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Occurrence and Biological Activity of Palmitoleic Acid Isomers in Phagocytic Cells

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ABBREVIATIONS: 16:1*n-7*, palmitoleic acid (*cis*-9-hexadecenoic acid); 16:1*n-9*, *cis*-7-hexadecenoic acid; 16:1*n-10*, sapienic acid (*cis*-6-hexadecenoic acid); DMDS, dimethyl disulfide; GC/MS, gas chromatography/mass spectrometry, LC/MS, liquid chromatography/mass spectrometry; LPS, bacterial lipopolysaccharide; PC, choline glycerophospholipids; PE, ethanolamine glycerophospholipids; PI, phosphatidylinositol; PS, phosphatidylserine.

ABSTRACT

Recent studies have highlighted the role of palmitoleic acid, *cis*-9-hexadecenoic acid (16:1*n-7*), as a lipid hormone that coordinates cross-talk between liver and adipose tissue, and exerts anti-inflammatory protective effects on hepatic steatosis and insulin signaling in murine models of metabolic disease. More recently, a palmitoleic acid isomer, *cis*-7-hexadecenoic acid (16:1*n-9*) that also possesses marked anti-inflammatory effects, has been described in human circulating monocytes and monocyte-derived macrophages. By using gas chromatographic/mass spectrometric analyses of dimethyl disulfide derivatives of fatty acyl methyl esters, we describe in this study the presence of a third 16:1 isomer, 6-*cis*-hexadecenoic acid (16:1*n-10*), or sapienic acid, in phagocytic cells. Cellular levels of 16:1*n-10* appear to depend not only on the cellular content of linoleic acid, but also on the expression level of FADS2, thus revealing a complex regulation both at the enzyme level, via fatty acid substrate competition, and directly at the gene level. However, unlike 16:1*n-7* and 16:1*n-9*, 16:1*n-10* levels are not regulated by the activation state of the cell. Moreover, while 16:1*n-7* and 16:1*n-9* manifest strong anti-inflammatory activity when added to the cells at low concentrations (10 μM), notably higher concentrations of 16:1*n-10* are required to observe a comparable effect. Collectively, these results suggest the presence in phagocytic cells of an unexpected variety of 16:1 isomers which can be distinguished on the basis of their biological activity and cellular regulation.

INTRODUCTION

Recent studies have revealed that in animal models of metabolic disease, the adipose tissue releases the monounsaturated fatty acid palmitoleic acid (*cis*-9-hexadecenoic acid; 16:1*n-7*), which suppresses hepatic steatosis and improves insulin sensitivity in the whole body [1, 2]. Thus this fatty acid has taken the spotlight as a promising anti-inflammatory lipid that may help ameliorate metabolic disorders [1, 2]. There is a large body of literature suggesting that monounsaturated fatty acids are beneficial to human diet [3]. The ratio of saturated to unsaturated fatty acids constitutes an important property of the phospholipid composition of biological membranes. Changes to this proportion are thought to exert deleterious effects on cells and, in particular, reduction in the number of unsaturated fatty acids in membranes may contribute to the development of a number of pathophysiological states such as cardiovascular disease, diabetes, and cancer [3]. From a biophysical point of view, monounsaturated fatty acids are 'good' to biological membranes because, being liquid at body temperature yet not easily oxidized, help maintain membrane fluidity within the appropriate limits.

However, aside from, or in addition to these biophysical effects, there appears to be something else to 16:1*n-7* that makes it unique in terms of biological activity, leading to the concept of this fatty acid serving as a lipid hormone, or 'lipokine' that coordinates metabolic responses between tissues [1, 2]. Work in murine models generally supports a role for 16:1*n-7* as a novel anti-inflammatory mediator and a positive correlate of insulin sensitivity [4]. Accordingly, in cells of murine origin, circulating 16:1*n-7* increases basal glucose uptake and activates insulin signaling, enhances β-cell function, improves glycemic control, increases glucose transport into skeletal muscle, and prevents palmitate-induced endoplasmic reticulum stress and apoptosis. [1, 2, 4-7]. Studies in humans, however, have suggested different outcomes [8, 9]. Circulating levels of 16:1*n-7* in humans have been reported to correlate positively with the degree of hepatic steatosis, as well as adiposity that promotes fat deposition in hepatocytes [10-11]. A recent study has found a positive correlation between circulating 16:1*n-7* levels and markers of inflammation in young healthy Canadians [13].

Thus, although a unifying mechanistic hypothesis that may help reconcile the variety of effects that 16:1*n-7* appears to serve has yet to emerge, the recent finding that innate immune cells contain an isomer of 16:1*n-7*, namely *cis*-7-hexadecenoic acid (16:1*n-9*) that also displays potent anti-inflammatory activity [14], has raised the challenging possibility that this multiplicity of actions and the compartmentalized manner in which they occur could be due, at least in part, to the overlapping actions of such isomers being present at the same or neighboring locations. 16:1*n-9* is synthesized by human phagocytic cells via β-oxidation of oleic acid, and its levels are elevated in the neutral lipids of lipid droplet-ladden monocytes, suggesting the possibility that detection of increased levels of this fatty acid in monocytes may constitute a biomarker for foamy cell formation [14]. When added exogenously, 16:1*n-9* exhibits potent anti-inflammatory actions *in vitro* and *in vivo* that are distinguishable from that of 16:1*n-7*, and comparable in magnitude to that exerted by *n-3* fatty acids [14].

In the current study we report that human phagocytic cells also express low levels of a third 16:1 isomer, *cis*-6-hexadecenoic acid (sapienic acid, 16:1*n-10*). Despite the common assumption that 16:1*n-10* is restricted to humans [15], we found relatively high levels of this fatty acid also in murine macrophages and in murine macrophage-like cell lines. In these cells, 16:1*n-10* levels appear to be controlled by complex mechanisms of regulation, including substrate competition and gene expression. Of note, 16:1*n-10* shows anti-inflammatory activity when added to cells, but the activity is observed at higher concentrations (>25 μM) than those required for 16:1*n-7* and 16:1*n-9* to exert similar effects.

MATERIALS AND METHODS

Cell isolation and culture conditions

Human monocytes were isolated from buffy coats of healthy volunteer donors obtained from the

Centro de Hemoterapia y Hemodonación de Castilla y León. Written informed consent was obtained from each donor. In brief, blood cells were diluted 1:1 with PBS, layered over a cushion of Ficoll-Paque, and centrifuged at 750 x *g* for 30 min. The mononuclear cellular layer was recovered and washed three times with PBS, resuspended in RPMI 1640 medium supplemented with 40 μg/ml gentamicin, and allowed to adhere in sterile dishes for 2 h at 37°C in a humidified atmosphere of CO_2/air (1:19). Nonadherent cells were removed by washing extensively with PBS, and the remaining attached monocytes were used the following day [14, 16, 17]. Human macrophages were obtained by incubating plastic-adhered monocytes in RPMI with heat-inactivated 5% human serum and 40 μg/ml gentamicin for 2 weeks in the absence of exogenous cytokine mixtures [16]. All procedures involving human samples were undertaken in accordance with the Spanish National Research Council Committee on Bioethics, under the guidelines established by the Spanish Ministry of Health and the European Union.

Murine resident peritoneal macrophages from Swiss mice (University of Valladolid Animal House, 10–12 week old) were obtained by peritoneal lavage using 5 ml of cold PBS, as described elsewhere [18, 19]. The cells were plated at 2 x 10^6 per well (six-well plates) in 2 ml of RPMI 1640 medium with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin, and allowed to adhere for 20 h in a humidified atmosphere of 5% CO_2 at 37°C. Wells were extensively washed with PBS to remove nonadherent cells. Adherent macrophages were then used for experimentation. All the protocols and procedures were approved by the Institutional Animal Care and Usage Committee of the University of Valladolid, and are in accordance with the Spanish and European Union guidelines for the use of experimental animals. The macrophage-like cell lines RAW264.7, $P388D_1$, and THP-1 were maintained in culture and handled exactly as described elsewhere [20-27].

For experiments, the cells were incubated in fresh serum-free medium for 1 h before addition of the various stimuli for different times. Unstimulated cells received vehicle. Opsonized zymosan

was prepared exactly as described [28]. No endogenous phospholipase A_2 activity was detected in the zymosan batches used in this study, as assessed by *in vitro* assay. Total cellular protein was measured utilizing a commercial kit (BioRad Protein Assay). Protein levels did not significantly change over the course of the experiments.

GC/MS of fatty acid methyl esters

The samples, corresponding approximately to 10^7 cells, were washed with PBS, lysed with water, and sonicated in a tip homogenizer twice for 15 s. Afterward, 10 nmol of 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine and 4 nmol of 1,2,3-triheptadecanoylglycerol was added, and total lipids were extracted according to Bligh and Dyer [29]. Lipid classes were separated by thin-layer chromatography. For neutral lipid separation, a solvent mixture consisting of *n*-hexane/diethyl ether/acetic acid (70:30:1, v/v/v) was used as the mobile phase [30]. For analysis of phospholipid subclasses, the plates were predeveloped with chloroform/methanol (1:1, v/v) and, after air-dried, they were impregnated with 1.5% boric acid in ethanol/water (1:1, v/v). Phospholipids were separated twice with chloroform/methanol/28% ammonium hydroxide (60:37.5:4, v/v/v) as the mobile phase [31]. The various lipid classes were scraped and extracted from the silica with 1 ml of chloroform/methanol (1:2, v/v) followed by 1 ml chloroform/methanol (2:1, v/v). The lipid extracts thus obtained were treated with 500 µl of 0.5 M KOH in methanol for 60 min at 37°C to obtain fatty acid methyl esters. To neutralize, 500 µl of 0.5 M HCl was added. Extraction of the fatty acid methyl esters was carried out with 2 ml *n*-hexane, and 1 µl was subjected to gas chromatography/mass spectrometry analysis as previously described [14, 16, 32], using an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass-selective detector operated in electron impact mode (EI, 70 eV), equipped with an Agilent 7693 autosampler, and an Agilent DB23 column (60 m length x 0.25 mm internal diameter x 0.15 µm film thickness). Data analysis was carried out with the Agilent G1701EA MSD Productivity Chemstation software, revision E.02.00.

Dimethyl disufide (DMDS) derivatization of fatty acid methyl esters

Derivatization of fatty acid methyl esters to DMDS adducts was carried out according to the procedure described by Sansone *et al*. [33], with slight modification. Briefly, the sample, containing the fatty acid methyl esters in *n*-hexane (70-100 µl) was placed in a screw amber cap vial with teflon-lined cap. 100 µl of dimethyl disulfide (Sigma Aldrich) was added, together with 50 µl of a 6% solution of iodine in diethyl ether. The vial was capped under nitrogen. The reaction was stirred at 40°C overnight. Afterward, 1 ml of *n*-hexane and 1 ml of 5% sodium thiosulfate solution were added. After mixing vigorously, the sample was centrifuged, the organic phase was collected, dried with anhydrous sodium sulfate, concentrated, and subjected to gas chromatography/mass spectrometry analysis. DMDS derivatives were analyzed with the same protocol as fatty acid methyl esters, with slight modifications of the oven conditions. The final ramp temperature was set at 10°C/min up to 250ºC, and this temperature was held for 20 min to allow the DMDS derivatives of 16:1 isomers elute. Identification of 16:1 isomers was carried out by elution time of the different DMDS adducts, and by specific fragments of the mass spectra generated by cleavage of the C-C bond that originally contained the double bond (ω-fragment, Δ-fragment and Δ-fragment with the loss of methanol).

LC/MS of 16:1-containing phospholipids

A cell extract corresponding to 5 x 10^6 cells was used for these analyses. The following internal standards were added: 600 pmol each of 1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine, 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoinositol, and 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine, before lipid extraction according to the method of Bligh and Dyer [29]. After evaporation of the organic solvent under vacuum, lipids were redissolved in 100 µl methanol/water (9:1, v/v), and injected into a high-performance liquid chromatograph equipped with a binary pump Hitachi LaChrom Elite L-2130 and a Hitachi Autosampler L-2200 (Merck). The column was a Supelcosil LC-18 (5 µm particle size, 250 × 2.1 mm) (Sigma-Aldrich) protected with a Supelguard LC-18 (20 × 2.1 mm) guard cartridge (Sigma-Aldrich). Mobile phase was a gradient of

solvent A (methanol/water/n-hexane/32% ammonium hydroxide, 87.5:10.5:1.5:0.5, v/v/v/v) and solvent B (methanol/n-hexane/32% ammonium hydroxide, 87.5:12:0.5, v/v/v). The gradient was started at 100% solvent A; it was decreased linearly to 65% solvent A, 35% solvent B in 20 min, to 10% solvent A, 90% solvent B in 5 min, and to 0% solvent A, 100% solvent B in an additional 5 min. Flow rate was 0.5 ml/min, and 80 µl of the lipid extract was injected. The liquid chromatography system was coupled online to a Bruker esquire6000 ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The total flow rate into the column was split and 0.2 ml/min entered into the electrospray interface of the mass spectrometer. Nebulizer gas was set to 30 psi, dry gas to 7 l/min and dry temperature to 325°C. PE, PI and PS were detected in negative ion mode with the capillary current set at +3500 V over the initial 25 min as $[M-H]^-$ ions. PC species were detected over the elution interval from 25 to 35 min in positive ion mode, as $[M+H]^+$ ions, with the capillary current set at −3500 V. 16:1-Containing PE, PI and PS species were identified by comparison with previously published data [34-39]. Cut off parameter was set at m/z 150 and fragmentation amplitude at 1 arbitrary unit. Because of the lability of vinyl ether linkages in acid media, plasmanyl (1-alkyl) and plasmenyl (1-alk-1'-enyl) glycerophospholipids were distinguished by acidifying the samples before lipid extraction. For the identification of acyl chains of 16:1-containing PC species, ionization was carried out in negative mode with post-column addition of acetic acid at a flow rate of 100 µl/h as $[M+CH_3CO_2]^-$ adducts, and acyl chains were identified by MS^3 experiments [34-39].

Real-Time PCR

Total RNA was extracted using Trizol reagent (Ambion). The cDNA templates were synthesized using Verso cDNA Synthesis Kit (Thermo Scientific), following the manufacturer's instructions. Quantitative PCR (qPCR) was carried out with an ABI 7500 machine (Applied Biosystems, Carlsbad, CA) using Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies, Santa Clara, CA) as previously described [40].

Immunoblot analysis

After the different treatments, the cells were lysed for 30 min in ice-cold buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 100 mM Na_3VO_4, 1 mM phenyl methyl sulfonyl fluoride, and protease inhibitor cocktail (Sigma). Total protein (50 μg) was resolved on 10–12% SDS-PAGE gels and transferred to a PVDF membrane. After transfer, nonspecific binding sites were blocked with 5% bovine serum albumin in PBS containing 0.1% Tween-20 at room temperature for 1 h. The membranes were then probed with the corresponding antibodies followed by HRP-conjugated secondary antibodies in blocking solution. β-Actin was used as a load control. The immunoblots were visualized using enhanced luminescence. Densitometry was performed on scanned images using Quantity One® software (Bio-Rad Laboratories), and values were normalized for the corresponding controls of each experiment.

Statistical Analysis

All experiments were carried out at least three times with incubations in duplicate or triplicate, and the data are expressed as mean ± SE. Statistical analysis was carried out by Student's *t*-test, with $p < 0.05$ taken as statistically significant.

RESULTS

We have previously shown that human monocytes and macrophages contain significant amounts of *cis*-7-hexadecenoic acid (16:1*n-9*), an unusual isomer of palmitoleic acid (16:1*n-7*) [14]. We began the current study by investigating the possible occurrence of other unidentified 16:1 isomers in human phagocytic cells. For these experiments we prepared fatty acid methyl esters of cellular fatty acids, which were then converted into dimethyl disulfide (DMDS) derivatives. DMDS derivatization provides unambiguous analytical resolution of positional isomers, since methyl sulfide substituents add to the two carbon atoms of the double bond, thus preventing isomerization [41]. Fig. 1 shows a

gas chromatography/mass spectrometry analysis of a mixture of four commercial 16:1 isomers, namely sapienic acid (16:1*n-10*), *cis*-7-hexadecenoic acid (16:1*n-9*), palmitoleic acid (16:1*n-7*), and palmitvaccenic acid (16:1*n-5*). All four isomers resolved well in gas chromatography, and each of them produced specific fragments with electron-impact ionization mass spectrometry, since fragmentation occurred at the level of the C–C bond where the sulfide groups are attached. In addition, a third fragment was detected, which corresponds to the loss of 32 mass units (methanol) from the fragments containing the methyl ester (Fig. 1). Collectively, these data indicate that analysis of fatty acyl DMDS derivatives by gas chromatography (retention time) coupled to electron impact mass spectrometry (detection of diagnostic peaks) allows unambiguous identification of the various isomers.

Application of this technique to analyze the occurrence of 16:1 isomers in human peripheral blood monocytes confirmed the predominant presence of 16:1*n-7* and 16:1*n-9*, in agreement with our previous estimates (Fig. 2) [14]. Interestingly, significant amounts of 16:1*n-10* could also be detected, albeit at lower amounts than those of the other isomers (Fig. 2A). Remarkably, the analysis of human monocyte-derived macrophages showed a different pattern of expression; while 16:1*n-7* and 16:1*n-9* were readily detected, the levels of 16:1*n-10* were much lower in comparison. Traces of 16:1*n-5* were also detected in macrophages (Fig. 2B). In human serum, 16:1*n-7* was, by far, the major isomer detected. Lower amounts of 16:1*n-9* and even lower of 16:1*n-10* were also detected. No 16:1*n-5* was found in serum (Fig. 2C). Collectively, these results suggest that production of 16:1*n-10* by human cells could be cell-type specific, being detectable at significant amounts only in monocytes. On the other hand, since 16:1*n-5* was found only in macrophages and at trace levels, this isomer was not considered further.

The occurrence of 16:1 isomers was also studied in monocytes activated with different stimuli. In the first place we used arachidonic acid, which can be secreted by damaged endothelium and possesses monocyte-activating properties [42]. We have recently shown that human monocytes

exposed to this fatty acid (10 μM, 2 h) acquire a foamy phenotype which is due to the accumulation of cytoplasmic lipid droplets [14, 16]. Lipid loading of the monocytes under these conditions is due to activation of the *de novo* pathway of lipid biosynthesis by arachidonic acid, and ultimately results in the accumulation of 16:1*n-9* and 16:1*n-7* in various lipid fractions [14, 16]. In keeping with our previous observations, monocytes activated by arachidonic acid demonstrated a time-dependent increase in the levels of both 16:1*n-9* and 16:1*n-7* (Fig. 3A). In contrast, the cellular levels of 16:1*n-10* did not significantly change. Other monocyte stimuli such as bacterial LPS (Fig. 3B), yeast-derived zymosan (Fig. 3C) or the Ca^{2+} ionophore A23187 (Fig. 3D) all failed to increase the levels of any of the three 16:1 isomers. Thus, while there are activation conditions that result in up-regulation of 16:1*n-9* and 16:1*n-7* levels. i.e. these leading to the formation of lipid-laden monocytes, 16:1*n-10* levels appear not to be regulated upon any state of activation of the cells. Note as well that differentiation of monocytes to macrophages also does not up-regulate 16:1*n-10* levels (*cf*. Fig. 2A and 2B).

Importantly, 16:1*n-10* was found at quite significant levels in cells of murine origin such as resident peritoneal macrophages (Fig. 4A). Moreover, when we analyzed RAW264.7 cells, a murine cell line which is widely used as a paradigm for studies of macrophage lipid signaling and metabolism [43], 16:1*n-10* was detected at levels comparable to those of 16:1*n-7*, and much higher than those of 16:1*n-9* (Fig. 4B). High levels of 16:1*n-10* were also observed in another murine-macrophage-like cell line, $P388D_1$ (Fig. 4C). When the study was extended to a human cell line, THP-1, 16:1*n-10* was also found at high levels (Fig. 4D).

The distribution of 16:1 fatty acids among phospholipids of RAW264.7 cells was measured by LC/MS. 16:1-Containing phospholipids were identified by determining the formation of a m/z 253 fragment in MS^n experiments, corresponding to a 16:1 acyl chain. Structural identification of the glycerophospholipids was achieved by looking at the fragments and/or neutral losses obtained in MS^2 experiments for PE, PI and PS, or MS^3 experiments for PC [34-39]. Fatty chains within phospholipids

are designated by their number of carbons : double bonds. A designation of O- before the first fatty chain indicates that the sn-1 position is ether-linked, while a P- designation indicates a plasmalogen form (*sn-1* vinyl ether linkage) [44].

A total of 15 glycerophospholipid species were found to contain 16:1 acyl chains, with PC species being, by far, the most enriched in these fatty acids. Remarkably, a single PC species, PC(16:0/16:1), comprised almost half of total cellular 16:1. By class, PC contained ~70-80% of total 16:1, the remainder being present in PE (~8-10%), PS (~7-9%), and PI (~2%) (Fig. 5A & 5B). GC/MS was utilized next to differentiate among 16:1 isomers within the different phospholipid classes. Approximately 50% of total 16:1 in PC was found to correspond to the *n-7* isomer, while the *n-10* comprised about 40%. 16:1*n-7* was also the most abundant isomer in PE (~70%), and 16:1*n-10* was the most abundant one in PI (~60%) and PS (~85%). 16:1*n-9* was detected at significant levels only in PC and PE (Fig. 5C, 5D, 5E & 5F). This distribution did not appreciably change when the cells were stimulated with bacterial LPS.

As a consequence of long term culture, many cell lines are known to contain low levels of essential fatty acids compared with primary cells [45], and we confirmed this to be true as well for the cell lines utilized in this study (Fig. 6). While the double bond of both 16:1*n-9* and 16:1*n-7* is introduced by stearoyl-CoA desaturase (Δ^9 desaturase), 16:1*n-10* is produced by a different enzyme, fatty acid desaturase 2 (FADS2, Δ^6 desaturase). In keeping of these observations, incubation of the cells with the selective Δ^6 desaturase inhibitor SC-26196 [46, 47] strongly inhibited 16:1*n-10* levels, while leaving 16:1*n-9* and 16:1*n-7* levels unchanged (Fig. 7A).

Since FADS2 also converts linoleic into γ-linolenic acid within the *n-6* fatty acid synthesis pathway, we reasoned that high levels of 16:1*n-10* in cultured cells could reflect, at least in part, an increased access of Δ^6 desaturase to palmitic acid due to the relative scarcity of linoleic acid. In keeping with this observation, we noted that the palmitic acid (16:0) to linoleic acid (18:2*n-6*) mol ratio in cultured cell lines is several fold higher than that of primary cells (Fig. 7B). Further, long-

term incubation of the cells with exogenous linoleic acid led to a significant nmol reduction of 16:1*n-10* levels, which corresponded with a similar nmol increase of *n-6* products in cells (Fig. 7C). Importantly, analyses of *FADS2* expression by qPCR clearly demonstrated higher expression levels of the gene in cultured cell lines with respect to primary cells (Fig. 7D). Thus, high expression levels of *FADS2*, together with higher 16:0/18:2*n-6* ratios (i.e. low 18:2*n-6* levels) would explain why cell lines produce more 16:1*n-10* than primary cells. Thus, increased 16:1*n-10* levels in cultured cells is not only a consequence of favored desaturation of palmitic acid over linoleic acid due to reduced levels of the latter, but also to an increased expression of the Δ^6 desaturase.

We have recently shown that both 16:1*n-7* and 16:1*n-9* possess potent anti-inflammatory activity both *in vivo* and *in vitro* [14]. To extend these results to 16:1*n-10*, we prepared cells enriched in the various isomers by incubating them with a low concentration (10 μM) of the corresponding fatty acid for 14 h in serum-free medium [14]. This procedure results in the cells taking up the fatty acids and accumulating them in various cellular lipids, mainly phospholipids (Fig. 8A). In keeping with our previous data [14], subtle differences were found between 16:1*n-7* and 16:1*n-9* with regard to their incorporation into cellular lipid classes; the latter manifesting greater tendency to accumulate into neutral lipids than the former. Significant amounts of 16:1*n-10* were also found in neutral lipids (Fig. 8A). Further analyses of 16:1 incorporation into phospholipid classess revealed that, in keeping with the distribution of the endogenous fatty acids, most of the exogenous fatty acids incorporated into PC, with lesser amounts being found in PE. Exogenous 16:1*n-7* was found to be a little more enriched in PE than 16:1*n-10* or 16:1*n-9*; the relative distribution of the latter two being very similar (Fig. 8B).

After the incubations with the fatty acids, the cells were stimulated with bacterial LPS, and the effect on the expression of a number of proinflammatory genes was investigated. In agreement with our previous data [14], cells enriched with 10 μM 16:1*n-7* or 16:1*n-9* showed significant inhibitory effects on the expression level of a number of inflammatory genes. Remarkably, these

effects were not observed in the 16:1*n-10*-enriched cells at this concentration (Fig. 9A,B,C); significantly higher concentrations of 16:1*n-10* (>25 μM) were necessary to observe a similar anti-inflammatory effect, as shown in dose-response studies (Fig. 9D). At doses at which 16:1*n-10* was ineffective (<25 μM), it did not alter the anti-inflammatory effect of 16:1*n-9* (Fig. 9E). These results show that, although all 16:1 isomers present in macrophages may manifest anti-inflammatory activity, there is a marked difference between 16:1*n-7* and 16:1*n-9* on the one hand, and 16:1*n-10* on the other with regard to the range of concentrations at which an effect is demonstrated. Interestingly, when yeast-derived zymosan was used instead of LPS to stimulate the cells, none of the fatty acids exerted significant effects on proinflammatory gene expression (Fig. 9F). Thus the anti-inflammatory effects of 16:1 fatty acids may be specific for LPS stimulatory conditions, suggesting an interference with signals arising from TLR4 engagement. In this regard, a hallmark of LPS activation of macrophages is the rapid phosphorylation activation of the extracellularly-regulated mitogen-activated protein kinases p42 and p44 [48]. Fig. 10 shows that 10 μM 16:1*n-9* blunted the LPS-induced phosporylation of the two kinases in a time-dependent manner, while 16:1n-*10* was ineffective.

DISCUSSION

In this study we show that human and murine phagocytic cells contain, in addition to 16:*1n-7* and 16:1*n-9*, a third 16:1 isomer, namely 16:1*n-10*. Originally described in human sebum and later in hairs and nails, 16:1*n-10* was commonly thought not to be present anywhere else in the human body [15, 49, 50]. However, 16:1*n-10* has recently been identified in erythrocyte membranes [41]. Thus, our identification in circulating human monocytes was certainly no surprise, although the low levels at which the fatty acid presents in monocytes and macrophages make it difficult to envision a biological role for it in innate immunity and inflammation. More unexpected was, however, to identify 16:1*n-10* at relatively high levels in mouse peritoneal macrophages and, especially, cultured

macrophage cell lines, because this fatty acid is conventionally considered to be unique to humans (hence its trivial name, sapienic acid, deriving from the root *sapiens*).

While the double bond present in 16:1*n-7* and 16:1*n-9* is introduced by a Δ^9 desaturase enzyme (stearoyl-CoA desaturase), 16:1*n-10* is produced by the action of a different enzyme, Δ^6 desaturase (fatty acid desaturase 2, FADS2). Our results indicate that, consistent with these different metabolic origins, cellular regulation of 16:1*n-10* levels differs from that of 16:*1n-7* and 16:1*n-9* in several key respects. The levels of the latter two fatty acids may be regulated during lipid loading conditions, owing to stimulus-induced activation of *de novo* fatty acid biosynthesis [14, 16, 51, 52]. In contrast, the levels of 16:1n-*10* appear not to depend on the activation state of the cells, but rather on complex, possibly interacting mechanisms involving both substrate competition reactions and increased expression of FADS2.

It has been long thought that desaturation at carbon 6 is greatly favored when the fatty acid substrate already contains a double bond at position 9, as is the case of linoleic acid [53]. Accordingly, in cells, desaturation of linoleic acid to γ-linolenic acid would be preferred over desaturation of palmitic acid to 16:1*n-10*, which would easily explain why cells containing high endogenous levels of linoleic acid produce 16:1*n-10* at very low amounts, and *vice versa*. While this idea is fully consistent with our results using human phagocytic cells and cultured cell lines, it is worth noting that mouse peritoneal macrophages, which contain large amounts of linoleic acid and, in general, of *n-6* fatty acids, amounting to approximately 25% of total cellular fatty acids [54, 55], also contain relatively high levels of 16:1*n-10*. Moreover, our studies using macrophage cell lines, which contain low levels of linoleic acid, show much greater expression of FADS2 relative to primary cells, suggesting that not only the palmitic acid to linoleic acid mol ratio but also the amount of enzyme present in the cell constitutes another key factor regulating 16:1*n-10* levels. Experiments are currently in progress to explore questions such as whether FADS2 operates constitutively at different efficiency depending on cell type, or whether there are cell-specific co-factors that may stimulate FADS2

preference for palmitic acid utilization. It is also possible that, depending upon cell type, linoleic acid is diverted to other reactions, i.e. serving as substrate of lipoxygenases for oxylipin formation, thus enabling the enzyme to use palmitic acid to a larger extent.

A striking feature of this work is that, unlike 16:1*n-7* and 16:1*n-9*, 16:1*n-10* is devoid of anti-inflammatory activity when used at low concentrations (up to 25 μM), as judged by its inability to prevent the increased expression of pro-inflammatory cytokines by LPS-stimulated macrophages. Higher concentrations of 16:1*n-10* are required to observe an effect. It is worth mentioning in this context that high concentrations of 16:1*n-10* (100 μM and above) have been demonstrated to possess antimicrobial activity in skin by a mechanism involving partitioning of the fatty acid into the cell membrane [56, 57]. While a change in cellular lipid composition due to the incorporation of the various 16:1 isomers may itself have an effect on receptor-mediated responses, our initial studies on the mechanism of regulation of LPS signaling by 16:1*n-9* show a selectively reduced phosphorylation of the extracellular-signal regulated kinases p42 and p44 by LPS in the presence of the fatty acid, thus suggesting genuine interaction of the fatty acid with recepor-activated signaling. It is also striking that 16:1 fatty acids blunt LPS-mediated signals only and not those from other innate immune receptors such as TLR2, dectin-1, or mannose receptors, which recognize yeast-derived zymosan [58], emphasizing selective receptor-mediated effects. This situation is reminiscent of recent work by Snodgrass *et al*. [59] on the inhibitory effect of docosahexaenoic acid, an *n–3* fatty acid, on TLR-mediated responses in monocytes. In this study, the fatty acid was found to impair receptor dimerization, blunting in this way subsequent signaling [59]. In our previous studies we showed that docosahexaenoic acid is as effective as 16:1 fatty acids in inhibiting LPS-mediated gene expression responses [14].

Our results also raise provocative questions as to the cellular utilization of the various lipid pools that may function as reservoirs for anti-inflammatory 16:1 fatty acids. In preliminary work, we noted that the anti-inflammatory effects of both 16:1*n-7* and 16:1*n-9* can be abrogated if the

incorporation of the fatty acids into cellular lipids is prevented by the presence of the general acyl-CoA synthetase inhibitor triacsin C (C.M. & J.B., unpublished results), suggesting that formation of an ester of the corresponding 16:1 fatty acid is necessary for biological activity. Hence, the initial lipid localization where the fatty acid is esterified after incubating it with the cells may determine its subsequent biological activity. It is remarkable in this regard that 70-80% of total 16:1 in untreated cells is found in PC and that almost half of it is present in just a single phospholipid species, namely PC(16:0/16:1). These observations become even more relevant in the light of the data showing that, regardless of isomer, the bulk of exogenous fatty acid added to the cells gets incorporated into PC. Given that recent work has demonstrated that certain PC species with defined fatty acyl ester composition have biological activity on their own [60, 61], it will be of interest to determine whether pure phospholipid species containing either 16:1*n-7*, or 16:1*n-9*, or 16:1*n-10* as one of the acyl substituents are also biologically active. While it is clear that much work needs to be carried out to unravel the effects of 16:1 fatty acids on innate immune reactions, the elucidation of intracellular metabolic routes utilizing these fatty acids might provide interesting opportunities of intervention to ameliorate the inflammatory response.

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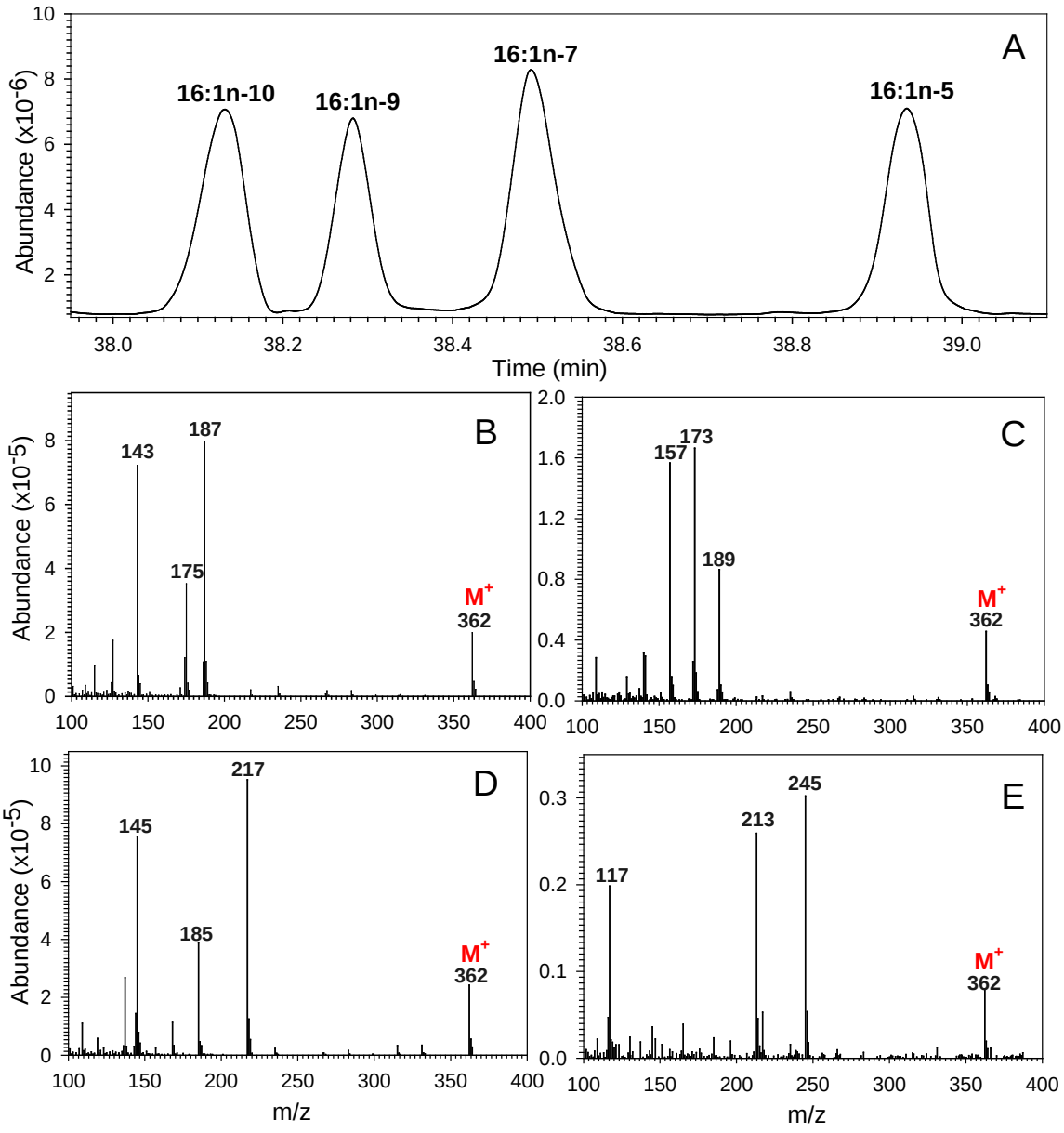

Figure 1. *Gas chromatography/mass spectrometry analyses of 16:1 isomers.* Authentic 16:1 fatty acid standards were methylated and transformed into DMDS adducts as described under 'Materials and Methods'. (**A**) Chromatographic separation of 16:1 fatty acid isomers; (**B**), (**C**), (**D**) and (**E**) mass spectra corresponding to the *n-10*, *n-9*, *n-7*, and *n-5* isomers respectively, showing the molecular ion (M^+) and the three diagnostic peaks (fragment corresponding to the terminal methyl part of the molecule, fragment containing the carboxyl group, and the latter with the loss of methanol).

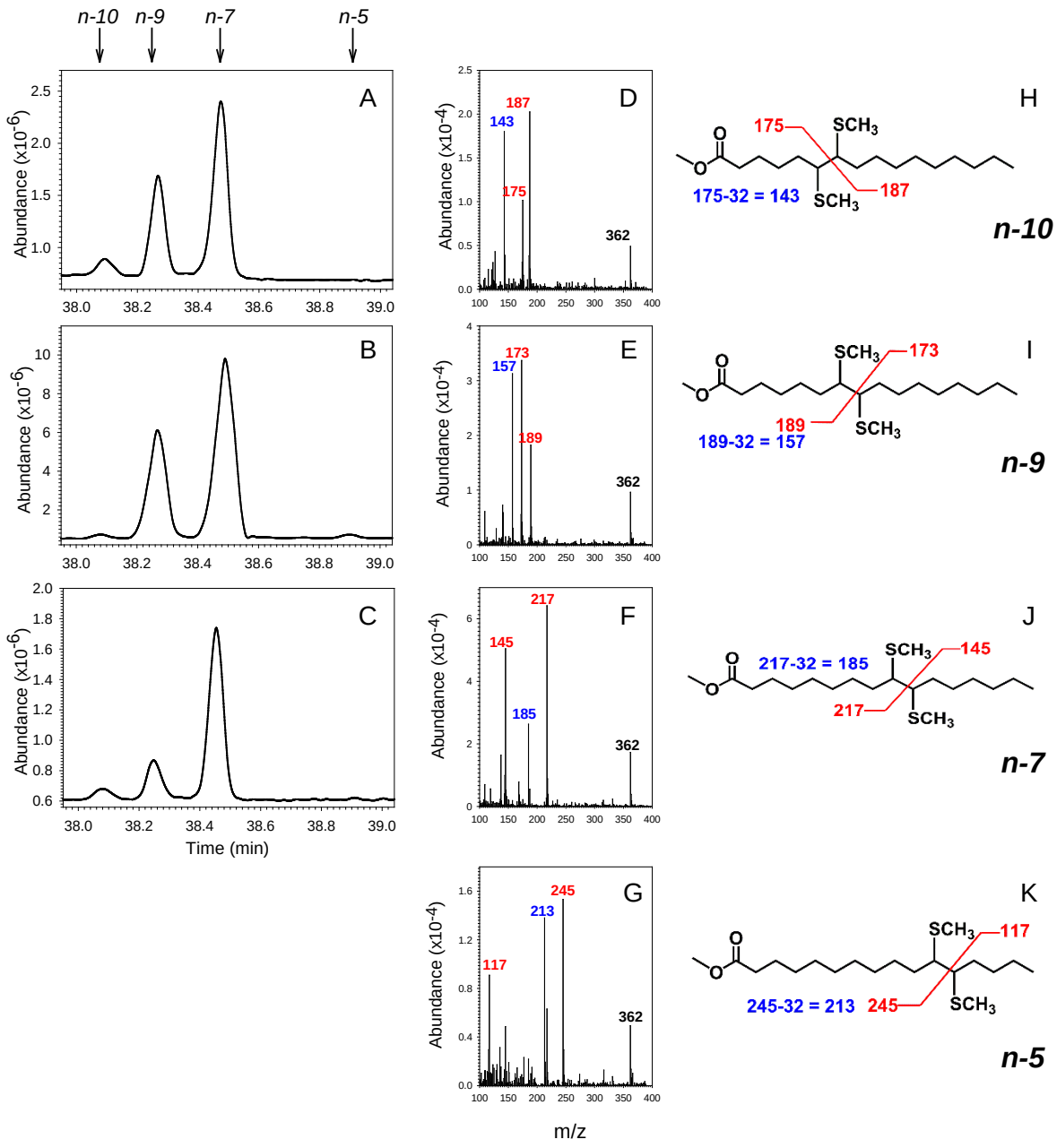

Figure 2. *Analysis of 16:1 isomers in human cells*. After sample collection and lipid extraction, total lipids were transmethylated. The resulting fatty acid methyl esters were derivatized with DMDS, and DMDS adducts of 16:1 isomers measured by gas chromatography/mass spectrometry. Chromatographic region showing the main 16:1 fatty acid isomers present in human monocytes (**A**), human monocyte-derived macrophages (**B**), and human serum (**C**). The mass spectra corresponding to the *n-10* (**D**, **H**), *n-9* (**E**, **I**), *n-7* (**F**, **J**), and *n-5* (**G**, **K**) isomers, indicating the molecular ion (M^+) and the three diagnostic peaks (fragment corresponding to the terminal methyl part of the molecule, fragment containing the carboxyl group, and the latter with the loss of methanol), are also shown.

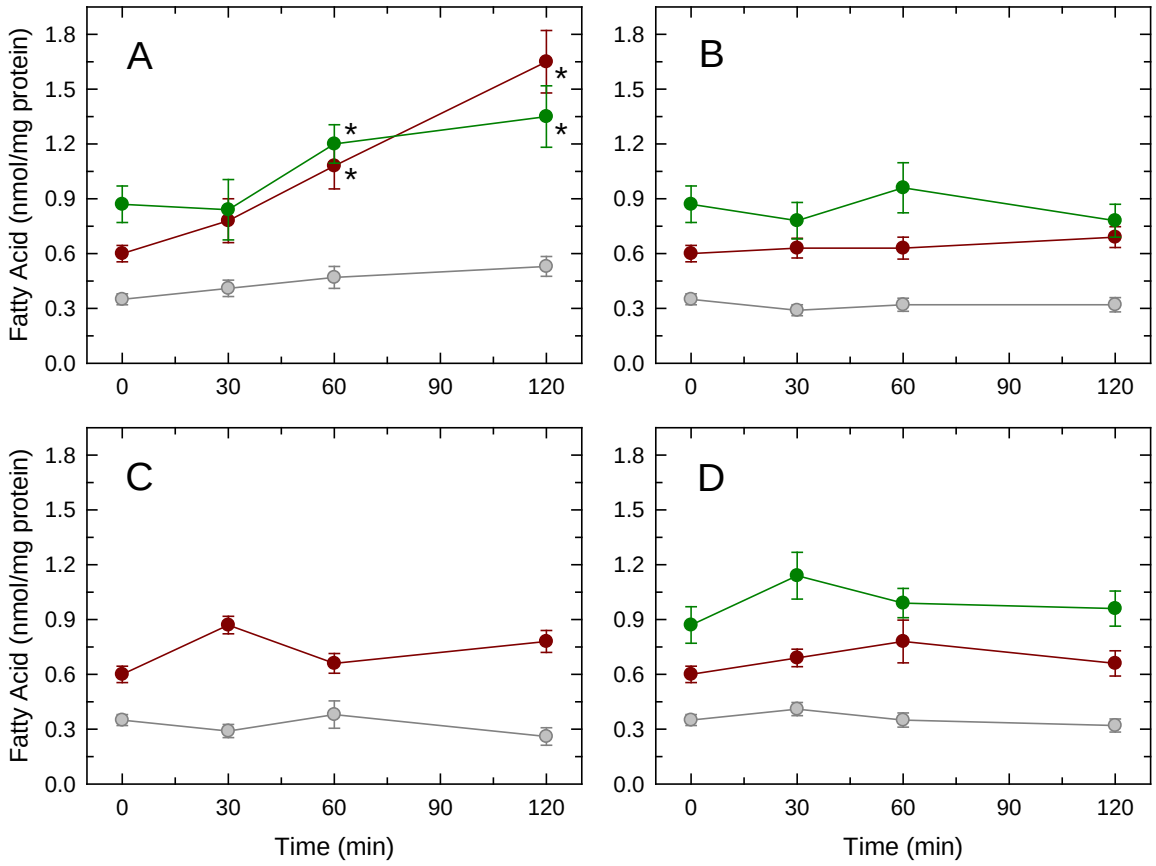

Figure 3. *Analysis of 16:1 fatty acids in activated cells*. Human monocytes were treated with 10 µM arachidonic acid (**A**), 100 ng/ml LPS (**B**), 1 mg/ml zymosan (**C**), or 2 µM calcium ionophore A23187 (**D**) for the times indicated. Afterward, cellular content of 16:1*n-10* (gray), 16:1*n-9* (dark red) and 16:1*n-7* (green) was measured by gas chromatography/mass spectrometry as described under 'Materials and Methods'. Results are shown as means ± SE of three independent experiments with incubations in duplicate. *$p < 0.05$, significantly different from the corresponding fatty acid in the unstimulated state (zero time). Note that in the experiments utilizing zymosan, no data on 16:1*n-7* can be shown because zymosan particles contain relatively high amounts of this fatty acid.

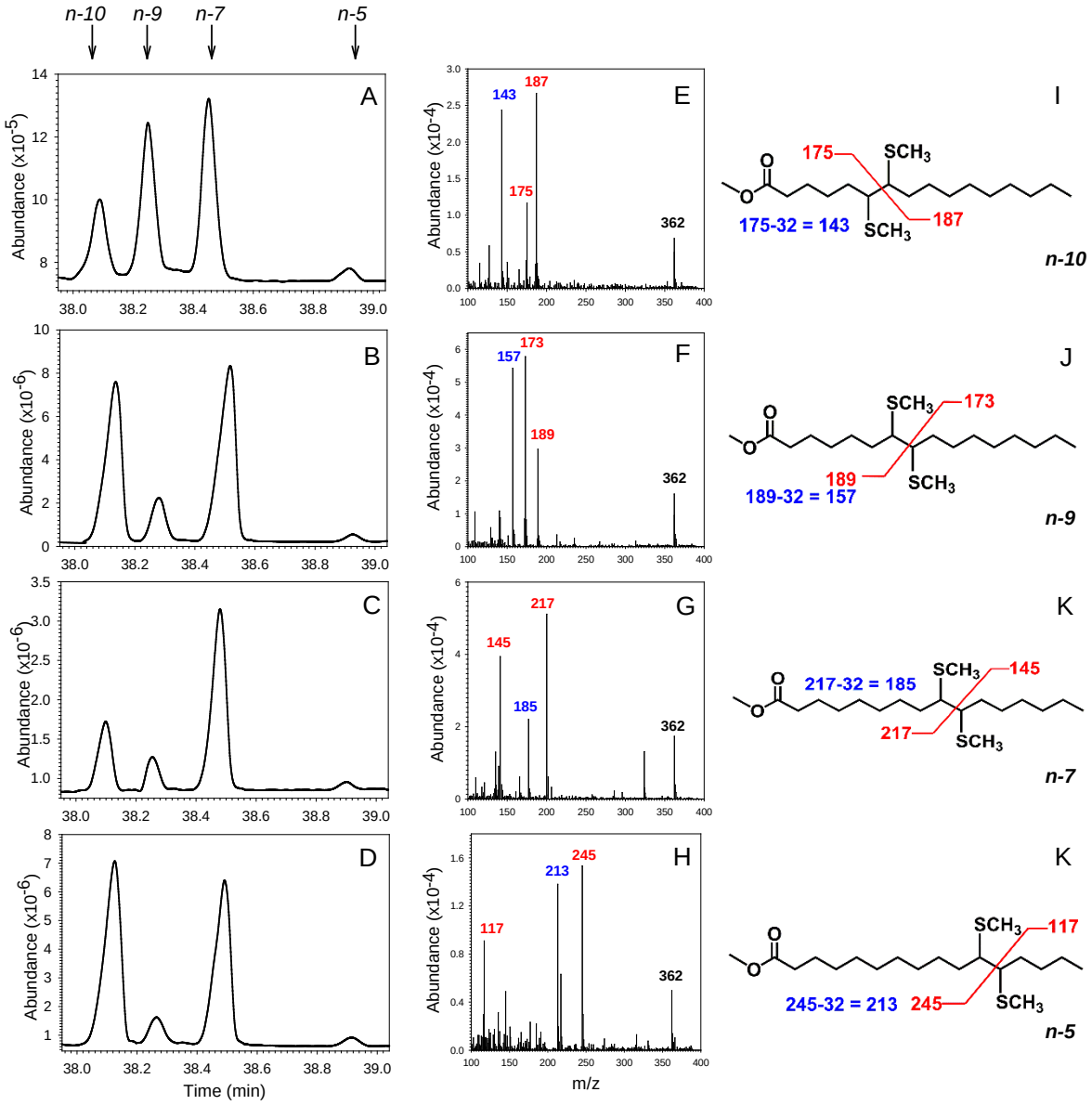

Figure 4. *Phagocytic cells of murine origin show elevated levels of 16:1n-10.* After sample collection and lipid extraction, total lipids were transmethylated. The resulting fatty acid methyl esters were derivatized with DMDS, and DMDS adducts of 16:1 isomers measured by gas chromatography/mass spectrometry. Chromatographic region showing the main 16:1 fatty acid isomers present in mouse peritoneal macrophages (**A**), RAW264.7 cells (**B**), P388D$_1$ cells (**C**) and THP1 cells (**D**). The mass spectra corresponding to the *n-10* (**E**, **I**), *n-9* (**F**, **J**), *n-7* (**G**, **K**), and *n-5* (**H**, **L**) isomers, indicating the molecular ion (M^+) and the three diagnostic peaks (fragment corresponding to the terminal methyl part of the molecule, fragment containing the carboxyl group, and the latter with the loss of methanol), are also shown.

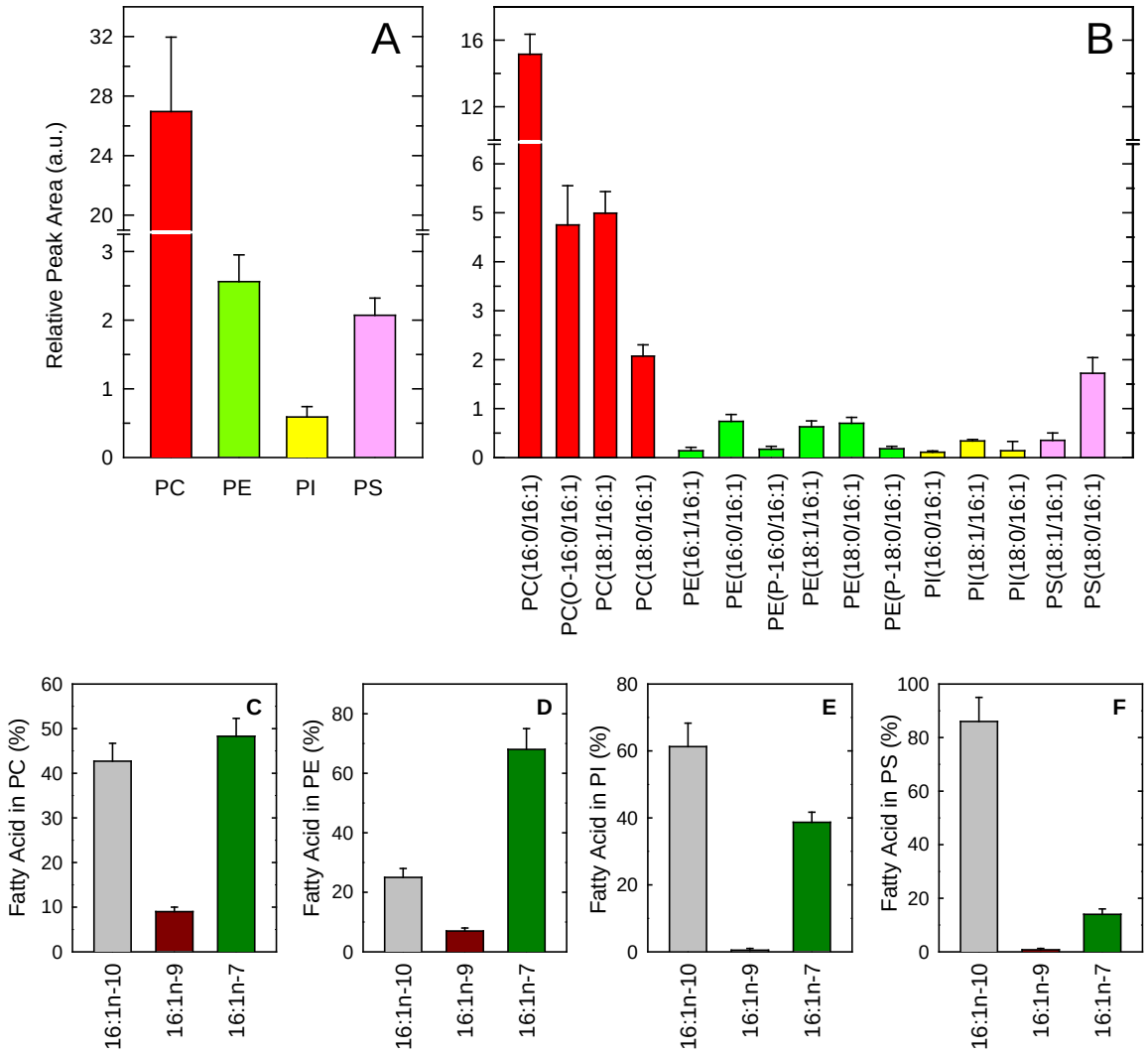

Figure 5. Distribution of 16:1 fatty acids between phospholipids. **A**: Total content of 16:1 fatty acids in phospholipids, and **B**: profile of 16:1-containing PC (red), PE (light green), PI (yellow), and PS (pink) species in unstimulated RAW264.7 cells, as determined by liquid chromatography coupled to mass spectrometry. Data in panel A are the sum of values of the various molecular species of each subclass shown in panel B. **C**, **D**, **E**, **F**: Identification of 16:1 isomer composition of the different phospholipid subclasses (see ordinate axes) of RAW264.7 cells, as determined by gas chromatography coupled to mass spectrometry. The data are given as % of the total 16:1 mol mass levels determined for each phospholipid subclass. Results are shown as mean ± SE of three independent experiments with incubations in duplicate.

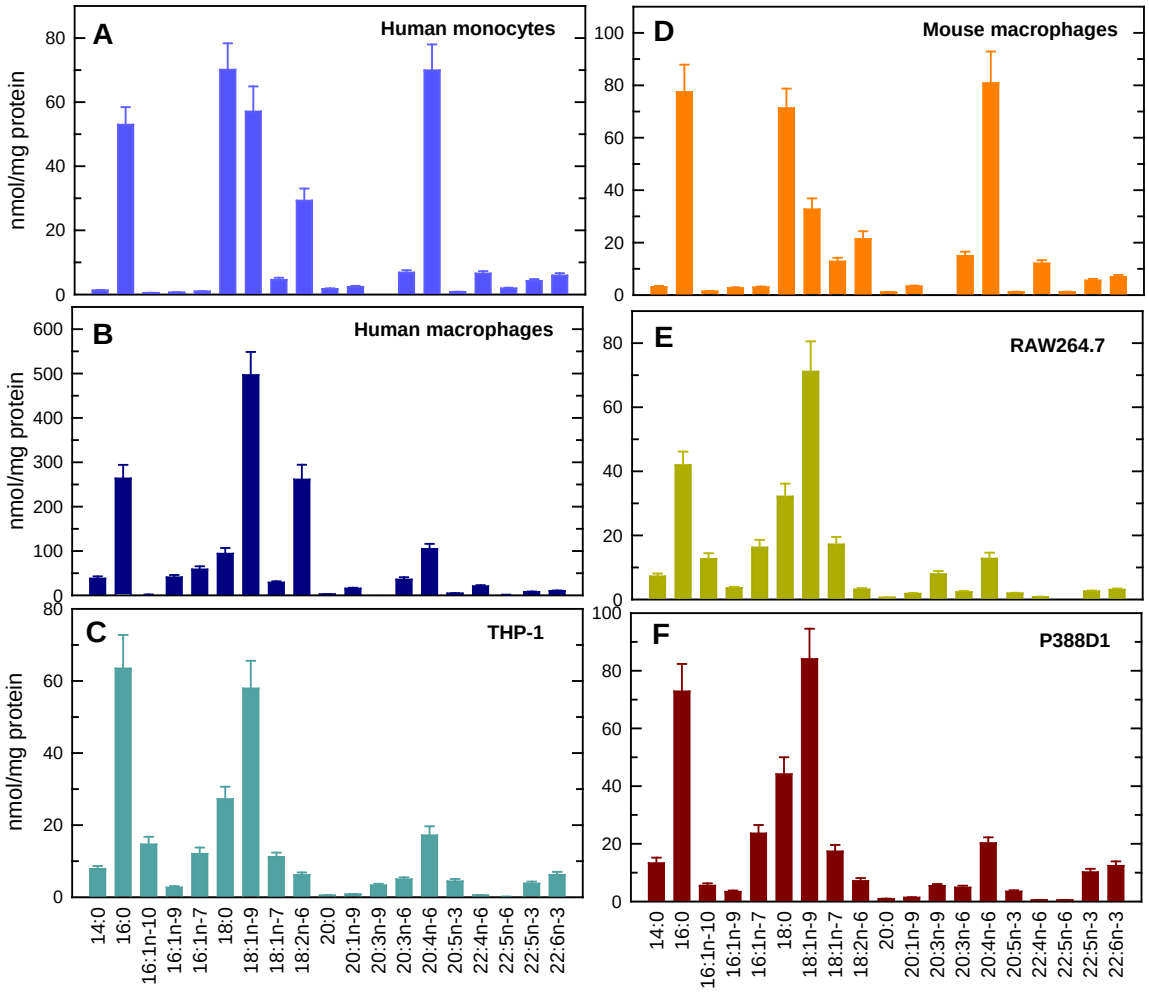

Figure 6. *Fatty acid composition of human and murine phagocytic cells.* The total fatty acid profile cells was determined by gas chromatography/mass spectrometry after converting the fatty acid glyceryl esters into fatty acid methyl esters. Approximately 10^7 cells were utilized for these determinations. Results are shown as mean ± SE of three independent experiments with incubations in duplicate.

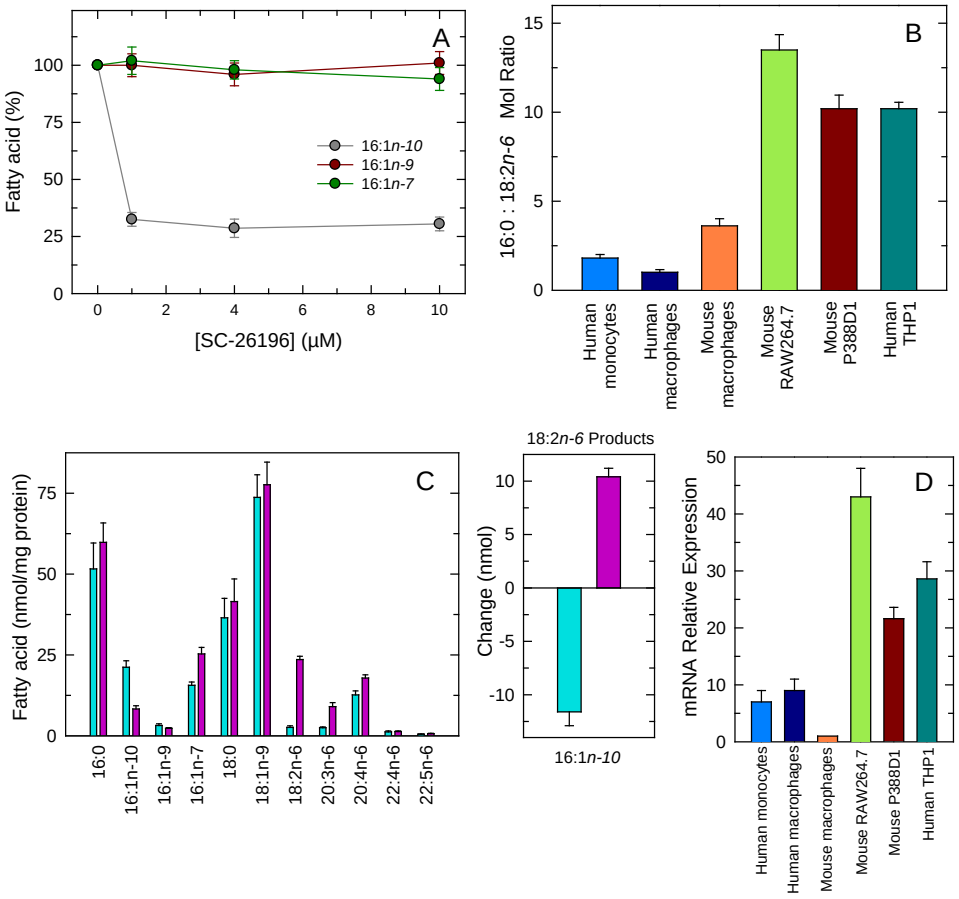

Figure 7. *Levels of 16:1n-10 are influenced by the cellular content of 18:2n-6.* **A**: Effect of the FADS2 inhibitor SC-26196 on 16:1 fatty acid levels. RAW264.7 cells were incubated with the indicated concentrations of the inhibitor for 24 h. Afterward, the content of 16:1*n-10* (gray circles), 16:1*n-9* (dark red circles), and 16:1*n-7* (green circles) was determined by gas chromatography/mass spectrometry. Results are referred to the amount of fatty acid determined in the absence of inhibitor (11.8 ± 1.8 nmol/mg, 2.6 ± 0.4 nmol/mg, and 16.6 ± 2.1 nmol/mg for 16:1*n-10*, 16:1*n-9*, and 16:1*n-7*, respectively). **B**: 16:0 to 18:2*n-6* mol ratio in different phagocytic cells. Total lipids were transmethylated and fatty acid methyl esters were measured by gas chromatography/mass spectrometry. **C**: Enrichment of cells with linoleic acid decreases the content of 16:1*n-10* in RAW264.7 cells. Cells were incubated without (cyan bars) or with 200 μM linoleic acid (complexed with albumin at a 2:1 ratio) (pink bars) for 48 h. Lipid extracts were transmethylated, and fatty acid methyl esters were measured by gas chromatography/mass spectrometry. **D**: Expression of FADS2 gene in different cell types. Results are shown as means ± SE of three independent experiments with incubations in duplicate (A, C) or triplicate (B, D).

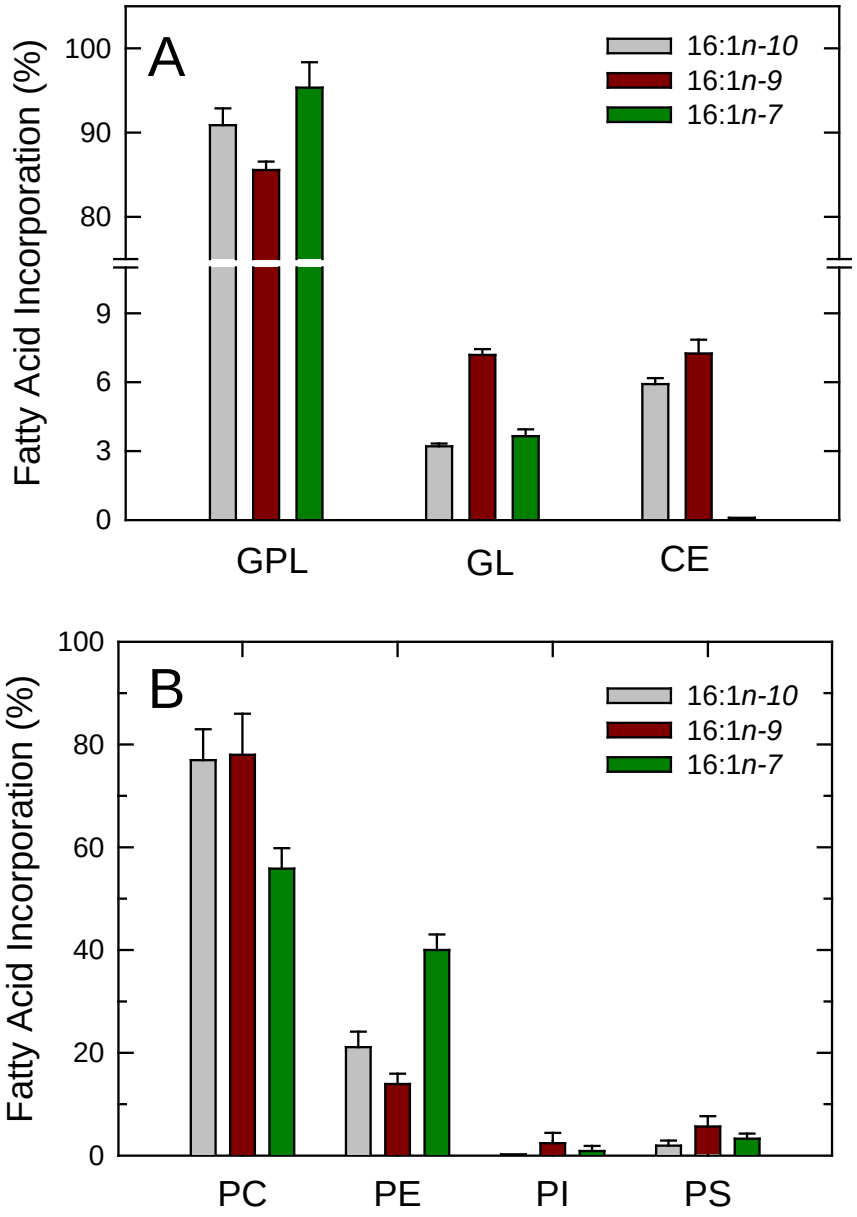

Figure 8 – *Incorporation of exogenous 16:1 fatty acids into cellular lipids*. RAW264.7 cells were incubated with either 10 µM of 16:1*n-10* (gray bars), or 16:1*n-9* (red bars), or 16:1*n-7* (green bars) for 14 h. Afterward, various celular lipid fractions were isolated and the 16:1 fatty acid content was determined by gas chromatography/mass spectrometry (**A**). The phospholipid fraction was further separated into subclasses and the 16:1 content was determined by gas chromatography/mass spectrometry (**B**). Results are shown as means ± SE of three independent experiments with incubations in duplicate. GPL, glycerophospholipids; GL, glycerolipids; CE, cholesterol esters.

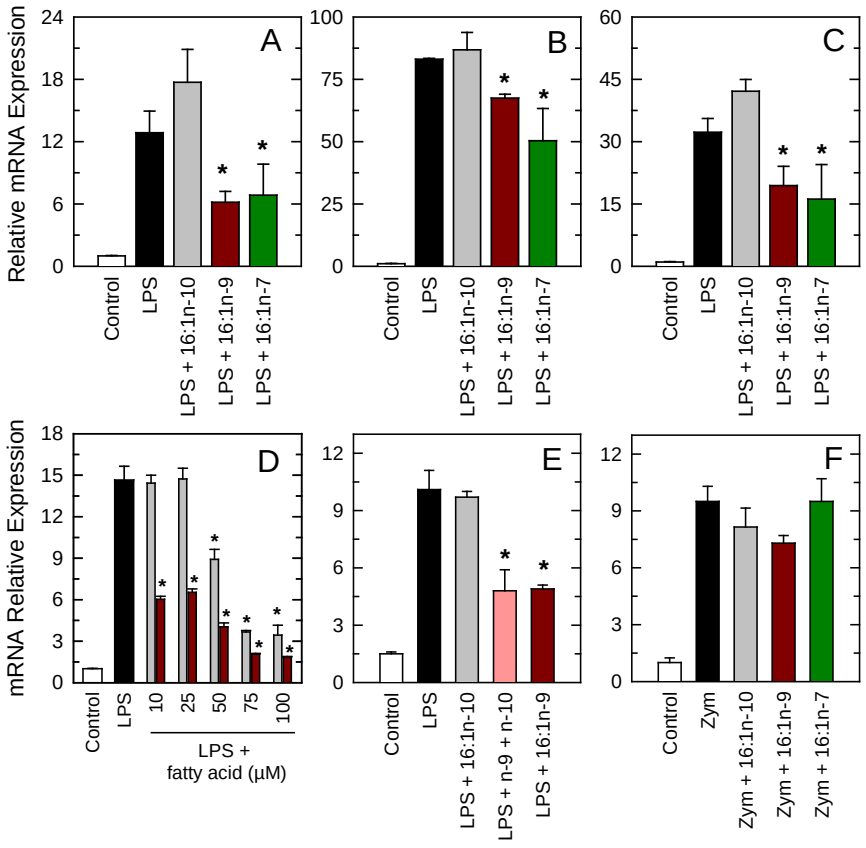

Figure 9. *Anti-inflammatory effect of 16:1 fatty acids*. **A**, **B**, **C**: The cells were incubated with 10 μM 16:*1n-10* (gray bar), 16:1*n-9* (dark red bar), 16:1*n-7* (green bar) or neither (black bar) for 14 h. Afterward the cells were stimulated by 100 ng/ml LPS for 6 h, and expression of *Il6* (A), *Ptgs2* (B), and *Ccl2* (C) was measured by qPCR. **D**: Dose-response determinations. The cells, incubated with the indicated fatty acid concentrations (16:1*n-9*, dark red bars; 16:1*n-10*, gray bars) were stimulated by 100 ng/ml LPS, and gene expression (*Nos2*) was measured by qPCR. Responses of control incubations, receiving the different fatty acid concentrations but not LPS, were no different than control unstimulated cells (open bar) and are omitted for clarity. **E**: 16:1*n-10* does not alter the effect of 16:1*n-9*. The cells, incubated with 10 μM 16:1*n-10* (gray bar), 16:1*n-9* (dark red bar), both (pink bar), or neither (black black), were stimulated by 100 ng/ml LPS for 6 h, and gene expression (*Ccl2*) was measured by qPCR. **F**: Effect of 16:1 fatty acids on zymosan-induced gene expression. The cells were incubated with 10 μM of 16:1*n-10* (gray bar), 16:1n-*9* (dark red bar), 16:1*n-7* (green bar) or neither (black bar) for 14 h. Afterward the cells were stimulated by 200 μg/ml zymosan for 6 h, gene expression (*Ccl2*) was measured by qPCR. Results are shown as means ± SE of three independent experiments with incubations in triplicate. *$p < 0.05$, significantly different from incubations with LPS without fatty acids.

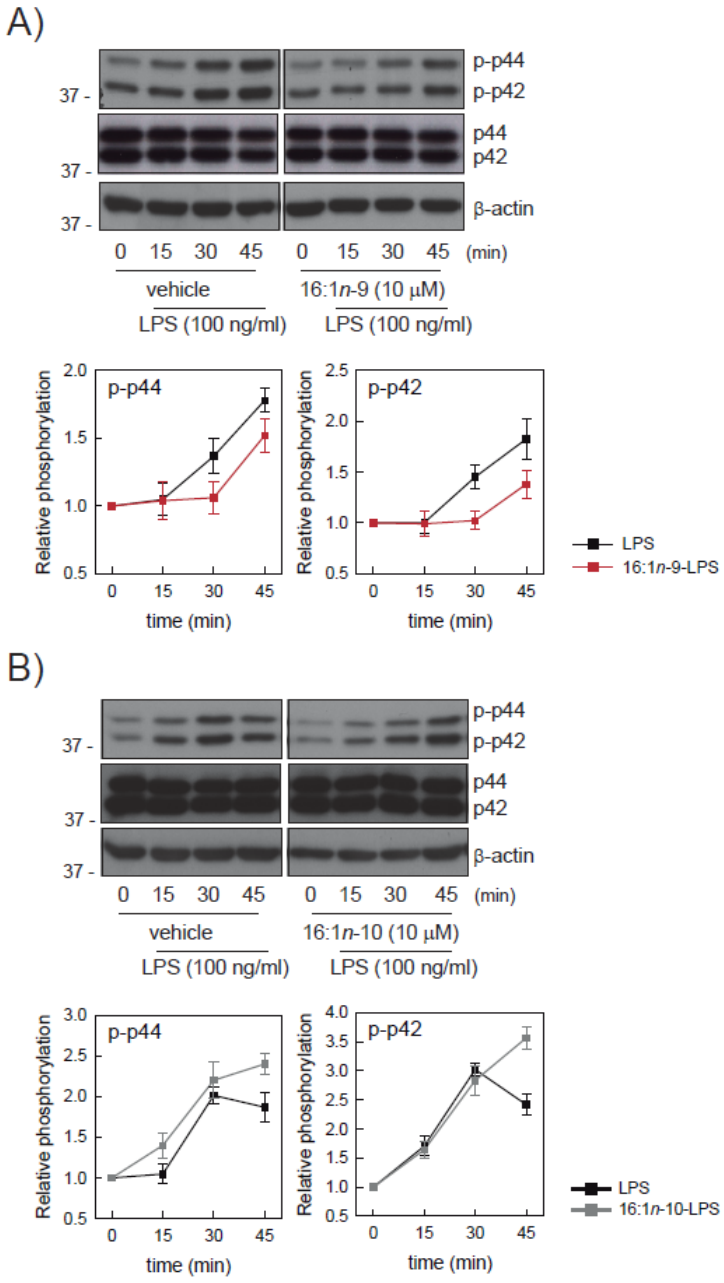

Figure 10. *Effect of 16:1 fatty acids on LPS-induced signaling*. RAW264.7 cells were incubated with 10 μM 16:*1n-9* (**A**), or 16:1*n-10* (**B**), or neither as indicated (vehicle) for 14 h. Afterward the cells were stimulated by 100 ng/ml LPS for the indicated times, and the phosphorylation of extracellular-regulated kinases p44 and p44 was studied by immunoblot. Blots shown are representative of three independent experiments, and the figures below the blots show the relative quantifications of p44 and p42 phosphorylation with respect to β-actin. Note that the blots corresponding to total p44 and p42 are from the same samples as phosphorylated p44 and p42, but from a different gel run in parallel.