA when COX was inhibited at the same time. This dramatic change (Fig. 7C, 7D) shows the role of iPLA2 for AA phospholipid reacylation. Therefore, in the presence of COX inhibitors, blockage of iPLA2 deprives the cell of phospholipid acceptor molecules and subsequently augments the intracellular AA concentration (75).

Given that free AA in cells induces apoptosis (29), the pronounced increase of free cellular AA may also explain some of the differential antiproliferative effects of gingerols and shogaols on cancer cells (86, 87). In contrast, PEA levels were not affected by ginger phenylpropanoids. Its biosynthesis mainly relies on N-acyltransferases, N-acylphosphatidylethanolamine phospholipase D, and fatty acid amide hydrolases, and only to a minor extent on lyso-phospholipase D and sPLA2, but not on other PLA2 classes (88, 89). Consequently, our data indicate selectivity toward i/cPLA2 of the ginger extract and its main constituents and exclude unspecific perturbation of lipid homeostasis. The action of ginger phenylpropanoids is in line with the already known antioxidant and radical scavenging effects of ginger (90, 91) and may further increase its effect on iPLA2 in cells. Reactive oxygen species produced by cyclooxygenases are known to either activate iPLA2 in a positive feedback loop (42) or act as mediator between cPLA2 and sPLA2 (92–95).

The ginger rhizome and its extracts have been shown to be safe, as exemplified by its widespread dietary use in Asia (1). In contrast, many tested synthetic i/cPLA2 inhibitors exert unwanted side effects due to unspecific toxicity/reactivity (e.g., BEL or MAFP), poor selectivity (e.g., MAFP and arachidonil trifluoromethyl ketone), or lack of oral availability [e.g., EXPLIS (96)]. Ginger extracts or isolated compounds show similar in vitro potencies against iPLA2 enzymes as standard PLA2 inhibitors, but are...
nontoxic. Ginger as a botanical drug has a great acceptance in the population, and might therefore be used as a physically and mentally well-tolerated augmentation to conventional anti-inflammatory medication in cases where first-line therapy is not sufficient. In particular, treatment of inflammatory bowel syndrome and celiac disease in which IL-1β and PGE_2 play a major role (97) or autoimmune inner ear disease (98) could be novel therapeutic applications of ginger. Overall, the inhibition of PLA2 enzymes provides a rational basis for several reported properties of ginger [anti-inflammatory, antipyretic, analgesic, or cardiovascular effects (86, 87)].

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The authors have no financial conflicts of interest.

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