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

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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 bromoenol lactone with 10-gingerol/-shogaol being most effective. Furthermore, a ginger extract (20 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: 4140–4150.

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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-dibromocetophenone; 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; thio-PC, 1-hexadecyl-2-arachidonoylthio-2-deoxy-sn-glycero-3-phosphorylcholine; TXB2, thromboxane B2.

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The rhizome of ginger (Zingiber officinale) is widely used in diet, but also in Asian traditional medicine, where it is employed therapeutically to treat mild forms of rheumatoid arthritis, fever, and upper respiratory tract infections (1). In conventional Western medicine, ginger preparations are administered clinically to treat emesis, nausea, and migraine headache (2, 3). Based on the widespread therapeutic use of ginger in Asia for the treatment of inflammatory diseases, several studies have been dedicated to the elucidation of potential anti-inflammatory mechanisms (4–7). At relatively high μM concentrations, ginger constituents such as gingerols, shogaols, and diarylheptanoids have been reported to weakly inhibit components of proinflammatory signal transduction pathways in vitro, such as NF-κB, protein kinase C, and MAPKs (8, 9). Moreover, ginger constituents have been shown to inhibit inducible NO synthase, cyclooxygenase (COX)-1/-2, and lipoxygenase in vitro (9–12). A significant inhibition of PGE2 formation by ginger constituents has already been shown in vivo (13). Furthermore, different gingerols and shogaols at low μM concentrations have been shown to directly bind to and partially activate the cation channel transient receptor potential vanilloid 1 (14–16), the molecular clue for the spiciness of ginger, as well as the serotonin 5-HT1A G protein-coupled receptor (17). Although the latter studies have elucidated feasible molecular mechanisms of action of the major biologically active ginger constituents, they cannot explain the range of anti-inflammatory effects observed in vivo. In particular, the reported antipyretic effects of ginger remain poorly understood.

In this study, we have profiled a standardized food-grade commercial ginger extract in a high content assay using freshly isolated human peripheral whole blood. Human blood was incubated with discrete stimuli targeting specific or nonspecific signal transduction pathways, and the resulting cytokine patterns were quantified. The assay was validated using blood from different donors and by profiling the effects of anti-inflammatory drugs with known targets. Using this profiling setup, we were able to identify calcium-independent phospholipase A2 (iPLA2) and cytosolic phospholipase A2 (cPLA2) enzymes as major biological targets of ginger phenylpropanoids.

PLA2 enzymes play a central role in inflammatory processes (18, 19) and are classified into three main groups, as follows: secretory phospholipase A2 (sPLA2), cPLA2, and iPLA2. These distinctly different enzymes hydrolyze the sn-2 ester bond of
phospholipids, thus liberating lysophospholipids and free fatty acids, which are precursors for a variety of different mediators of physiological and pathological processes (20, 21). PLA2 iso-
zymes are not only involved in the generation of free arachidonate (AA), which is metabolized by cyclo- and lipo-
genases to eicosanoids (22), but also in the incorporation of free AA into membranes and redistribution into specific compart-
ments (remodeling) (23, 24). Besides regulatory functions in lipid catabolism/metabolism, PLA2 enzymes are critically involved in signal transduction, phospholipid and cell membrane bilayer remodeling (25), store-operated cation channels and Ca2+ release-
activated Ca2+ channels (26), Fas-induced apoptosis (27), cellular proliferation and differentiation (28, 29), glucose-induced insulin secretion (30), and, most important for this study, they play a central role in acute (18) and chronic inflammation (31).

Both iPLA2 and cPLA2 are crucial for IL-1β maturation and secretion (membrane translocation and fusion of storage vesicles) (32, 33). IL-1β is mainly secreted by monocytes/macrophages and dendritic cells, but also fibroblasts; 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 in-
fammary bowel disease (34, 35). IL-1β further induces COX-2 expression in endothelial cells and is involved in edema forma-
tion, where it synergizes with PGE2 (36). IL-1β expression in endothelial cells and is involved in edema forma-
dreadnamic changes in arachidonate-phospholipid remodeling, due to extracts in vitro. Moreover, ginger phenylpropanoids induce dra-
ments (remodeling) (23, 24). Besides regulatory functions in lipid AA into membranes and redistribution into specific compart-
mengases to eicosanoids (22), but also in the incorporation of free

Chemicals to eicosanoids, the cells were washed and incubated in serum-
free medium (see below).

Cytokine measurements. Cytokine quantification in whole blood was done with a cytometric bead array (CBA) human inflammatory cytokine kits (BD kit 551811) for IL-8, IL-1β, IL-6, IL-10, TNF-α, IL-12p70, and the BD CBA human alarmin 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-1β Flex Set (558279), according to the manufacturer’s manuals, measured with a FACSscan flow cytometer equipped with an argon laser, and evaluated with the CBA software V1.4 (all by BD Biosciences).

ELISA measurements of thrombomodulin B2, and leukotriene B4. The throm-
boxon B2 (TXB2) and leukotriene B4 (LTB4) ELISA colorimetric kit (EHTLB4) 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 $2 \times 10^6$ U937 macrophages were stimulated with LPS (1 μg/ml) to measure TXB2 and $10 \times 10^6$ primary CD14+ monocytes/macrophages to measure LTB4. Cells were preincubated with DMSO (vehicle) or test compounds for 30 min prior to stimulation with LPS (1 μg/ml) and for full LTB4 release costimulation with fMLP (1 μM). LTB4 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, according to the manufacturer’s recommendations, using 10 μl samples dissolved in LDS buffer. Blotting was done on nitrocellulose mem-

branes in a NuPage blotting chamber, according to the instruction manual.

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-1β; Abnova MaxTab and Sigma-Aldrich) were used at 2 μl/ml; and secondary Ab (anti-mouse IgG [goat] HRP labeled by GE Healthcare) at 1/8000 dilution. Chemiluminescence detection was done with ECL Plus Western blotting detection reagents (Amersham Biosciences by GE Healthcare). For IL-1β detection with Western blot analysis, isolated monocytes, in 96-well plates at 200

μl/100 μl of MOPS buffer for gel electrophoresis.

Ethidium bromide uptake to measure P2X7-mediated ion fluxes

Ethidium+ 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 × 10^6 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 of phosphatidylcholine by quantitative extraction of phosphate-containing lipids, a molybdenum blue spray solution, and 2 µM thio-PC, self-forming at 80°C until measurement. Lyophilisates were reconstituted in 10 µl hypotone buffer (10 mM HEPES, 0.3 mM EDTA, 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 (10^6 cells/ml) were kept in culture medium, according to the recommendations by American Type Culture Collection. Two weeks before monomeric factor C2 was triphospholipid 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 (Waters Alliance HT with separation module 2795 coupled to a dual em 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 phosphatase-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 × 10^6 cells/ml and aliquoted at 50 µl into 96-well plates. The 50 µl diluted stimuli were added as 2× stock and incubated at 37°C for the indicated time. Conditions for p38 phosphorylation were 10 µl/ml anti-CD3 Ab and 2 nM PMA for 20 min; for ERK1/2 phosphorylation, 10 nM PMA for 30 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 3 min at 4°C. The pellets were lysed with 5 µl diluted BD denaturation buffer containing phosphatase inhibitors (4 mM sodium p-tauussium tartrate, 2 mM imidazole, 1.15 mM sodium molybdate, 1 mM sodium orthovanadate, and 1 mM sodium fluoride). Lysates were stored with no detectable decomposition for 3 d at -80°C.

Buffer. The final phospholipase buffer consisted of 10 µl lystate and 10 µl mixed micelle buffer (for iPLA2; 190 mM MOPS, 152 mM KOH, 3 mM DTT, 2 mM ATP, 1 mg/ml BSA, and for cPLA2; 190 mM MOPS, 152 mM KOH, 3 mM DTT, 1.2 mM CaCl2, 1 mg/ml BSA, 20 µM bromoelactone lactone (BEL)) containing mixed micelles (16 mM PK, 2 mM Tween 20, and 2 mM tio-PC, self-forming at T > 35°C). (Final concentrations are therefore 100 mM Good’s Buffer adjusted with KOH to pH 7.4 at 40°C. 170 mM sucrose, 2 mM DTT, 0.5 mg/ml BSA, 8 mM PK, 1 mM Tween 80, and 1 mM tio-PC, and either 1 mM ATP and 0.5 mM EDTA or 0.1 mM free calcium and 10 µM BEL.)

Mixed micelles. Stock solutions of PK in chloroform, Tween 80, and phospholipid in ethanol were mixed and dried under a nitrogen flow. The mixed lipid film was hydrated in mixed micelle buffer at 40°C, briefly sonicated to detach film from glass surface if necessary, and gently (to avoid foam formation) shaken until the solution became totally clear.

Size and ζ potential of the micelles were analyzed on a zetasizer, giving one single sharp peak in both cases with an average size distribution of 32.3 ± 9.6 nm by volume (area of 99.3%) and 27.9 ± 6.25 nm by number (area of 100%) and an average ζ potential of −39.4 ± 3.25. Estimated by their size, they consist of ~500 detergent molecules (quod est demonstrandum), and their ζ potential most likely masks c/iPLA2 in the spotting mode. Moreover, these micelles are self-forming, sonicating is not mandatory, and they are readily hydrolyzed by all three phospholipase subtypes tested.

**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 DTT (100 mM aq. sol.; for reduction of dithio-lyso-phosphatidylcholine), 0.2 µl Cs4EDTA (100 mM aqeous 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 pH 1 with 2 M trichloroacetic acid 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 (Waters Alliance HT with separation module 2795 coupled to a dual em 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 phosphatase-containing lipids, a molybdenum blue spray solution and charring were used.

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Proliferation was determined with the BD FICT 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. Palmitotyl-6-ascorbic acid 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 palmitotyl-6-ascorbate 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; extractor 1 V; and RF lens 0.2 V. 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**

Gas chromatography/mass spectrometry

Mass spectrometry was performed by a gas chromatography/mass spectrometry method. A total of 5 × 10^6 U937 cells was cultured in complete RPMI 1640 medium and differentiated with 2 nM PMA for 48 h and washed with PBS, and the medium was replaced with PBS-free cells. Cells were
incubated with test compounds (DMSO, 2 μM 10-phenacol, 2 μg/ml ginger Hot Flavor extract, 50 and 100 μM aspirin, 0.5 μM methyl arachidonyl 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 5 with hydrochloric acid and the samples added to C 18 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 PBS (0.25 ml), and then centrifuged for 5 min at 1000 rpm in a Heraeus Biofuge fresco (rotor 3325).

Selected examples of effects on differentially stimulated whole blood by different inhibitors (10 μM) and the experimental samples ginger Hot Flavor extract, two H. procumbens extracts, and a Salix sp. extract (each 50 μg/ml). Human whole blood from different donors was treated with inhibitors and incubated for 18 h with LPS (312 ng/ml) (upper two graphs) and PMA/αCD3 (1 μg/ml) (lower two graphs), respectively. Data are mean values (+SD) of blood samples from at least three different donors, each measured in triplicates. *p < 0.05, **p < 0.01, ***p < 0.001.

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'-dibromomacetophenone (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 mg/ml) was analyzed in this assay. Additional anti-inflammatory medicinal plant extracts, like Harpagophyllum procumbens and Salix spp., were tested in the same setup. The ginger extract potently inhibited IL-1β expression with little interassay variability, whereas the H. procumbens and Salix extracts (both 50 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 (parthenoloid 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 less 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 expressions; less pronounced IL-12, IL-6, and IL-10 are only partially inhibited. IL-8 expression was not modulated by ginger. Data are the mean values from SD was 20%.

**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%.

**FIGURE 3.** A. Relative expression of IL-1β in culture medium of human monocytes. Cells were incubated for 4 h with vehicle control, LPS (312 ng/ml), ginger Hot Flavor extract (10 µg/ml), or both, and then stimulated for 30 min (e.g., with vehicle control, ATP [2 mM], ginger Hot Flavor extract [10 µg/ml], or both). Data show mean values + SD (n = 4); *p < 0.05. B, Representative graphs of the time-resolved (x-axis) ethidium bromide fluorescence (y-axis) of vehicle control (1) and ginger Hot Flavor extract (10 µg/ml) in LPS-primed and ATP-stimulated monocytes (ATP added at 0 s). The time versus fluorescence mean value plots were identical, and an inhibition of either ATP at its receptor or fast ion fluxes (potassium out, calcium in) can be excluded. C, Western blot for IL-1β after 60-min stimulation in glaconate basal salt solution in the cytosol (a) and in supernatant medium (b) of isolated monocytes. Upper row, 31-kDa pro-IL-1β; lower row, 17-kDa mature IL-1β. Lane 1, Vehicle control; lanes 2–4, priming with ginger extract, LPS, and ginger extract prior to LPS; lane 5, LPS priming plus ginger extract stimulation; lane 6, only ATP stimulation; lanes 7–9, LPS priming with ATP stimulation; alone, with ginger extract prior to LPS, and ginger extract prior to ATP; and lane 10, pro- and mature IL-1β standards.
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 × 10^5) 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

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**FIGURE 4.** Chemical structures of ginger phenylpropanoids. The alkyl side chain length varies by two carbon atoms between 6-(n = 1), 8-(n = 2), and 10-(n = 3) gingerol and shogaol, respectively.

**FIGURE 5.** A, Fluorescence-coupled PLA2 assay. The substrate thio-PC is incorporated into mixed micelles and incubated with a PLA2 containing cell lysate. The reaction is quenched with methanol, and the product, a free thiol, is coupled to monobromobimane and quantified by HPLC with fluorescence detection. B, Size and homogeneity of mixed micelles were measured by zetasizer. C, Absolute activities of iPLA2 (left) and cPLA2 (right) in absence and presence of either 10 μg/ml ginger Hot Flavor extract or 10 μM pure compounds or controls are shown as mean values + SD (n = 3). If not indicated otherwise, mean values are significantly different from controls (p < 0.05). D, Concentration-dependent inhibition of 10-shogaol (μM) and ginger extract (μg/ml) on iPLA2 and cPLA2, showing positive controls BEL and MAFP, respectively. Data show mean values ± SD (n = 3).
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 PL2 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 PL2 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 molar percent 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 iPLA2. 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 prostaglandin secretion and modulation of arachidonate-phospholipid remodeling by ginger phenylpropanoids**

Based on the finding that ginger phenylpropanoids (Fig. 4) inhibit the PL2 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 PGE_2 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/1 × 10^7 cells) and in the thoroughly washed cellular fraction (0.4 nmol/1 × 10^7 cells) of undifferentiated U937 cells, PGE_2 was only found in the supernatant of U937 macrophages. U937 macrophages constitutively released PGE_2 (0.1 nmol/1 × 10^7 cells) even without LPS stimulation (Fig. 7A). Upon stimulation by LPS, the PGE_2 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 PGE_2 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 PGE_2 and the prostanoi metabolite TXB_2 by >50% (Figs. 7A, 7B, 8). In nonstimulated U937 macrophages, 10-shogaol inhibited constitutive PGE_2 expression by >50% (Fig. 7A). Noteworthily, even concentrations of ginger extract as low as 2 μg/ml showed a significant inhibition of PGE_2 and TXB_2 release, whereas LTB_4 was only weakly inhibited. As expected, BEL, which more specifically inhibits iPLA2, had no effect on PGE_2, TXB_2, or LTB_4 production, whereas MAFP also inhibited LTB_4 (Figs. 7, 8). Moreover, PEA, which is also constitutively released by U937 macrophages (0.1 nmol/1 × 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 PGE_2, AA, and PEA levels in LPS-stimulated U937 macrophages (PMA differentiated). A and B, PGE_2 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, PGE_2, and PEA and the intracellular level of AA and PEA were measured by GC/MS. 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.
the free AA levels in U937 macrophages in the cellular fraction and in the supernatant under both untreated and LPS-stimulated conditions (Fig. 7C–E). A PLA2 inhibitor would be expected to rather decrease free AA levels. M AFP reduced free AA in the supernatant, but aspirin, as expected, did not influence free AA levels. Because the ginger extract and 10-shogaol inhibit both iPLA2 and COX, we speculated that this double effect may lead to an increase of free AA from the pool that constitutively feeds COX catalyzing prostanoid synthesis. We therefore combined the iPLA2 inhibitor BEL and aspirin. As shown in Fig. 7C and 7D, this combination led to an increase of free AA similar to what was observed with ginger phenylpropanoids. We concluded that the disruption of arachidonate-phospholipid remodeling, that is, the blockage of phospholipid reacylation by iPLA2 inhibition, and the concurrent inhibition of COX is responsible for the unexpected effect on free AA by ginger phenylpropanoids (Supplemental Fig. 3).

Discussion

A validated high-content whole blood assay was established to profile robust cytokine modulating effects of reported immunomodulatory agents, including botanical drugs. This has led to the elucidation of a new anti-inflammatory molecular mechanism of action of gingerols and shogaols, which are the major phenylpropanoids in dietary ginger (67, 68). Our study indicates that the potent inhibition of i/cPLA2 by phenylpropanoids is related to the specific inhibition of IL-1β secretion from monocytes/macrophages by ginger (Supplemental Fig. 2). Moreover, our data confirm the inhibition of COX (10, 11, 67–69) by ginger phenylpropanoids. This is shown by the inhibition of LPS-stimulated PGE2 and TXB2 secretion (Figs. 7, 8) from U937 macrophages, indicating non-selective inhibition of COX enzymes. LPS-stimulated LTb4 from primary human monocytes/macrophages (U937 macrophages did not secrete LTb4) was only weakly inhibited by the ginger extract. The double inhibition of prostanoid secretion and IL-1β by ginger phenylpropanoids is interesting because IL-1β is known to reinforce PGE2 synthesis (70), which plays multiple roles in inflammatory processes (71, 72).

Our study further shows that the significant cellular inhibition of prostanoid production by ginger phenylpropanoids together with the inhibition of iPLA2 leads to a dramatic and unexpected 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 deacytlation/reacytlation 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., lysophosphatidylcholine) (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 M AFP 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 isomor 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 antioxidative and radical scavenging effects of ginger (90, 91) and may further increase its effect on iPLA2 in cells. Reactive oxygen species produced by cyclo-oxigenases 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 M AFP), poor selectivity (e.g., MAFP and arachidonyl 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 PGE2 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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References


