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Lipin-2 Reduces Proinflammatory Signaling Induced by Saturated Fatty Acids in Macrophages*

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Background: Lipin-2 is a lipid metabolic enzyme.
Results: Lipin-2 levels control the generation of proinflammatory factors in macrophages overloaded with saturated fatty acids.
Conclusion: Lipin-2 has an anti-inflammatory action under fatty acid overload conditions.
Significance: Lipin-2 is involved in the cross-talk between lipid metabolism and inflammation.

Lipin-2 is a member of the lipin family of enzymes, which are key effectors in the biosynthesis of lipids. Mutations in the human lipin-2 gene are associated with inflammatory-based disorders; however, the role of lipin-2 in cells of the immune system remains obscure. In this study, we have investigated the role of lipin-2 in the proinflammatory action of saturated fatty acids in murine and human macrophages. Depletion of lipin-2 promotes the increased expression of the proinflammatory genes *Il6*, *Ccl2*, and *Tnfα*, which depends on the overstimulation of the JNK1/c-Jun pathway by saturated fatty acids. In contrast, overexpression of lipin-2 reduces the release of proinflammatory factors. Metabolically, the absence of lipin-2 reduces the cellular content of triacylglycerol in saturated fatty acid-overloaded macrophages. Collectively, these studies demonstrate a protective role for lipin-2 in proinflammatory signaling mediated by saturated fatty acids that occurs concomitant with an enhanced cellular capacity for triacylglycerol synthesis. The data provide new insights into the role of lipin-2 in human and murine macrophage biology and may open new avenues for controlling the fatty acid-related low grade inflammation that constitutes the *sine qua non* of obesity and associated metabolic disorders.

Chronic low grade inflammation produced in the adipose tissue during obesity is due, at least in part, to the increased levels of fatty acids released from adipocytes, especially saturated fatty acids, that are able to stimulate tissue resident macrophages and change their polarization state from an anti-inflammatory (M2) to a proinflammatory state (M1) (1). Persistent activation of inflammatory pathways and the interplay of macrophages/adipocytes are important events in the pathogenesis of insulin resistance, which can herald the onset of type 2 diabetes (2). For these reasons, there is much interest in the study of macrophage activation by saturated fatty acids and the molecular mechanisms that control their proinflammatory action.

Saturated fatty acids can affect cellular homeostasis by different pathways that could eventually cross-talk. They can activate TLR4 and TLR2, two members of the Toll-like receptor family that recognize microorganisms and trigger host responses (3). The interaction of TLR4/2 receptors with their canonical ligands (Gram-negative bacterial lipopolysaccharide/lipopeptides) induces an intracellular signaling cascade that activates kinases of the mitogen-activated protein kinase (MAPK) family and adaptor molecules and ends in the activation of key proinflammatory transcription factors such as AP-1 and NF-κB (4–6). Among the MAPK family of enzymes, JNK1 is a relevant player in the control of the activation of hypertrophic adipose tissue infiltrating macrophages by fatty acids (3, 7). JNK1 expression in hematopoietic cells governs diet-induced inflammation and insulin resistance without affecting obesity (8). Moreover, high saturated fatty acid concentrations show the ability to induce endoplasmic reticulum stress, triggering the unfolded protein response, a complex cellular reaction mounted to restore cellular homeostasis that may ultimately affect the induction of proinflammatory genes via JNK activation (8–10).

Saturated fatty acids, like other fatty acids, are also internalized into the cells and incorporated into triacylglycerol (TAG).[5] Fn5
Recently, it has been shown that the capacity of macrophages to store saturated fatty acids into TAG correlates inversely with their capacity to express proinflammatory genes and undergo M1 polarization, perhaps by reducing the intracellular concentration of free saturated fatty acids and hence their modulation of proinflammatory pathways (11).

Along the biosynthetic pathway of TAG, lipins, also known as Mg^{2+}-dependent phosphatidic acid phosphatases, are in charge of the generation of diacylglycerol pools to be acylated by the action of diacylglycerol:acyl-CoA acyltransferases (12).

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[5] The abbreviations used are: TAG, triacylglycerol; UPR, unfolded protein response; EGFP, enhanced GFP.

Interestingly, before their enzymatic activity was revealed, lipins had been described as proteins implicated in the development of fat deposits (13–15). The diacylglycerol pools generated by lipins can also be used for the synthesis of the major membrane phospholipids phosphatidylcholine and phosphatidylethanolamine (16). Perhaps because of their key cellular roles, very few known illnesses associated with genetic alterations of lipins have been described (17). Among the three different genes that encode lipin proteins (lipin-1, -2, and -3), mutations in *LPIN2* result in Majeed syndrome, a recessive Mendelian rare disease characterized by recurrent episodes of fever and inflammation in bone and skin and congenital dyserythropoietic anemia (18). Psoriasis, a skin inflammatory disease, is also associated with *LPIN2* mutations (19). Thus, human diseases that have been linked to mutations of *LPIN2* seem to have an inflammatory foundation. These observations led us to speculate about a role for lipin-2 in the regulation of inflammatory processes. In this study, we have identified lipin-2 as an unexpected player in the proinflammatory action of saturated fatty acids in both monocyte-derived human macrophages and the murine macrophage cell line RAW 264.7. Our studies indicate that lipin-2 is an anti-inflammatory enzyme that controls TAG synthesis, JNK/AP-1 pathway activation, and ultimately the up-regulation of proinflammatory genes.

EXPERIMENTAL PROCEDURES

Reagents—Fatty acids, antibody against β-actin, SP600125, and PD98059 were obtained from Sigma. Antibodies against ERK, phospho-ERK (Thr^{202}–Tyr^{204}), JNK, phospho-JNK (Thr^{183}–Tyr^{185}), phospho-c-Jun, and c-Jun, were purchased from Cell Signaling (Danvers, MA). BODIPY 493/503 was purchased from Invitrogen. Human macrophage Nucleofection solution was from Amaxa (Gaithersburg, MD). ON-Target *plus* siRNAs against mice mRNAs were obtained from Dharmacon, and siRNAs *Silencer Select* against human mRNAs was obtained from Ambion.

Cells—Human blood monocyte-derived macrophages were obtained from buffy coats of healthy volunteer donors obtained from the Centro de Hemoterapia y Hemodonación de Castilla y León (Valladolid, Spain), as described previously (20–22). Murine RAW 264.7 macrophages were maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine at 37 °C in a 5% CO_2 humidified incubator. Palmitic acid and oleic acid were complexed to BSA (2:1 molar ratio) as described (23). For gene expression analysis, an 8-h time treatment of cells with fatty acids was used, based on time course experiments.

PCR—RNA was extracted using TRIzol reagent method (Invitrogen) according to the manufacturer's protocol. First strand cDNA was then obtained by using the Moloney murine leukemia virus reverse transcriptase from 1 μg of RNA. PCR was then performed using specific primers for *Lipin1α*, *-1β*, *-2*, and *-3* mRNA (see below).

Real Time PCR—RNA was extracted using TRIzol reagent method (Invitrogen). First strand cDNA was synthesized from 2 μg of total RNA using the Transcriptor First Strand cDNA synthesis kit (Roche Applied Science) and random primers. The cDNA was amplified by real time PCR using the KAPA SYBR master mix quantitative PCR (Roche Applied Science) and specific primers for each gene (24). Sequences of the primers used for murine genes were as follows: *Cyclophilin,* 5′-TGGAAGAGCCAAGACAGACA-3′ and 5′-GCCGGAGTCGACAATGAT-3′; *Lpin1,* 5′-CTCCGCTCCCGAGAGAAG-3′ and 5′-TCATGTGCAAATCCACGGACT-3′; *Lpin1α,* 5′-GGTCCCCAGCCCCAGTCCTT-3′ and 5′-GCAGCCTGTGGCAATTCA-3′; *Lpin1β,* 5′-CATGCTTCGGAAAGTCCTTCA-3′ and 5′-GGTTATTCTTTGGCGTCAACCT-5′; *Lpin2,* 5′-AGTTGACCCCATCACCGTAG-3′ and 5′-CCCAAAGCATCAGACTTGGT-3′; *Lpin3,* 5′-TGGAATTGGGATGACAAGGT-3′ and 5′-CCCAAAGCATCAGACTTGGT-3′; *Tnfα,* 5′-ACGGCATGGATCTCAAAAGAC-3′ and 5′-AGATAGCAAATCGGCTGACG-3′; *Ccl2,* 5′-AGGTCCCATGTCATGCTTCTGG-3′ and 5′-CTGCTGCTGGTGATCCTCTTG-3′; and *Il6,* 5′-TAGTCCTTCCTACCCCAATTTCC-3′ and 5′-TTGGTCCTTAGCCACTCCTTC-5′. Primers for human genes were as follows: *Cyclophilin,* 5′-CAGACAAGGTCCCAAAGACAG-3′ and 5′-TTGCCATCCAACCACTCAGTC-3′; *LPIN1α,* 5′-TGCTGGAGAGCAGCAGAACTC-3′ and 5′-GAACCGGAAGGACTGGGAGTG-3′; *LPIN1β,* 5′-TGCTGGAGAGCAGCAGAACTC-3′ and 5′-AAGACTGTGGAGGGCAAGAAC-3′; *LPIN2,* 5′-CCTCTCCTCAGACCAGATCG-3′ and 5′-GGAGAATCTGTCCCAAAGCA-3′; *LPIN3,* 5′-CACTCCACCCTCCACTCCTA-3′ and 5′-ACAGGTAGATGGTGGCCTTG-3′; *TNFα,* 5′-ATGAGCACTGAAAGCATGATCC-3′ and 5′-GAGGGCTGATTAGAGAGAGGTC-3′; *CCL2,* 5′-CAGCCAGATGCAATCAATGCC-3′ and 5′-TGGAATCCTGAACCCACTTCT-3′; and *IL6,* 5′-AAATTCGGTACATCCTCGACGG-3′ and 5′-GGAAGGTTCAGGTTGTTTTCTGC-3′. Primers for LPIN1α and 1β were from Ref. 25. The relative mRNA abundance for a given gene was calculated using the ΔΔ*CT* method, using cyclophilin as the internal standard (26).

Constructs and Transfections—Lipin2-EGFP plasmid was generated by introducing the cDNA sequence of the mouse *lpin2* (Thermo Scientific, clone 5101211) in the EGFP expression vector pEGFP-N3 (Clontech) by using HindIII and SalI restriction enzymes. The primers used were as follows: 5′-CACACAAAGCTTAAATGAATTATGTGGGCCAGCT-3′ and 5′-CACACAGTCGACAGCCAGGTCATCCAGGTCC-3′. Confirmation of the correct insertion of the cDNA was performed by sequencing. Plasmids (EGFP or lipin2-EGFP) were transfected using Lipofectamine™ LTX and PLUS™ reagents following the manufacturer's instructions. After 24 h of transfection, fluorescent cells were selected by cell sorting using a FACS Aria. Cells were allowed to recover for 48 h in the presence of 1 mg/ml geneticin and then used for experiments. At that time, more of 90% of the cells exhibited fluorescence.

Small Interfering RNA (siRNA) Transfection—RAW 264.7 cells (2×10^5) were transiently transfected with siRNAs (20 nM) in the presence of 5 μl/ml Lipofectamine™ RNAiMAX (Invitrogen) under serum-free conditions for 5 h. Afterward, 5% serum was added, and the cells were maintained at normal culture conditions for 48 h. A scrambled siRNA was used as a negative control. By using a negative fluorescent siRNA, we estimated that 95% of the cells were transfected. Also, the siRNAs produced a 70–90% reduction in the expression of the corresponding targets. Transfection of human macrophages was achieved by the Nucleofection method as described previously (20).

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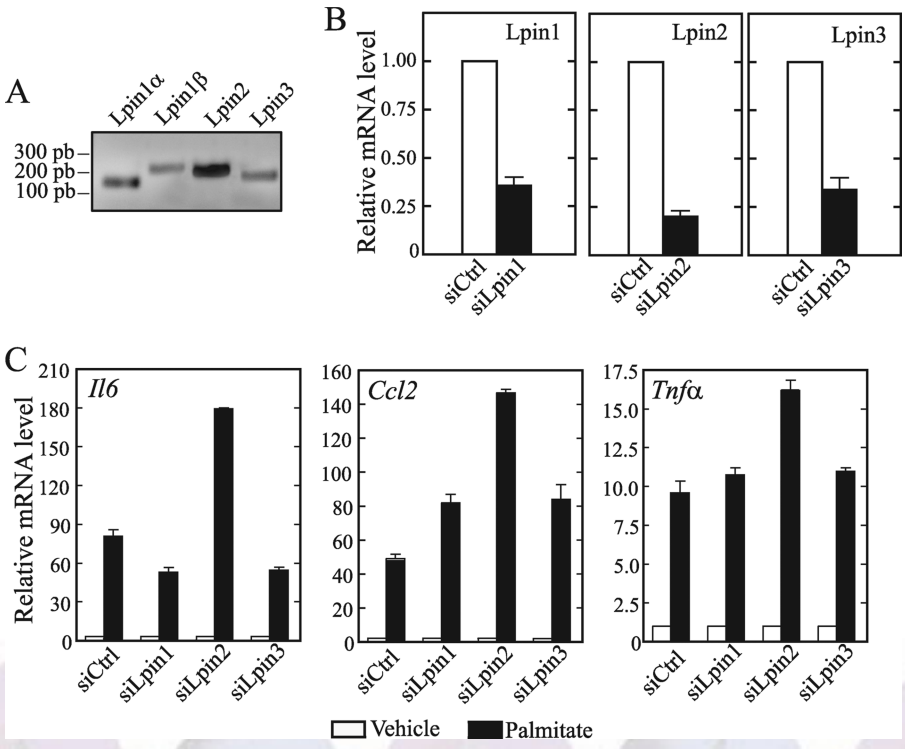


FIGURE 1. **Expression of lipins and role in macrophages.** *A*, PCR analysis of *Lpin1α, -1β, -2,* and *-3* mRNA expression in RAW 264.7 macrophages. *B,* cells were transfected with control siRNA (*siCtrl., open bars*) or siRNA against *Lpin1, -2,* or *-3* (*siLpin1, -2,* or *-3, black bars*), and after 48 h, the levels of mRNA expression for each lipin gene was analyzed. *C,* cells transfected with control siRNA or siRNA against *Lpin1, -2,* or *-3* were treated with vehicle (*open bars*) or 300 μM palmitic acid (*black bars*) for 8 h, and mRNA levels for *Il6*, *Ccl2,* and *Tnfα* were analyzed by real time PCR. Data are representative of at least three independent experiments. *Error bars* represent ± S.E. ($n = 3$).

Immunoblot—After cellular stimulation, cells were lysed with 20 mM Tris-HCl (pH 7.4), containing 150 mM NaCl, 0.5% Triton X-100, 1 mM Na_3VO_4, 150 mM NaF, 1 mM PMSF, and a protease inhibitor mixture (Sigma) at 4 °C. Homogenates were then clarified by centrifugation at 13,000 × *g* for 10 min. Protein from the supernatants was quantified by the Bradford protein assay kit (Bio-Rad), and 100 μg of protein was analyzed by immunoblot using specific antibodies. Detection of immunoreactive bands was conducted by chemiluminescence (ECL™, Amersham Biosciences) using a Bio-Rad VersaDoc 5000 system. The resulting digital images were analyzed for quantitative band densitometry at different time exposures within the linear response defined by Quantity One software (Version 4.5.2; Bio-Rad).

IL-6, TNF-α, and MCP-1 Quantification—After cellular activation, supernatants were used for quantification of IL-6, TNF-α, and MCP-1 by specific ELISA kits (eBioscience, San Diego) following the manufacturer's instructions.

Flow Cytometry—After the various treatments, cells were scraped, washed with phosphate-buffered saline, and stained with 0.2 μg/ml BODIPY 493/503 for 5 min as described previously (20). Cells were washed, and fluorescence was analyzed by flow cytometry in a FACS Gallios (Beckman Coulter) using the FL1 detector. Fluorescence data were measured in linear scale.

DNA Binding Assays—The DNA binding activity of nuclear c-Jun was assayed by a commercial kit (Active Motif) following the manufacturer's instructions. Nuclear extracts from activated cells were prepared as described previously (27).

Microscopy—After treatments, cells were stained with 2 μg/ml BODIPY 493/503 for 5 min. The cells were then washed with PBS, fixed with 4% paraformaldehyde, and mounted using an antifade medium. Fluorescence was monitored by confocal microscopy using a Bio-Rad Radiance 2100 laser-scanning system coupled to a Nikon TE-2000U. The objective was CFI Plan Apo ×60, 1.4 numerical aperture, oil immersion. The fluorescence of BODIPY 493/503 was monitored at 488 nm argon excitation using the combination of the long pass filter HQ500LP and the short pass filter HQ560SP.

TAG Analysis by Mass Spectrometry—Analysis of the fatty acids of TAG was performed by gas chromatography/mass spectrometry. Briefly, the total cellular TAG fraction isolated by thin layer chromatography with hexane/ether/acetic acid (70:30:1, v/v/v) as a mobile phase, was transmethylated with 500 μl of 0.5 M KOH in MeOH for 30 min at 37 °C. One volume of 0.5 M HCl was added to neutralize, and fatty acid methyl esters were extracted twice with 2 volumes of *n*-hexane. Analysis of fatty acid methyl esters was carried out in an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass selective detector operated in electron impact mode (70 eV), equipped with an Agilent DB23 column (60 m × 0.25 mm inner diameter × 0.15 μm film thickness), under the conditions described previously (28, 29). Data acquisition was carried out both in scan, for identification, and selected ion monitoring mode for quantitation, using 74 and 87 fragments for saturated, 83 for monounsaturated, 67 and 81 for diunsaturated, and 79 and 91 for polyunsaturated fatty acid methyl esters. A 37-component mixture from Supelco was used for calibration curves, and nonadecanoic acid was used as an internal standard. Data

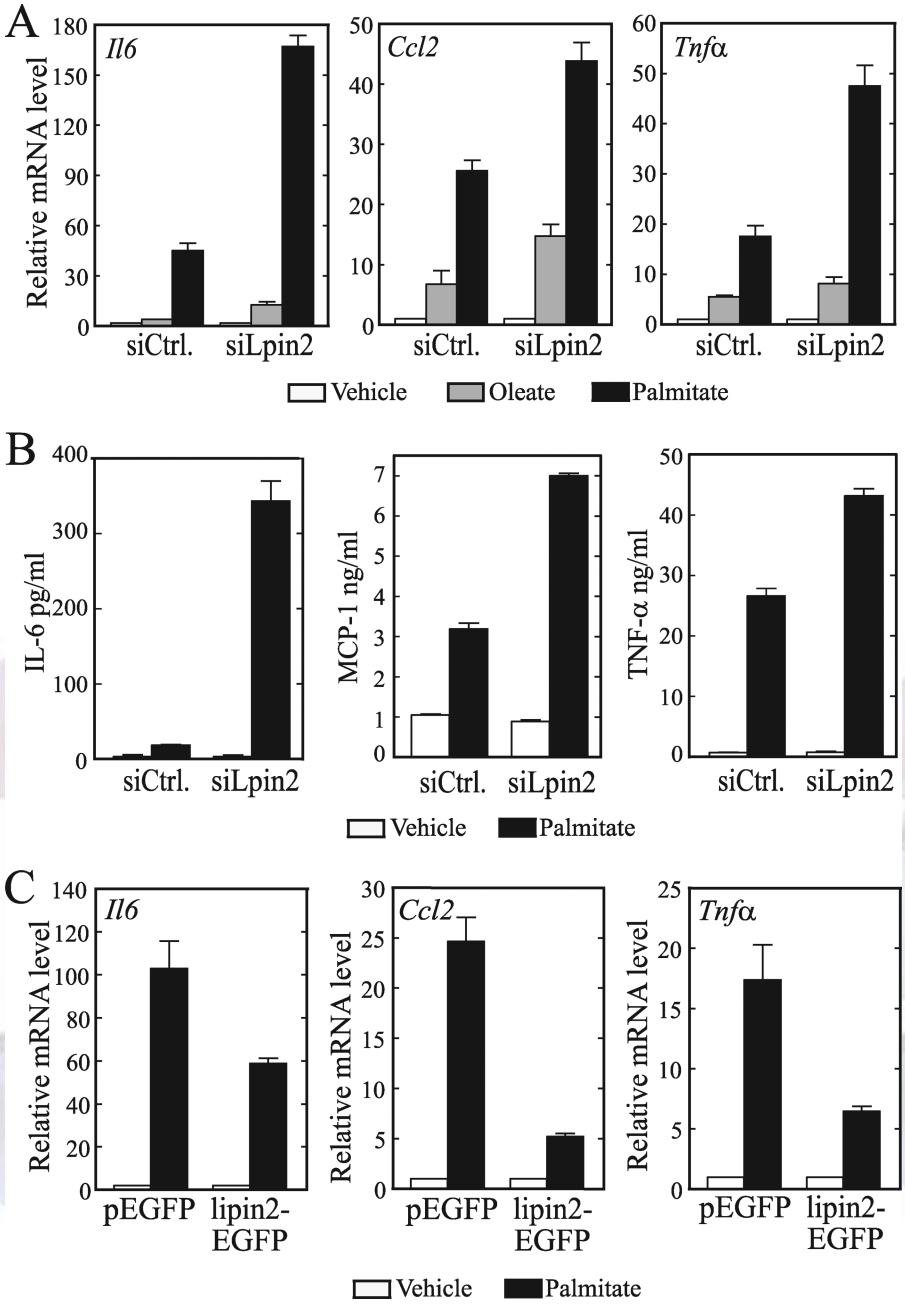


FIGURE 2. **Lipin-2 levels regulate the expression of proinflammatory genes in macrophages.** *A,* RAW 264.7 cells were transfected with control siRNA (*siCtrl*) or siRNA against *Lpin2* (*siLpin2*) as indicated, treated with vehicle (*open bars*), 300 μM oleic acid (*gray bars*), or 300 μM palmitic acid (*black bars*) for 8 h, and mRNA levels for *Il6, Ccl2,* and *Tnfα* were analyzed by real time PCR. *B,* cellular supernatants from cells transfected as in *A* and treated with vehicle (*open bars*) or 300 μM palmitic acid (*black bars*) for 24 h were assayed for the indicated proinflammatory factors by using ELISA. *C,* cells expressing either the pEGFP or the lipin2-EGFP plasmids were activated with vehicle (*open bars*) or 300 μM palmitic acid (*black bars*) for 8 h, and the levels of mRNA for *Il6, Ccl2,* and *TNFα* were analyzed by real time PCR. Data are representative of at least three independent experiments. *Error bars* represent ± S.E. ($n = 3$).

analysis was carried out with the Agilent G1701EA MSD Productivity Chemstation software, revision E.02.00.

Statistical Analysis—Experiments were carried out at least three times in duplicate or triplicate. Statistical analysis was carried out by the Student's *t* test, with p values <0.05 taken as statistically significant.

RESULTS

Lipin-2 Levels Regulate the Expression of Proinflammatory Genes—Saturated fatty acids activate macrophages and induce the production of proinflammatory cytokines (30). Because lipins can participate in macrophage signaling cascades by impacting on cellular concentrations of diacylglycerol and phosphatidic acid (31–33), we began our study by testing whether lipins could be involved in the up-regulation of proinflammatory genes under pathophysiologically relevant conditions, *i.e.* exposure of the cells to high concentrations of palmitic acid. For this purpose, lipin levels were decreased by siRNA technology. RAW 264.7 macrophages express the three types of lipins known to date, lipin-1, -2, and -3 (Fig. 1*A*), and conditions were established to achieve silencing of all three forms (Fig. 1*B*). Inhibition of lipin-2 expression but not of F1

lipin-1 or lipin-3 resulted in a strong enhancement of the induction of the proinflammatory genes *Il6, Ccl2*, and *Tnfα* by palmitic acid (Fig. 1*C*). In the absence of palmitic acid stimulation, reduction of the expression of any of the lipins did not alter the basal expression level of the genes under analysis. Importantly, the enhancing effect of lipin-2 depletion on the expression of proinflammatory genes was found to be selective for palmitic acid stimulation, because treating the cells with oleic acid, a monounsaturated fatty acid, showed a much lower effect (Fig. 2*A*). ELISA measurements confirmed that the increased expression of *Il6, Ccl2*, and *Tnfα* in lipin-2-depleted and palmitic acid-stimulated cells did result in increased synthesis of their protein products, IL-6, MCP-1, and TNFα, respectively (Fig. 2*B*). To characterize further the effect of lipin-2 on proinflammatory gene expression, cellular levels of this protein were increased by transfecting the cells with a lipin-2-EGFP plasmid. The induction of proinflammatory cytokines by palmitic acid was reduced in cells overexpressing lipin-2 with respect to control cells transfected only with the EGFP plasmid (Fig. 2*C*). Collectively, these data suggest that lipin-2 plays a key regulatory role in proinflammatory gene expression by macrophages exposed to saturated fatty acids.

Lipin-2 Regulates Proinflammatory Cytokine Production via JNK1—Members of the MAPK family, especially JNK, have been implicated in the proinflammatory stimulation of macrophages by fatty acids (3, 8, 30). Therefore, the impact of lipin-2 on the phosphorylation/activation status of different MAPKs after palmitic acid treatment was studied. Treatment of the macrophages with palmitic acid significantly induced the phosphorylation of ERKs (p42/p44) and JNK1 (Fig. 3). When the cells were transfected with siRNA against lipin-2, palmitic acid treatment promoted a clear enhancement of the phosphorylation of the above-mentioned kinases (Fig. 3). These results demonstrate that lipin-2 limits the activation of ERK and JNK induced by palmitic acid in macrophages.

The involvement of JNK and ERK in the increased production of proinflammatory genes by lipin-2-depleted and palmitic acid-treated cells was studied next. The inhibitors SB600125 (specific for JNK (34)) and PD98059 (specific for MEK1/2, *i.e.* the kinase that phosphorylates and activates the ERKs (35)) were initially used for this purpose. As shown in Fig. 4*A*, the expression of *Tnfα*, *Ccl2*, and *Il6* was strongly inhibited by SB600125 in palmitic acid-treated cells. Importantly, SB600125 was also capable of inhibiting the up-regulation of these genes in lipin-2-depleted cells (Fig. 4*A*). Confirmation of this effect at the protein level was obtained by measuring protein levels of IL-6, MCP-1, and TNF-α by specific ELISAs (Fig. 4*B*). In contrast to the data obtained with SB600125, the effect of PD98059 was not as pronounced, suggesting a lesser role for ERKs in mediating these processes.

To further substantiate the above findings, siRNAs against JNK1 were utilized next. Cells depleted of JNK1 demonstrated a strong inhibition of mRNA and protein levels of the aforementioned proinflammatory factors in response to palmitic acid, both in control siRNA-transfected cells and in lipin-2-depleted cells (Fig. 5, *A* and *B*). From these data, it is apparent that JNK1 constitutes an obligatory key component of the signaling cascade triggered by palmitic acid, which leads to the induction of proinflammatory genes, and that this cascade can be overstimulated when lipin-2 is decreased.

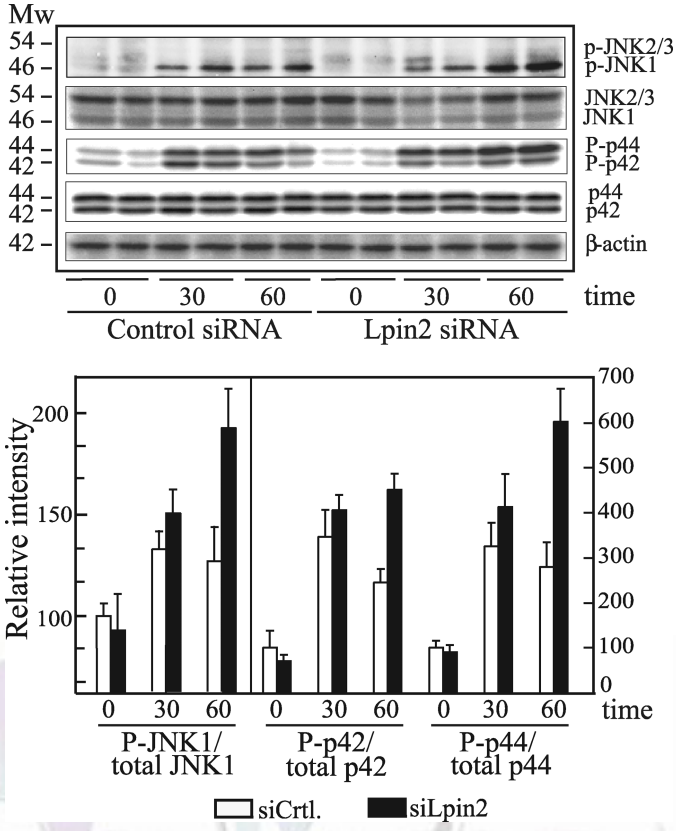


FIGURE 3. **Lipin-2 controls the phosphorylation levels of JNK1 and ERK.** RAW 264.7 macrophages were transfected with control siRNA or siRNA against *Lpin2* as indicated and treated with 300 μM palmitic acid for the indicated periods of time. Phosphorylation levels and total protein for JNK and ERK were assayed by Western blot (*upper panel*). β-Actin levels were also assayed for protein loading. Relative intensity of phosphorylated bands against total protein was analyzed for JNK1, ERK p42, and ERK p44, and the results are represented in the *lower panel*. Data are representative of three independent experiments.

Lipin-2 Regulates the Activation of c-Jun—Among many other proteins, JNK phosphorylates c-Jun, a member of the Jun family of proteins that forms part of the transcription factor AP-1, implicated in the transcriptional regulation of different proinflammatory genes (36). Thus, the possible role of c-Jun during palmitic acid activation and its regulation by lipin-2 was analyzed. Interestingly, it was appreciated that phosphorylation of c-Jun increased in cells lacking lipin-2 at almost all the time points tested (Fig. 6*A*). Nuclear c-Jun activation was assayed using a commercially available kit (Active Motif), and a clearly enhanced activation of c-Jun by palmitic acid was observed when lipin-2-depleted cells were used (Fig. 6*B*). Such enhancement was inhibited by SP600125, providing further evidence that JNK is critically involved under these conditions (Fig. 6*B*). By using siRNA technology, the effect of c-Jun on the capacity of the cells to produce proinflammatory mediators in response to palmitic acid was analyzed next. Depletion of cellular c-Jun levels inhibited the up-regulation of mRNA for *Il6, Ccl2*, and *Tnfα*, an effect that was particularly striking when lipin-2-depleted cells were used (Fig. 6*C*). Thus, these data demonstrate that the extent of JNK-mediated c-Jun activation in palmitic acid-treated cells is controlled by lipin-2.

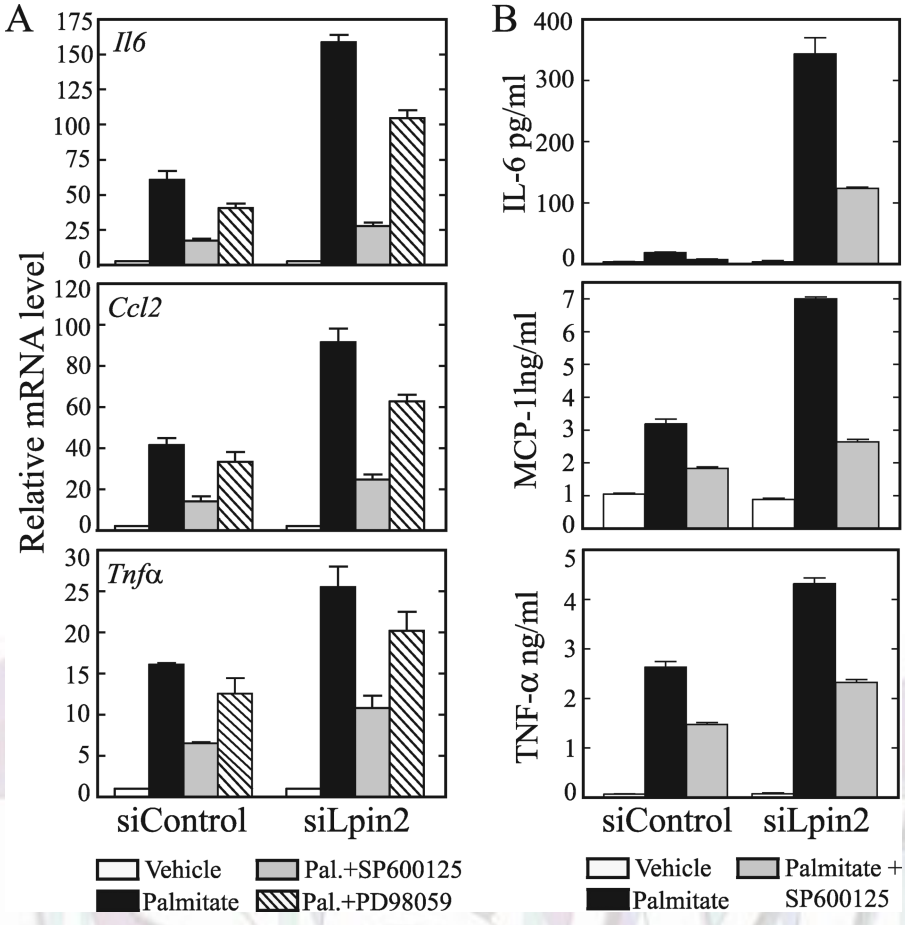


FIGURE 4. **Effect of JNK and ERK inhibition on the up-regulation of proinflammatory genes in lipin-2-deficient macrophages.** *A,* real time PCR analysis of the mRNA expression levels of *Il6*, *Ccl2,* and *Tnfα* in RAW 264.7 cells transfected with control siRNA or siRNA against *Lpin2*. The cells were treated with vehicle (*open bars*) or 300 μM palmitic acid alone (*black bars*) or 300 μM palmitic acid plus 10 μM SP600125 (*gray bars*) or 10 μM PD98059 (*striped bars*) for 8 h. *B*, cellular supernatants from cells transfected with control siRNA or siRNA against *Lpin2* and treated exactly as in *A* for 24 h were assayed for the indicated proinflammatory factors by specific ELISAs. Data are representative of at least three independent experiments. *Error bars* represent ± S.E. ($n = 3$).

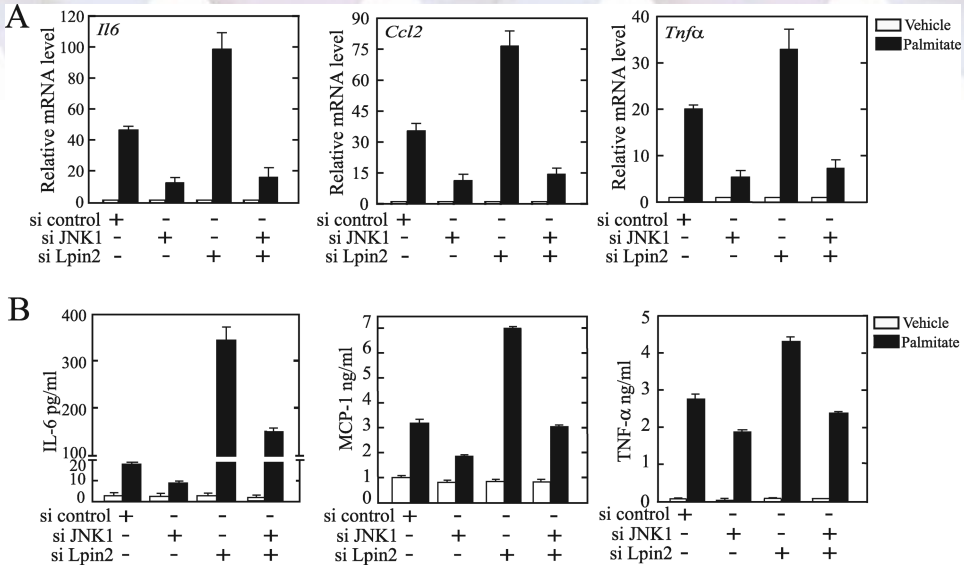
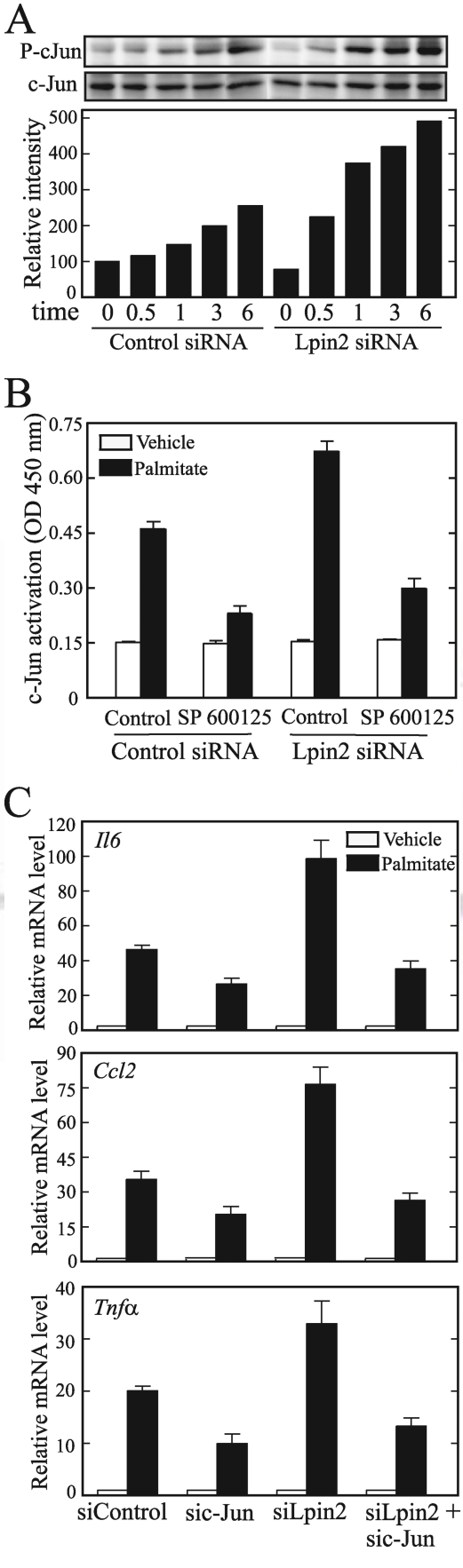


FIGURE 5. **JNK1 mediates the up-regulation of proinflammatory genes in lipin-2-deficient macrophages.** RAW 264.7 cells were transfected with control siRNA, siRNA against *JNK1* (*si JNK1*), against *Lpin2* (*si Lpin2*), or both and treated with vehicle (*open bars*) or 300 μM palmitic acid (*black bars*). *A*, real time PCR analysis of the expression of *Il6*, *Ccl2,* and *Tnfα* mRNA after 8 h. *B*, cellular supernatants after 24 h treatment were assayed for the indicated proinflammatory factors by specific ELISAs. Data are representative of at least three independent experiments. *Error bars* represent ± S.E. ($n = 3$).



F7

F8

Lipin-2 Regulates the Incorporation of Fatty Acids in TAG in Macrophages—The enzymatic activity of lipins (Mg^{2+}-dependent phosphatidic acid phosphatases) is centrally involved in the *de novo* synthesis of lipids (37). Because the specific role of the various lipin isoforms in lipid metabolism in macrophages and other cells is not well defined, studies were conducted to assess whether lipin-2 plays a regulatory role in TAG synthesis by macrophages exposed to saturated fatty acids. Because TAG molecules are stored in cytoplasmic lipid droplets, these organelles were studied by microscopy after staining with BODIPY 493/503. As shown in Fig. 7*A*, depletion of lipin-2 by siRNA resulted in a considerable reduction of the lipid droplet content of macrophages. These results were confirmed by flow cytometry, which allows quantification (Fig. 7*B*). Direct measurements of total TAG mass were carried out by mass spectrometry. Cell treatment with palmitic acid increased cellular TAG content by about 30% (Fig. 7*C*). However, in the absence of lipin-2, palmitic acid-induced TAG synthesis was strongly reduced, thus demonstrating the key role of lipin-2 in this process. Analyses of the fatty acid composition of TAG in the RAW 264.7 macrophages revealed that this neutral lipid was composed almost exclusively of palmitic acid (16:0) and stearic acid (18:0). The content of both fatty acids increased in TAG after exposure of the cells to palmitic acid but that did not happen when lipin-2-depleted cells were used (Fig. 7*D*).

Human Macrophages Exhibit an Exacerbated Proinflammatory Response in Absence of Lipin-2—Experiments were also conducted with human blood monocyte-derived macrophages to assess whether our observations with RAW 264.7 macrophages could be extended to primary cells and thus bear pathophysiological relevance. Like RAW 264.7 macrophages, human macrophages also express *LPIN1*α, *-1*β, *-2,* and *-3* mRNAs (Fig. 8*A*). Treatment of human macrophages with palmitic acid induced the expression of the proinflammatory genes *Il6* and *Tnf*α, and the lack of lipin-2 promoted a very marked up-regulation of them (Fig. 8*B*). In these cells, a quantitative depletion of lipin-2 (>80%) was achieved by siRNA technology (Fig. 8*C*). Also, in agreement with the results mentioned above, TAG mass measurements by mass spectrometry confirmed that depletion of lipin-2 strongly reduced the TAG content in human macrophages after palmitic acid treatment (Fig. 8*D*). Note that the basal TAG content in human macrophages is very high. In these cells, nine fatty acids were readily detectable in TAG (Fig. 8*E*). Treatment of the cells with palmitic acid increased the content of this fatty acid in TAG and, curiously,

FIGURE 6. **c-Jun is overactivated in lipin-2-deficient macrophages.** *A,* RAW 264.7 macrophages were transfected with control siRNA or siRNA against *Lpin2* and treated with 300 μM palmitic acid for the indicated periods of time. Phosphorylation levels and total c-Jun were assayed by Western blot. The *lower panel* represents the quantification of the relative intensity of the phosphorylated bands against total c-Jun protein. *B,* nuclear c-Jun DNA binding activity was assayed by a specific kit in cells transfected with either control siRNA or siRNA against *Lpin2* and then treated with vehicle (*open bars*) or 300 μM palmitic acid (*black bars*) in the absence or presence of 10 μM SP600125 for 3 h, as indicated. Absorbance at 450 nm is shown. *C,* real time PCR analysis of the expression of *Il6*, *Ccl2,* and *Tnf*α mRNA in cells transfected with control siRNA or with siRNA against c-*Jun* (*sic-Jun*), *Lpin2,* or both and treated with vehicle (*open bars*) or 300 μM palmitic acid (*black bars*) for 8 h. Data are representative of at least three independent experiments. *Error bars* represent ± S.E. (n = 3).

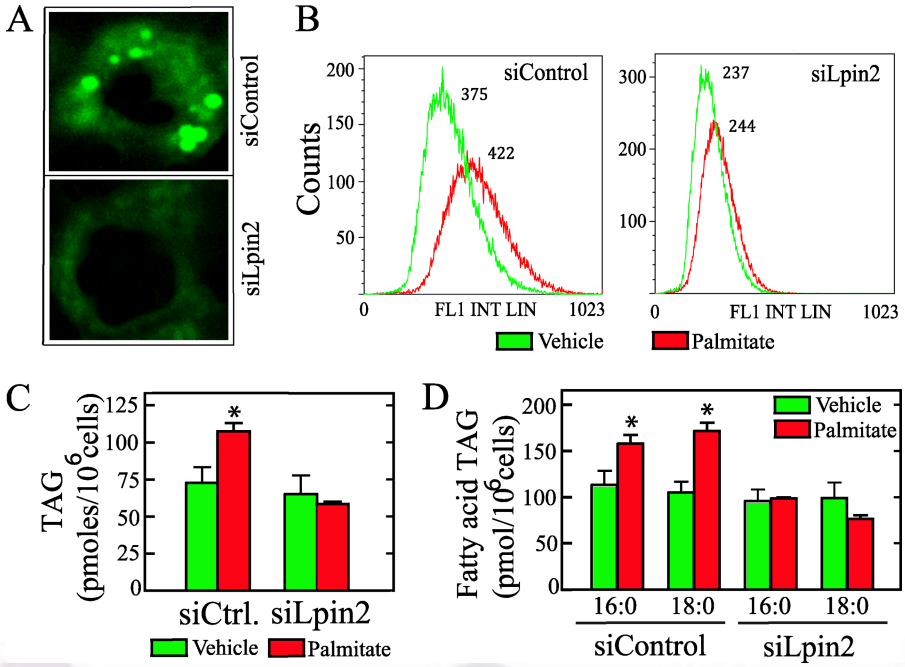


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FIGURE 7. **Lipin-2-deficient macrophages exhibit a diminished capacity to produce TAG.** *A*, RAW 264.7 macrophages transfected with control siRNA or siRNA against *Lpin2* were treated with 300 μM palmitic acid for 16 h. Cells were stained with BODYPI 493/503 and visualized by fluorescence microscopy. *B*, cells treated as in *A* were analyzed by flow cytometry. *Green traces* refer to untreated cells and *red traces* to palmitic acid-treated cells. *Numbers* close to the traces represent the mean fluorescence intensity for each condition. *C* and *D*, cells transfected with control siRNA or siRNA against *Lpin2* were treated with vehicle (*green bars*) or 300 μM palmitic acid (*red bars*) for 16 h. Fatty acids present in TAG were analyzed by mass spectrometry as described under "Experimental Procedures." Total cellular TAG is represented in *C*, and quantification of palmitic acid (16:0) and stearic acid (18:0), accounting for more than 90% of total fatty acids in TAG, is represented in *D*. Data are representative of at least three independent experiments. *Error bars* represent $\pm$ S.E. ($n = 3$) (*, $p < 0.05$).

decreased the content of oleic acid (18:1) and linoleic acid (18:2). Whether this finding bears any biological significance remains to be elucidated. Analysis of TAG fatty acid content in lipin-2-depleted cells revealed a general decrease in all fatty acids (Fig. 8*E*). After palmitic acid treatment, the TAG fraction of lipin-2-depleted cells did not experience the enrichment in this fatty acid that occurred in control cells (Fig. 8*E*). Collectively, these results indicate that human macrophages mount a proinflammatory response to saturated fatty acids, the extent of which appears to be controlled by lipin-2, possibly by driving the incorporation of these fatty acids into TAG.

DISCUSSION

Macrophages exhibit marked cellular responses to saturated fatty acids and play an important role in metabolic disorders that involve increased lipid exposure such as atherosclerosis or obesity (1–3). Saturated fatty acids are also known to promote endoplasmic reticulum stress and the activation of the unfolded protein response that ultimately intersects with different signaling pathways to generate a low grade chronic inflammatory state (9). Although a great deal of effort has been put into unveiling the role of kinases, chaperones, and nuclear factors in the regulation and integration of all of these processes, very little is known about the role of lipid-modifying enzyme effectors (9). Our data provide insights into the role of lipin-2 as a key player in human and murine macrophage proinflammatory activation by saturated fatty acids. Here, we demonstrate that lipin-2 uncouples saturated fatty acid activation from proinflammatory gene expression. We also show that reduction of lipin-2 expression promotes an increased proinflammatory state through the overstimulation of the JNK1/AP-1 pathway. Finally, we show that lipin-2 controls the incorporation of free fatty acids into TAGs, especially during fatty acid overload conditions, thereby reducing the damage generated. A schematic representation of all these findings is shown in Fig. 9. Collectively, the data uncover lipin-2 as a new player in the proinflammatory activation driven by saturated fatty acids, which suggests new opportunities for pharmacological intervention for the treatment of pathological conditions associated with these processes.

F9

In our experiments, activation of JNK1/c-Jun appears to constitute the major cellular contributor to inflammatory signaling. This observation is in agreement with previous studies suggesting that hematopoietic JNK is the main pathway for the expression of proinflammatory factors and macrophage infiltration in the adipose tissue of mice on a high fat diet (3, 8).

Recently, it has been reported that deletion of yeast phosphatidate phosphatase makes the cells more sensitive to fatty acid-induced toxicity (38), a finding that concurs with the results presented here. Yeast possesses only one gene coding for phosphatidate phosphatase, whereas mammals possess three genes that encode for lipin proteins, and one of them, *Lpin1*, undergoes alternative splicing to generate two more lipin variants in mouse (lipin-1α and -1β) and three in humans (lipin-1α, -1β, and -1γ). Evidence is accumulating to indicate that some of these proteins may regulate distinct processes in cellular physiology. For example, lipin-1α and -1β appear to control different aspects of adipocyte differentiation (39). The protective anti-inflammatory role attributed to lipin-2 in this work

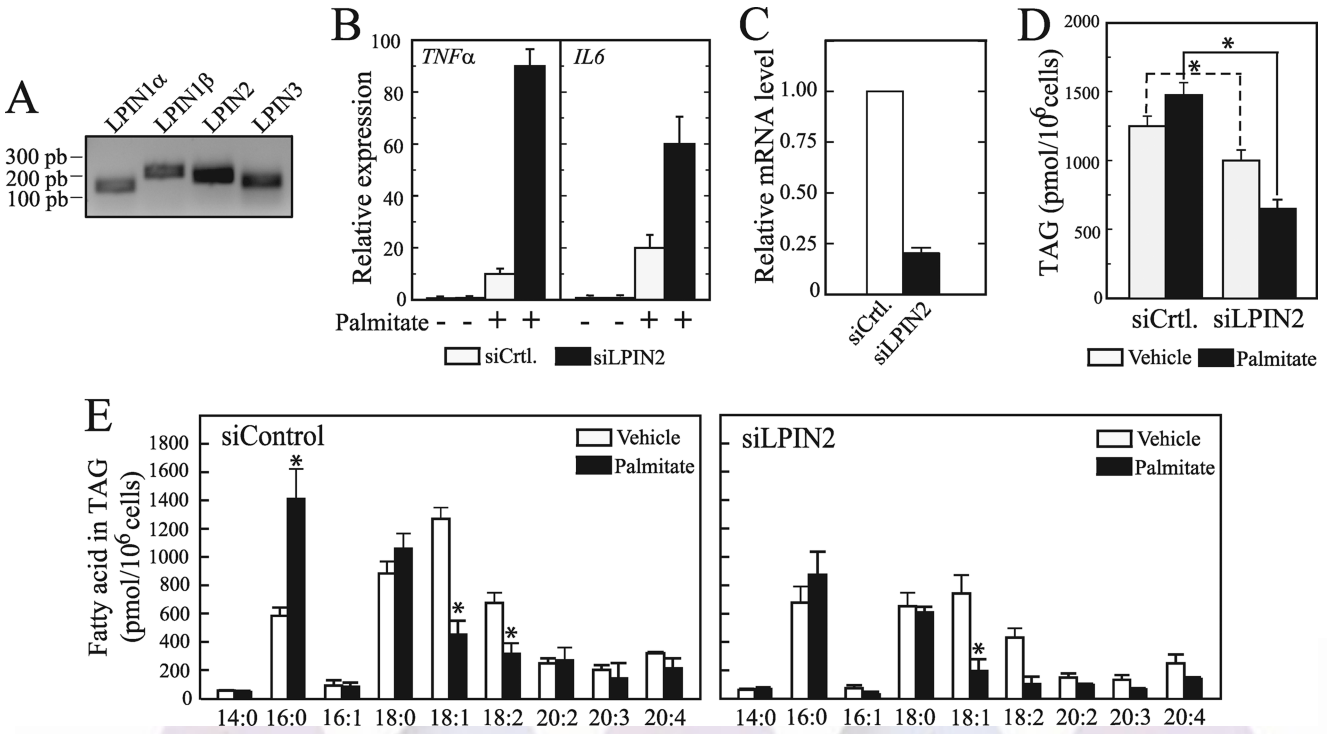

FIGURE 8. **Lipin-2 controls proinflammatory gene expression and TAG storage in human macrophages.** *A,* PCR analysis of *LPIN1α, -1β, -2,* and *-3* mRNA in human macrophages. *B,* human macrophages were transfected with a control siRNA (*open bars*) or siRNA against *LPIN2* (*black bars*) and then treated or not with 300 μM palmitic acid for 8 h as indicated. mRNA levels for *TNFα* and *IL6* were analyzed by real time PCR. *C,* mRNA expression levels of *LPIN2* in cells transfected with control siRNA (*open bar*) or against *LPIN2* (*black bar*) for 48 h. *D* and *E,* mass spectrometry analysis of fatty acids esterified into TAG of human macrophages transfected with control siRNA or siRNA against *LPIN2* and treated with vehicle (*open bars*) or 300 μM palmitic acid (*black bars*) for 16 h. Total TAG content is shown in *D* and fatty acid composition of TAG is shown in *E*. Data are representative of at least three independent experiments. *Error bars* represent ± S.E. ($n = 3$) (*, $p < 0.05$).

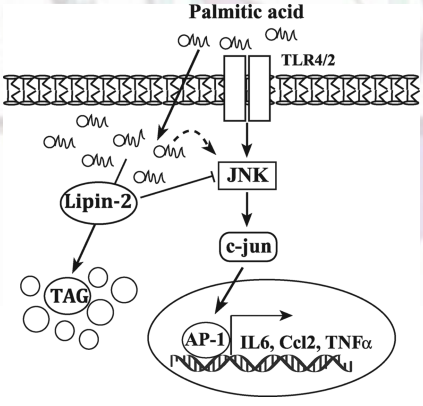

FIGURE 9. **Role of lipin-2 in the generation of proinflammatory mediators in macrophages stimulated with saturated fatty acids.** Saturated fatty acids such as palmitic acid impact on cellular homeostasis by two different mechanisms. In the first mechanism, saturated fatty acids act through TLR4/2 and turn on a cascade of signals that culminate in JNK/AP-1 activation. In the second mechanism, saturated fatty acids enter the cell and promote cellular damage. Both pathways end in the up-regulation of proinflammatory genes by macrophages. Lipin-2, by controlling the biosynthesis of TAG and hence the incorporation of fatty acids into neutral lipids, attenuates the activation of JNK/c-Jun and the levels of proinflammatory mediators such as IL-6, MCP-1, and TNFα.

appears to be specific for this protein, because depletion of either lipin-1 or lipin-3 does not show any appreciable effect during palmitic acid overload.

In previous work, we demonstrated that lipin-1 modulates size, amount, and TAG fatty acid composition of lipid droplets in human macrophages (20). However, TAG synthesis itself does not appear to be regulated by lipin-1, because depletion of this protein does not affect the rate of fatty acid incorporation into TAG (20). In this study, we have observed that TAG mass in lipin-2-depleted macrophages, either human or murine, is significantly decreased as compared with cells expressing normal lipin-2 levels, and this occurs under normal or fatty acid overload conditions. Putting all these observations together, it appears clear that in macrophages, lipin-1 and lipin-2 play different roles. On the one hand, lipin-1 regulates lipid droplet formation by impinging upon cytosolic group IVA phospholipase A_2-mediated signaling (20, 40–42); on the other hand, lipin-2 acts to regulate cellular TAG mass levels. Lipin-2 would therefore be a "metabolic" enzyme, producing diacylglycerol moieties to accommodate fatty acids in TAG, thereby antagonizing the stress produced by excess free fatty acids.

The results presented in this work might provide clues to explain, at least in part, the episodes of inflammation that occur in patients with Majeed syndrome and psoriasis, which are associated to lipin-2 mutations (18, 19). For example, in those patients who have an inflammatory process already ongoing, saturated fatty acids could produce an exacerbated inflammatory state in macrophages, generating more proinflammatory cytokines and contributing to the maintenance of inflammation. In the same line of thought, it has been described that obese patients with psoriasis are more difficult to treat than non-obese individuals (43, 44). Several trials have demonstrated that psoriatic patients have partially recovered after weight loss and that increases in weight worsen the symptoms (43, 44).

In a recent study with a Dutch population, researchers discovered a single nucleotide polymorphism (SNP9-rs3745012) within the 3′-untranslated region of LPIN2 that is significantly associated with type-2 diabetes in individuals with a high body mass index that affects fat distribution, making it a candidate to be a *thrifty allele* (45, 46). Because SPN9 is a noncoding polymorphism, the authors claim that it could produce its effects by changing the levels of expression of LPIN2. While waiting for the real consequences of this polymorphism, this observation is in agreement with our results suggesting that mutations that affect lipin-2 in humans may influence the development of conditions like type-2 diabetes, which are related to the adipose tissue inflammatory state of the patient.

In summary, results from this work have unveiled a role for lipin-2 as a key participant in the regulation of proinflammatory gene expression by saturated fatty acids in macrophages. Our observations show that the expression levels of cellular lipin-2 may have important consequences not only for the incorporation of fatty acids to TAG but also for the damage produced by those fatty acids to the cell and for the proinflammatory response in macrophages. Because we have extended our studies to human macrophages, these results could have relevance in the development of new strategies to treat lipid-related low inflammation conditions.

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