Controversial correlations between biological activity and concentration of the novel lipokine palmitoleate (9Z-hexadecenoate, 16:1) might depend on the formation of an active 16:1 metabolite. For its identification, we analyzed the glycerolphospholipid composition of mouse Swiss 3T3 fibroblasts in response to 16:1 using LC-MS/MS. 16:1 was either supplemented to the cell culture medium or endogenously formed when cells were stimulated with insulin or growth factors as suggested by the enhanced mRNA expression of 16:1-biosynthetic enzymes. The proportion of 1-acyl-2–16:1-phosphatidylinositol (16:1-PI) was time-dependently and specifically increased relative to other glycerolphospholipids under both conditions and correlated with the proliferation of fatty acid (16:1, palmitate, oleate, or arachidonate)-supplemented cells. Accordingly, cell proliferation was impaired by blocking 16:1 or 16:1-PI by the selective stearoyl-CoA desaturase-1 inhibitor CAY10566 and restored by supplementation of 16:1. The accumulation of 16:1-PI occurred throughout cellular compartments and within diverse mouse cell lines (Swiss 3T3, NIH-3T3, and 3T3-L1 cells). To elucidate further whether 16:1-PI is formed through the de novo or remodeling pathway of PI biosynthesis, phosphatidate levels and lyso-PI-acyltransferase activities were analyzed as respective markers. The proportion of 16:1-phosphatidate was significantly increased by insulin and growth factors, whereas lyso-PI-acyltransferases showed negligible activity for 16:1-coenzyme A. The relevance of the de novo pathway for 16:1-PI biosynthesis is supported further by the comparable incorporation rate of deuterium-labeled 16:1 and tritium-labeled inositol into PI for growth factor-stimulated cells. In conclusion, we identified 16:1 or 16:1-PI as mitogen whose biosynthesis is induced by growth factors.

During de novo biosynthesis of phospholipids (PLs), fatty acids are successively introduced into the sn-1 and sn-2 positions of the common PL precursor phosphatidic acid (PA) by glycerol-3-phosphate-1-acyltransferases and lyso-PA-acyltransferases, respectively (1). PA is either dephosphorylated to diacylglycerol (DAG) before the polar headgroup of phosphatidylcholine (PC) and -ethanolamine (PE) is introduced or is activated to cytosine diphosphate (CDP)-DAG for the synthesis of phosphatidylinositol (PI) and phosphatidylglycerol (PG) (2). sn-2 fatty acids are further exchanged in the remodeling pathway (Land's cycle) by the concerted action of PL- and acyl-CoA-specific lysophospholipid acyltransferases (1) and phospholipase A₂ (3). Generally, the remodeling pathway shapes the sn-2 fatty acid composition of PLs (1).

Palmitoleate (9Z-hexadecenoate, 16:1), a major product and marker of fatty acid biosynthesis, is synthesized by stearoyl-CoA desaturase (SCD) isoenzymes (SCD1–4 in mice) from palmitate (hexadecanoate, 16:0) and either elongated to oleate (9Z-octadecenoate, 18:1), the major monounsaturated fatty acid in mammalian cells or incorporated into triacylglycerols and membrane PLs. Plasma and adipose tissue levels of 16:1 are associated with risk for type 2 diabetes after lifestyle intervention for which

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**Background:** Conflicting reports about the function of palmitoleate might depend on the formation of bioactive metabolites.

**Results:** Palmitoleate induces cell proliferation and is formed during stimulation with growth factors and specifically incorporated into palmitoleoyl-phosphatidylinositol during de novo phosphatidylinositol biosynthesis.

**Conclusion:** Palmitoleate or palmitoleoyl-phosphatidylinositol is proposed as mediator during cell proliferation.

**Significance:** The study combines the mitogenic effect of palmitoleate with insights into its metabolism.
circulating 16:1 concentrations correlated with insulin sensitivity (9, 10) according to recent animal studies (11). 16:1 plasma levels were increased in aP2/mal1-deficient mice due to enhanced lipogenesis in adipocytes (11). The increase of 16:1 was found to protect against insulin resistance in liver and skeletal muscle by regulating a subset of metabolic enzymes (11). Along these lines, cellular studies found that 16:1 increases basal glucose uptake and activates insulin signaling (11–13), promotes β-cell proliferation and secretory function (14–16), regulates the expression and degradation of metabolic enzymes (11), and prevents 16:0-induced endoplasmic reticulum stress and apoptosis (17, 18). These unique characteristics of 16:1 among fatty acids led to the concept of 16:1 as lipid hormone coordinating metabolic responses (11). The mechanism by which plasma 16:1 regulates metabolic enzymes has not been identified so far. However, the detailed understanding of this mechanism will be required to understand fully the controversial functions of 16:1.

Our approach to elucidate the signaling mechanism of 16:1 is based on the assumption that not 16:1 directly but a highly regulated 16:1 metabolite mediates the lipokine functions that would explain misleading correlations when focusing on 16:1 alone. We found that 16:1 (or one of its metabolites) induces proliferation of Swiss 3T3 fibroblasts, a commonly used mouse model cell line. Using comprehensive liquid chromatography tandem mass spectrometry (LC-MS/MS)-based lipidomics, we identified 1-acyl-2–16:1-sn-phosphatidylinositol (16:1-PI) as strongly and specifically regulated membrane PL. 16:1-PI is specifically formed in response to exogenous 16:1 as well as to metabolic hormones and growth factors through induction of fatty acid and de novo PI biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Quantitative RT-PCR primers were obtained from Sigma-Aldrich or FASMAC Co. (Kanagawa, Japan). Lipid standards, lyso-PLs, and acyl-CoAs were bought from NOF Co. (Tokyo, Japan) or Avanti Polar Lipids (Alabaster, AL). Materials used were fetal calf serum (FCS), Invitrogen; DMEM, fatty acid-free BSA, 16:0, 18:0, arachidonic acid (5Z,8Z,11Z,14Z-eicosatetraenoic acid, 20:4), phorbol 12-myristate 13-acetate (PMA), dexamethasone, insulin from bovine pancreas, staurosporine, Sigma-Aldrich; 16:1, deuterium (d16)-16:1, prostanoid (PG)F2α, sphingosine 1-phosphate, Cayman (Ann Arbor, MI); recombinant mouse interleukin (IL)-1β, recombinant mouse tumor necrosis factor (TNF)α, recombinant human basic fibroblast growth factor (bFGF), R&D Systems (Minneapolis, MN); recombinant mouse platelet-derived growth factor (PDGF) BB, Invitrogen; mouse endothelin-1, rosiglitazone, A23187, Enzo Life Sciences (Farmingdale, NY); myo-[2-3H]inositol, American Radiolabeled Chemicals Inc. (St. Louis, MO); human recombinant transforming growth factor (TGF)-β, solvents, and all other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan) unless stated otherwise.

**Cells, Cell Viability Assay, and Cell Counting**—Mouse Swiss 3T3 fibroblasts, NIH-3T3 fibroblasts, and 3T3-L1 preadipocytes were cultured at 37 °C in a 5% CO2 incubator in DMEM containing 10% (v/v) heat-inactivated FCS. After 3–4 days, confluent cells were detached using 1 × trypsin/EDTA solution and resuspended at 1.7 × 105 cells in 5 ml of medium in 35-mm dishes. Quiescent cells, used throughout the study, were obtained by maintaining confluent cells in DMEM supplemented with 0.2% BSA for 24 h at 37 °C and 5% CO2. Cells were cultured for the indicated times with insulin (1 μM), PDGF (25 ng/ml), bFGF (10 ng/ml), TGF-β (2.5 ng/ml), rosiglitazone (10 μM), dexamethasone (1 μM), endothelin-1 (100 nm), IL-1β (1 ng/ml), PMA (200 nm), A23187 (2.5 μM), PGE2 (100 nm), TNFα (10 ng/ml), stauroscopein (250 nm), 1–181-sn-PA (10 μM), or sphingosine 1-phosphate (2 μM) at 37 °C and 5% CO2.

To compare the cellular effects of exogenous 16:0 and 16:1, cells were preincubated in the presence of the respective fatty acid (5 or 10 μM) for 4 h. Deuterium-labeled (d16)-16:1 was used in the lipidomic study at 5 μM. The concentration of organic solvents used as vehicle (methanol or dimethyl sulfoxide) was kept below 0.2% (v/v). Treatment of Swiss 3T3 cells with methanol or dimethyl sulfoxide (0.2%, v/v, each) was without effect on their molecular PL composition (PC, PE, phosphatidylserine (PS), PI, PG, or sphingomyelins) as confirmed by LC-MS/MS.

For the determination of cell viability, Swiss 3T3 cells (7,500/well) were seeded into 96-well plates in standard culture medium. After incubation at 37 °C and 5% CO2 for 24 h, cells were starved in DMEM supplemented with 0.2% BSA for 24 h. Then, cells were treated with fatty acids (5 or 10 μM) again for 24 h, and cell viability was determined by the reduction of the tetrazolium salt WST-8 through mitochondrial dehydrogenases using Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD) according to the manufacturer’s instructions.

To investigate the effect of CAY10566 on cell proliferation, Swiss 3T3 cells (80,000/well) were seeded into 12-well plates in DMEM containing 10% (v/v) charcoal-stripped FCS (PAA, Coelbe, Germany) and treated with CAY10566 (0.1 nm–10 μM) and 16:1 (50 μM) for 48 h at 37 °C and 5% CO2. Cell numbers were determined using a Vi-CELL Series Cell Counter (Beckman Coulter, Krefeld, Germany).

**Determination of LPIAT Activity**—Micromoles of cultured cells were isolated according to Ref. 19. In brief, cells (10-cm dish, confluent) were scraped into 0.6 ml of ice-cold buffer containing 20 mM Tris-HCl (pH 7.4), 5 mM mercaptoethanol, 1 mM Na2VO4, and a protease inhibitor mixture (homogenization buffer). After sonication of the cells on ice (3×30 s), the lysate was centrifuged at 9,000 × g for 15 min, the pellet was discarded, and the supernatant was centrifuged again at 100,000 × g for 1 h at 4 °C. Pellets were suspended in homogenization buffer, and the total protein concentration was determined.

To determine lysophosphatidylinositol acyltransferase (LPIAT) activity, enzyme (100,000 × g pellets, 1 μg) was added to mixtures of crude 1-acyl-sn-PI (50 μM) and multiple acyl-CoAs as indicated (in total 50 μM) in 20 mM Tris-HCl (pH 7.4), 5 mM mercaptoethanol, a protease inhibitor mixture, and 1 μM CaCl2. Reactions were stopped by adding 375 μl of chloroform/methanol (1:2, v/v) after 30 min at 37 °C, lipids were extracted using the method of Bligh and Dyer, and formed PLs were analyzed by LC-MS as described previously (20). 1,2-Dimyristoyl (14:0)-sn-PE (0.8 nmol) was used as internal standard. Endogenous PLs (determined from control incubations lacking lyso-PLs) were subtracted to calculate PL formation rates.
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expressed as units (change in MS signal intensity/min). The product nomenclature mentions first the fatty acid from the 1-acyl-sn-PL acceptor and second from the acyl-CoA donor.

Subcellular Fractionation—Nuclear, mitochondrial, membrane, and cytosolic fractions of Swiss 3T3 cells were separated as described in the Abcam protocol (Abcam, Cambridge, MA). Briefly, Swiss 3T3 cells (0.4 × 10^6) in 100 μl of ice-cold 20 mM Tris-HCl (pH 7.4), 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 2 mM mercaptoethanol, and a proteinase inhibitor mixture (subcellular fractionation buffer) were passed through a 25-gauge needle (10 ×), incubated on ice for 20 min, and centrifuged at 720 × g for 20 min (4 °C). The pellet was resuspended in subcellular fractionation buffer, homogenized again (25-gauge needle, 10 ×) and centrifuged (720 × g for 20 min, 4 °C) to obtain the nuclear pellet. The supernatant was subjected to differential centrifugation at 10,000 × g for 20 min (mitochondrial pellet) and 100,000 × g for 1 h (membrane pellet and cytosolic supernatant).

Incorporation of Tritium-labeled Inositol into the Lipid Fraction of Swiss 3T3 Cells—Quiescent Swiss 3T3 cells (confluent, 12-well plate) in DMEM plus 0.2% BSA were treated with bFGF (10 ng/ml) and/or tritium-labeled inositol (2 μCi/ml) for 24 h at 37 °C and 5% CO₂. The incorporation of tritium-labeled inositol into PLs was determined according to Ref. 21. In brief, cell layers were washed extensively with ice-cold phosphate-buffered saline (pH 7.4), exposed to 5% (m/v) trichloroacetic acid (500 μl) for 5 min on ice, and scraped. After centrifugation (20,000 × g, 5 min, 4 °C) and washing with 5% (m/v) trichloroacetic acid, the pellet was dissolved in 1% (m/v) aqueous sodium dodecyl sulfate (100 μl). An aliquot (20 μl) was used for protein determination using DC protein assay kit (Bio-Rad). The remainder was extracted according to the method of Bligh and Dyer (22) and mixed with Rotiszint eco plus (5 ml; Carl Roth GmbH, Karlsruhe, Germany) for liquid scintillation counting in a Packard TRI-CARB 2100TR Liquid Scintillation Analyzer.

Quantitative RT-PCR—Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA), and first-strand cDNAs were synthesized using Superscript III (Invitrogen). PCR (LightCycler System; Roche Applied Science) was conducted according to (20) using FastStart DNA Master SYBR Green I (Roche Applied Science). Sense and antisense primers (0.5 mM, each) are shown in supplemental Table S1. cDNA levels were quantified using the second derivative maximum method of the LightCycler analysis software. Results, given as arbitrary units, were normalized to β-actin mRNA levels.

Extraction of PLs and Sample Pretreatment for LC-MS and LC-MS/MS—PLs and DAGs from cultured cells (4 × 10⁵) and PLs from in vitro activity assays were extracted according to the method of Bligh and Dyer (22). 1,2-Di-14:0-sn-PC and 1,2-di-14:0-sn-PE (0.4 nmol, each) were added to improve recovery and as internal standards. The extracted lipids were dissolved in 100 μl of methanol, diluted, and applied to LC-MS/MS analysis.

For Figs. 3, B, C, F, and G, 4A, and 5E, anionic PLs (PS, PI, PG, and PA) and free fatty acids were extracted according to Ogiso et al. (23). In brief, anionic lipids of cultured cells (4 × 10⁵ in 500–μl aqueous phase) were extracted by n-butyl alcohol (500 μl), mixed with 0.5 ml of methanol/chloroform (1:1, v/v), and separated by anion exchange chromatography using an equilibrated DEAE-cellulose (750-μl) column. Bound PLs were eluted with chloroform/methanol/28% aqueous ammonia/28% acetic acid (200:100:3.0:9, v/v/v/v), dried under vacuum, and redissolved in 100 μl of methanol. 1,2-Di-14:0-sn-PE, 1,2-di-14:0-sn-PS, and 1,2-di-14:0-sn-PG (0.75 nmol each) were added to improve recovery and as internal standards.

Reversed Phase Liquid Chromatography—PLs and free fatty acids (10-μl injection) were separated on an Acquity™ UPLC BEH C₈ column (130 Å, 1 × 100 mm for analysis of PLs; 2.1 × 30 mm for in vitro LPIAT activity assays and analysis of DAGs; Waters, Milford, MA) using an Acquity™ Ultra performance LC system (Waters). Flow rates (0.1 ml/min for BEH C₈, 1 × 100 mm and 0.8 ml/min for BEH C₈, 2.1 × 30 mm), column temperature (45 °C) and gradient (BEH C₈, 1 × 100 mm: 80% 20 mM aqueous ammonium bicarbonate (A)/20% acetonitrile (B) to A/B = 5/95 within 20 min and A/B = 5/95 for 10 min; BEH C₈, 2.1 × 30 mm: A/B = 80/20 to A/B = 5/95 within 8.5 min and A/B = 5/95 for 1.5 min) were adjusted as described (20). DAGs were separated on an Acquity UPLC BEH C₈ column (2.1 × 30 mm) at a flow rate of 0.8 ml/min using a gradient of A/B = 30/70 to A/B = 5/95 within 8.5 min.

Mass Spectrometry—After separation by LC, lipids were detected by a TSQ Vantage Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific) equipped with an HESI-II electrospray ionization source. For the analysis of PLs and free fatty acids, the tune parameters were chosen as described (20). For DAG analysis, the ion spray voltage was set at 3,250 V in the positive ion mode, the heated capillary temperature at 180 °C, the vaporizer temperature to 180 °C, the sheath gas (nitrogen) pressure to 50 p.s.i., and the auxiliary gas (nitrogen) pressure to 10 p.s.i. The scan range of the instrument was set at m/z 150–1200 for PL and free fatty acid analysis and at m/z 500–750 for DAG analysis. For quantification, PC and sphingomyelins were analyzed as [M+H]^+ by m/z = 184 precursor ion scans (positive ion mode, collision energy: 35 V, scan time: 1 s), PS, PE, PI, PG, PA, and free fatty acids were quantified as [M−H]^− by full scans in the negative ion mode with a scan time of 1 s if not indicated otherwise or by single ion monitoring. The identity of the PL headgroup was confirmed for PE by m/z = 141.0 neutral loss scans (positive ion mode, collision energy: 25 V), PS by m/z = 87.0 neutral loss scans (negative ion mode, collision energy: 25 V), and PI by m/z = 241.0 precursor ion scans (negative ion mode, collision energy: 35 V). The fatty acid composition of PLs was determined by detecting the [M−H]^− fatty acid anions by single reaction monitoring (collision energy: 40–45 V) or product ion scans (m/z 200–400, collision energy: 40 V, scan time: 1 s). The higher signal intensity of sn-1 than sn-2 fatty acid anions was utilized to estimate the isomeric position of the fatty acids (24). DAGs were quantified as [M+NH₄]^+ by a full scan in the positive ion mode (scan time: 1 s), and their fatty acid composition was determined by product ion scans (m/z 200–400, collision energy: 20 V, scan time: 1 s) from monoacylglycerol fragment ions after neutral loss of the sn-1 or sn-2 fatty acid. Selective ion monitoring was used to detect PLs from in vitro activity assays (scan time: 0.25 s, scan width: 0.6 m/z units).

In variation with the general method, the LC system was coupled to a TSQ Quantum Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific) for time course (Fig. 3E) and
lipidomic studies using supplemented fatty acids (Fig. 1, D–G). The ion spray voltage was set at 5,500 V in the positive and negative ion mode, the heated capillary temperature to 300 °C, the sheath gas (nitrogen) pressure to 65 p.s.i., and the auxiliary gas (nitrogen) pressure to 5 p.s.i. Other parameters were set as described above.

Mass spectra were processed using the Xcalibur 2.0 Software (Thermo Scientific), and mass spectral peaks were assigned by manual integration and baseline correction. Data are given as relative intensity or total PI intensity. Relative intensities express the amount of molecular PL species as a percentage of the sum of all species detected in the respective PL subclass (= 100%). If isomeric lipids could not be separated by LC, their combined relative intensity is given. The species first mentioned is the major one according to the combined signal intensity of the sn-1 and sn-2 fatty acid anions detected by single reaction monitoring (see above). Brackets were used if the signal intensity of the minor sn-1 and sn-2 fatty acid anions was more than a factor of 10 lower than for the major molecular species (separated by “”). To assess the relative intensities of PL subgroups containing a specific fatty acid, the relative intensities of individual PL species from each dataset were summarized prior to averaging. Signal intensities based on more than one species were ascribed to the major species (not in brackets) of interest for this calculation. To assess the total PI intensity, the detected molecular PI intensities were normalized to the number of cells and the internal standard 1,2-di-14:0-sn-PE and summarized for each PL subclass. Please note that total PI intensities were not corrected for different ionization characteristics because focus was set on the comparison of samples and not on their absolute lipid mass.

Statistics—Data are presented as mean ± S.E. Statistical evaluation of the data were performed by one-way ANOVAs for independent or correlated samples followed by the Tukey HSD post hoc tests or by Student’s t test for paired and correlated samples. p values < 0.05 were considered statistically significant. All statistical calculations were performed using InStat 3.0 (GraphPad Software Inc., La Jolla, CA).

RESULTS

16:1 Specifically Increases Cellular 16:1-PI Levels and Induces Cell Proliferation—Controversial correlations were observed between 16:1 concentrations and associated biological activities with respect to study design and metabolic or nutritional conditions (4–8). From this, we speculated that 16:1 is metabolized to an active component that functions as a lipokine. First, we tested whether Swiss 3T3 fibroblasts are an appropriate model to study the lipokine character of 16:1. The effect of 16:1 on cell proliferation was determined for serum-starved subconfluent Swiss 3T3 cells cultivated for 24 h in the presence of fatty acids (16:0, 16:1, 18:1, or 20:4; 5 μM, each) or 10% FCS as control. Then, their mitochondrial dehydrogenase activity was assessed as a marker of cell viability. Supplementation with FCS increased significantly cell proliferation as expected (Fig. 1A, left panel). Among the fatty acids tested, 16:1 was most effective in promoting cell proliferation (Fig. 1A, left panel). This effect was even more pronounced when cells were stimulated with PDGF (Fig. 1A, right panel), which is a well-known activator of phospholipase C- and phosphatidylinositol-3-kinase-dependent signaling pathways in Swiss 3T3 cells (25, 26). To confirm further a role of 16:1 as mitogenic signaling molecule, we inhibited the biosynthesis of 16:1 (and 18:1) using the specific SCD-1 inhibitor CAY10566 (27). Swiss 3T3 cell proliferation was concentration-dependently decreased by CAY10566 (Fig. 1B; >10 nM) and restored by supplementation with 16:1 (50 μM, Fig. 1C). Please note that additive effects cannot be excluded.

Whether 16:1 may enhance cell proliferation by forming a highly regulated membrane metabolite was investigated by a lipidomic approach. Quiescent Swiss 3T3 cells were supplemented with 16:1 before PLs and free fatty acids were time-dependently assessed by LC-MS/MS. Because changes in the cellular PL composition rather than absolute values were in the focus of interest, results are provided as relative intensities that express the signal intensity of a molecular PL species as a percentage of the total PL subclass intensity. Deuterium-labeled (d14)-16:1 (5 μM) was time-dependently incorporated into all PL subclasses (Fig. 1D) in exchange for 16:1 or after elongation to d16:1-18:1 (28) in exchange for 18:1 (data not shown). However, only the relative intensity of 16:1-containing PL (labeled plus unlabeled) remarkably increased (Fig. 1, E and F). The accumulation in PL seems to be unique for 16:1 as suggested from the weak enrichment of the structurally related fatty acid 16:0 (even at the double concentration of 16:1) (Fig. 1G). The chromatogram with the extracted mass of PL(16:0/16:1) is shown in Fig. 2 for single ion monitoring in the negative ion mode (used to detect the [M–H]⁻ ion; panel A) and m/z = 241.0 precursor ion scans (used to confirm the inositol headgroup; panel B). The mass spectra from negative-ion full scans were averaged over the time window of the respective peak. Fragmentation of the [M–H]⁻ parent ion and detection of the fatty acid anions by product ion scans provide information about the fatty acid composition of the PI species. The chromatographic and mass spectrometric characterization of the 16:1-PI species PI(18:0/16:1) and PI(18:1/16:1) is presented in supplemental Figs. S1 and S2.

Insulin and Growth Factors Induce Enzymes of 16:1 Biosynthesis and Specifically Increase Cellular 16:1-PI Levels—Insulin is a well-known inducer of fatty acid biosynthesis (29). Using RT-PCR, we confirmed that enzymes involved in the biosynthesis of monounsaturated fatty acids (including 16:1), namely acetyl-CoA carboxylase-1, fatty acid synthase, SCD-1 and SCD-2, are induced in Swiss 3T3 cells by stimulation with insulin, PDGF, or bFGF (Fig. 3A). As observed for supplementation with 16:1 (Fig. 1F), insulin and the growth factors PDGF and bFGF increased the relative intensity of the 16:1- and 18:1-containing species PI(16:0/16:1), PI(18:1/16:1), PI(16:0/18:1), PI(18:0/18:1), PI(18:1/18:1), and PI(18:1/18:2) (Fig. 3, B–D), reaching maximum effects after 24 h of stimulation (Fig. 3E).

Comparable results were obtained for bFGF-treated cells independent of whether 16:1- and 18:1-PI species were quantified by full scans in the negative ion mode or by detecting their daughter [M–H]⁻ fatty acid anions using single reaction monitoring (Fig. 3, B and D). The accumulation of 16:1 is highly specific for PI among glycerol-PLs, sphingomyelins, and unsaturated free fatty acids during stimulation with bFGF (Fig. 3F) and occurs relative to polyunsaturated PI species whose pro-
The relative intensity of 16:1-PI strongly decreases (Fig. 3G). The relative intensity of 16:1-PI was comparable for intact cells and nuclear, mitochondrial, and cytosolic fractions but was significantly increased for the membrane fraction (locus of PL biosynthesis) under basal conditions (data not shown). Stimulation with bFGF, however, abolished these differences (data not shown). Total signal intensities of PI were calculated as a sum of individual PI species, normalized to the number of cells, and corrected for the efficiency of lipid extraction using 1,2-di-14:0-sn-PE as internal standard. Significant time-dependent differences in the total signal intensity of PI were not observed during 48 h of stimulation with bFGF (data not shown), indicating relative changes of individual PI species being absolute.

Hormonal stimulation increased the relative intensity of 16:1-PI comparable with supplementation with 16:1 (compare Figs. 3F and 1F). However, even further enhanced was the accumulation of 16:1-PI when bFGF and PDGF were applied together with exogenous 16:1 (4-h incubation, Fig. 1F) suggesting additional mechanisms being activated other than the induction of 16:1 biosynthesis. Additive effects by cotreatment of 16:1 and insulin were not observed (Fig. 1F).

**The Increase of 16:1-PI Is Insulin- and Growth Factor-specific and Not Limited to Swiss 3T3 Cells—**Distinct hormones induce 20:4 release and thus may affect the relative fatty acid composition of PI. In fact, stimulation of Swiss 3T3 cells with bFGF resulted in a rapid decrease of polyunsaturated PI thereby inducing a reversible and nonspecific increase of the remaining PI species (including 16:1-PI) within 10 min to 1 h (Fig. 3E). Levels of 16:1-PI recovered again (4 h) before they were subjected to a second more pronounced, 16:1-PI-specific and long
lasting increase (8–48 h, Fig. 3E). To compare the effects of cellular effectors on this second increase independent of a direct turnover of sn-2–20:4-PI, ratios of 16:1- and sn-2–18:1-PI were calculated. The ratio was significantly elevated for stimulation with insulin and the growth factors bFGF, PDGF, and TGF-β2 but not for other stimuli such as proinflammatory and apoptotic cytokines (IL-1β, TNFα), mitogens (PMA, lyso-PA, sphingosine 1-phosphate (S1P)), and diverse mediators known to affect lipid metabolism (PGF2α, rosiglitazone, dexamethasone, endothelin-1, staurosporine, Ca2+-ionophore A23187) (Fig. 4A).

All experiments so far have been performed with Swiss 3T3 cells. To investigate whether comparable effects also occur in other mouse cell lines, we determined the relative intensity of 16:1-PI in NIH-3T3 fibroblasts and 3T3-L1 preadipocytes after stimulation with bFGF for 24 h. bFGF increased significantly the relative intensity of 16:1-PI in both cell lines (Fig. 4B), indicating the up-regulation of 16:1-PI as the common mechanism.
Formation of 16:1-PI Is Based on the de Novo Pathway of PI Biosynthesis but Not on the Remodeling One—The specific incorporation of 16:1 into PI might be based either on an enhanced activity or altered specificity of LPIATs (which introduce sn-2 fatty acids during remodeling of PI) or on de novo 16:1-PI biosynthesis via formation of 16:1-PA. In vitro activity assays using Swiss 3T3 microsomes as a source of lysophospholipid acyltransferases exclude LPIATs to mediate the incorporation of 16:1 into PI. Preferentially 20:4 and less pronounced 18:1 were introduced into crude sn-2-lyso-PI, whereas 16:0 and 16:1 were hardly found as PI esters (Fig. 5A), and stimulation of Swiss 3T3 cells with bFGF, PDGF, or insulin for 24 h did not...
significantly increase microsomal LPIAT activity (Fig. 5B). However, bFGF comparably promoted the incorporation of tritium-labeled inositol into membrane lipids and of d14–16:1 into PI (Fig. 5C), suggesting the de novo rather than the remodeling pathway being involved in the accumulation of 16:1-PI. In accordance with this, insulin, bFGF, and PDGF were found to induce significantly the mRNA expression of glycerol-3-phosphate-1-acyltransferases (GPATs)-3 and -4 (Fig. 5D), the rate-limiting enzymes of PA biosynthesis (30), providing the enzymatic requirements for an efficient synthesis of 16:1-PA. In fact, limiting enzymes of PA biosynthesis (30), providing the enzymes PI synthase and PG synthase (Fig. 6), which would require different specificities of the downstream enzymes downstream of PA (see Fig. 6) discriminate PL precursors according to their fatty acid composition, or a specific PA pool is used for PI biosynthesis. One critical enzyme mediating specificity might be PA phosphatase that converts PA into DAG for PC, PE, and PS biosynthesis. Neither insulin nor growth factors significantly affected the relative intensity of 16:1-DAG, suggesting PA phosphatase to be incapable of translating an increase of 16:1-PA into elevated levels of 16:1-DAG (Fig. 6). However, whether the increase of PI species containing 16:1 or 18:1 actually underlies the mitogenic effects of 16:1 remains unanswered.

The unique metabolic fate of 16:1 seems to be related to the poor turnover of 16:1-CoA by remodeling LPIATs allowing the de novo PL biosynthesis of PI to determine the 16:1 profile. This is surprising because (i) the remodeling pathway and not de novo PL biosynthesis usually shapes the sn-2 profile of PLs and (ii) changes in PA are generally not specifically translated into single PLs (1). The mechanisms by which 16:1 is specifically introduced into PI are not fully understood. Either PL-biosynthetic enzymes downstream of PA (see Fig. 6) discriminate PL precursors according to their fatty acid composition, or a specific PA pool is used for PI biosynthesis. One critical enzyme mediating specificity might be PA phosphatase that converts PA into DAG for PC, PE, and PS biosynthesis. Neither insulin nor growth factors significantly affected the relative intensity of 16:1-DAG, suggesting PA phosphatase to be incapable of translating an increase of 16:1-PA into elevated levels of 16:1-DAG (Fig. 6). A subsequent redirection of 16:1-PA into CDP-DAG biosynthesis via CDP-DAG synthase might explain the preference of the CDP-DAG pathway but not the differences between PI and PG, which would require different specificities of the downstream enzymes PI synthase and PG synthase (Fig. 6).

It should be noted that insulin and growth factors induce enzymes of 16:1 and PA biosynthesis (Figs. 3A and 5D) and elevate the proportion of 16:1-PA (Fig. 5E) but leave the level of free 16:1 unchanged (Fig. 3F). A strict control of intracellular free fatty acid levels is well known for the eicosanoid precursor 20:4 (whose proportion remained unchanged under these assay conditions as well; data not shown) (31) and might also apply to 16:1.

Our study describes a correlation between 16:1-PI biosynthesis and cell proliferation. Supplementation of 16:1 specifi-
cally increased 16:1-PI levels, induced Swiss 3T3 cell proliferation, and restored the proliferation rate of cells whose 16:1 biosynthesis was blocked by inhibition of SCD-1 (28). However, our study cannot exclude that mechanisms other than the formation of 16:1-PI also contribute to the mitogenic potential of 16:1. Particularly the conversion of 16:1 to 18:1 and the enrichment of specific 18:1-containing PI species (Fig. 3D) might bear functional consequences.

The observed effects on 16:1-PI formation and cell proliferation occur at physiologically relevant 16:1 concentrations. Peak levels of 16:1 found in human plasma are considerably higher than the low micromolar concentrations of 16:1 applied in our cellular studies (32). The concentration of exogenous 16:1 is substantially changed through altered lipid metabolic states in contrast to the majority of fatty acids (5–8, 10, 11). (ii) Efficiency is already achieved at low micromolar concentrations (our study). An intermediate formation of 16:1-PI during 16:1 signaling would also explain the partly controversial results from human and animal studies describing correlations between biological functions and circulating concentrations of 16:1 (4–11). The cellular proportion of 16:1-PI would namely

![Diagram A](image1.png)

**FIGURE 5. The inducible 16:1-PI formation depends on de novo PI biosynthesis.** A, LPIAT activities of microsomes from unstimulated Swiss 3T3 cells were determined for crude lyso-PI (50 μM) and a mixture of 16:0-, 16:1-, 18:1-, and 20:4-CoAs (12.5 μM, each). B–F, Swiss 3T3 cells were treated with or without bFGF (10 ng/ml), insulin (1 μM), or PDGF (25 ng/ml) for 24 h. G, microsomal LPIAT activities were measured for crude lyso-PI (50 μM) and a mixture of 16:0-, 18:1-, and 20:4-CoAs (16.7 μM, each). C, left, the incorporation of tritium-labeled inositol (2 μCi/ml) into the cellular lipid fraction was determined after 24 h. Decays per minute (dpm) were normalized to the amount of protein in the cell lysate. Right, relative intensities of 1-d14-16:1-containing PI species are given for cells incubated in presence of d14-16:1 (5 μM). D, mRNA expression of enzymes of PA biosynthesis and PI remodeling was determined by RT-PCR and is given as a percentage of untreated cells; n.d., not detectable; GPAT, glycerol-3-phosphate acyltransferase; LPAAT, lyso phosphatic acid acyltransferase. E and F, relative intensity of 16:1-containing PA (E) and DAG (F) is shown. Data are given as means ± S.E. (error bars); n = 3–4. *, p < 0.05; **, p < 0.01; ***, p < 0.01 versus the untreated control (w/o); ANOVA + Tukey HSD post hoc tests.
not only be dependent on the plasma level of 16:1 but also on its organism-, tissue-, cell type-, and condition-specific metabolism. Future biological studies addressing the lipokine functions of 16:1 should therefore not only assess 16:1 plasma but also 16:1-PI tissue concentrations.

We set our focus on the investigation of the metabolism and signaling of 16:1 as lipokine. However, our study also provides novel insights into the signal transduction pathways of the tyrosine kinase receptor ligands, insulin, PDGF, and bFGF. These hormones induce enzymes of 16:1 biosynthesis and specifically increase the amount of 16:1-PI that might amplify their mitogenic signaling.

Taken together, the extensive research over the last years revealed unique features of 16:1 among fatty acids that rationalize its classification as lipid hormone. Conclusions drawn from these studies were partly conflicting and complicated by the lack of knowledge of the molecular mechanism through which 16:1 mediates its lipokine functions. Here, we characterized 16:1 as a mitogen, identified 16:1- and 18:1-PI as its major metabolites, and discussed a role of 16:1- and/or 18:1-PI in 16:1 signaling. Moreover, we described the inducible biosynthesis of 16:1-PI from which we can explain the unique metabolic fate of 16:1 compared with other fatty acids.

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