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A Jak2 inhibitor, AG490, reverses lipin-1 suppression by TNF-α in 3T3-L1 adipocytes

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A Jak2 inhibitor, AG490, reverses lipin-1 suppression by TNF-α in 3T3-L1 adipocytes

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Abstract

Lipin-1 is a multifunctional metabolic regulator, involving in triacylglycerol and bioactive glycerolipids synthesis as an enzyme, transcriptional regulation as a coactivator, and adipogenesis. In obesity, adipose lipin-1 expression is decreased. Although lipin-1 is implicated in the pathogenesis of obesity, the mechanism is still not clear. Since TNF-α is deeply involved in the pathogenesis of obesity, insulin resistance, and diabetes, here we investigated the role of TNF-α on lipin-1 expression in adipocytes. Quantitative PCR studies showed that TNF-α suppressed both lipin-1A and -1B isoform expression in time- and dose-dependent manners in mature 3T3-L1 adpocytes. A Jak2 inhibitor, AG490, reversed the suppressive effect of TNF-α on both lipin-1A and -1B. In contrast, NF-κB, MAPKs, ceramide, and β-catenin pathway tested were not involved in the mechanism. These results suggest that TNF-α could be involved in obesity-induced lipin-1 suppression in adipocytes and Jak2 may play an important role in the mechanism.

Keywords: Lipin; Obesity; TNF-α; Jak2; Adipocytes.

Introduction

Lipin-1 was identified as a responsive mutant gene in fatty liver dystrophy (*fld*) mouse at the year of 2001 and acts as phosphatidic acid phosphatase-1 which is involving in a synthesis of triacylglycerol [1,2]. The member of mammalian lipin family has been classified into lipin-1A, -1B, -2, and -3 with distinct tissue expression patterns [2,3]. Lipin-1 is predominantly expressed in adipose tissue and skeletal muscle. Transient overexpression of lipin-1A or -1B in mouse embryonic fibroblasts showed that lipin-1A is required for adipogenesis, whereas lipin-1B induces lipogenic genes [3,4]. In liver, lipin-1 is working as a transcriptional coactivator for peroxisome proliferator-activated receptors (PPARs) and PPAR-γ coactivator-1 thereby controlling of hepatic lipid metabolism [5]. Lipin-1 may also act on the assembly and secretion of hepatic very low density lipoproteins [6]. From these findings, lipin-1 should be considered as a multifunctional metabolic regulator instead of a lipid-regulating enzyme (reviewed in [7,8]).

In human studies, Yao-Borengasser *et al.* [9] showed that the expression level of lipin-1 in subcutaneous white adipose tissue is decreased in patients presenting impaired glucose tolerance and is inversely correlated to the body mass index more strongly in lipin-1B than -1A. Similar studies were reported that the expression level of lipin-1 in subcutaneous white adipose tissue is decreased in obesity or the patients representing metabolic syndrome and the decrease is recovered by weight reduction [10,11]. In contrast, adipocyte specific lipin-1 transgenic mice showed obesity phenotype but strikingly amelioration of insulin resistance [12]. Although these findings suggest that the expression level of adipose lipin-1 contributes to adipocyte functions, little is known about the mechanism of decreased lipin-1 expression in obesity. To elucidate the precise molecular mechanism of attenuated adipose lipin-1

expression in obesity will clue to a therapeutic target for obesity and insulin resistance.

Obesity triggers a chronic inflammatory state and cytokine release from either adipocytes or macrophages infiltrating adipose tissue [13,14]. Since TNF-α is addressed to a key cytokine for altering metabolic function of adipocytes, we made a hypothesis that TNF-α plays a role in decreased adipose lipin-1 expression in obesity. To clarify the above hypothesis, we investigated here a role of TNF-α on lipin-1 mRNA expression in 3T3-L1 adipocytes.

Materials and Methods

Chemicals

TNF-α and desipramine hydrochloride were purchased from Sigma (St. Louis, MO). SN50 and C_2-Ceramide were purchased from Biomol (Plymouth Meeting, PA). U0126, SB202190, SP600125, GSK-3β inhibitor VIII, and AG490 were purchased from Calbiochem (San Diego, CA) and were dissolved in dimethy sulfoxide (DMSO) with a final concentration of 0.1% DMSO in culture medium. Bovine serum albumin (BSA) used was free fatty acid-free grade (Sigma).

Cell culture

3T3-L1 fibroblasts were purchased from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) with 4.5 g/l glucose supplemented with 10% bovine serum (Invitrogen,

Carlsbad, CA). Before adipocytic induction, confluent fibroblasts were cultured to DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen) for 2 days. Differentiation of 3T3-L1 preadipocytes was induced by exposing the confluent cells to insulin (2 μM), 3-isobutyl-methyl-xanthine (0.5 mM), and dexamethasone (1 μM) for 2 days, and then to insulin (2 μM) alone for an additional 2 days. After incubation with these reagents, the medium containing 10% FBS was replenished every other day. Cells were used 13-14 days after differentiation induction when exhibiting more than 95% adipocytes phenotype. Before exposing TNF-α or each reagent indicated in the text, differentiated adipocytes were serum-starved in serum-free DMEM containing 0.1% BSA for 12 h.

Quantitative Real-time Reverse Transcription-PCR analysis

Total RNA was extracted from 3T3-L1 adipocytes using TRIzol reagent (Invitrogen). Complimentary DNA was synthesized with random primers using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time RT-PCR analysis was performed with an Applied Biosystems 7300 Sequence Detection System using TaqMan Gene Expression master mix according to the manufacturer's specifications (Applied Biosystems). Validated TaqMan Gene Expression Assays containing gene specific TaqMan probes and primers for mouse lipin-1A (assay identification no. Mm00522205_m1 corresponding to GenBank accession no. **NM_172950**), mouse lipin-1B (Mm01276800_m1, **NM_015763**) were used for assay-on-demand gene expression products (Applied Biosystems). To normalize the relative expression of the genes of interest, the Eukaryotic 18S rRNA (Hs99999901_s1, **X03205.1**) gene was used as an endogenous control. All samples were performed at least in triplicate. Amplification data were

analyzed by comparative threshold cycles (CT) method with a Sequence Detection Software version 1.4 (Applied Biosystems). The 2-DDCT method was used to calculate the relative mRNA expression [15].

Statistical Analysis

Data are expressed as the means ± S.E. Statistical analysis was performed by analysis of variance and subsequent Newman-Keuls multiple comparison tests using GraphPad Prism Software Version 4. $P < 0.05$ was considered statistically significant.

Results

First, we made to elucidate whether murine TNF-α suppresses lipin-1 mRNA expression in fully differentiated 3T3-L1 adipocytes. As demonstrated in Fig. 1A-D, TNF-α suppressed both lipin-1A and -1B mRNA expression in dose- and time-dependent manners.

To elucidate a possible mechanism by which TNF-α-induced lipin-1A and -1B gene suppressions, we examined if intracellular TNF-α signaling molecules such as nuclear factor-kappa B (NF-κB), mitogen-activated protein kinases (MAPKs), ceramide, and β-catenin/TCF pathway, may be implicated in the mechanism. To evaluate the effect of NF-κB, SN50, a cell-permeable peptide which inhibits translocation of the NF-κB active complex into nucleus [16], was tested. As illustrated in Fig. 2A and B, SN50 did not reverse the effect of TNF-α on lipin-1

expression. Mitogen-activated protein kinases (MAPKs) are a family of three distinct protein kinases termed MEK-ERK1/2, p38, and c-Jun N-terminal kinase (JNK) and involving in the intracellular signaling of TNF-α. To clarify whether each MAPK signaling is involved in the reduced expression of lipin-1 mRNA by TNF-α, we examined the effects of each kinase inhibitor for MEK (U0126), p38 (SB202190), or JNK (SP600125) on the inhibition of mRNA expression of lipin-1 by TNF-α in 3T3-L1 adipocytes. As demonstrated in Fig. 2C and D, pretreatment with each MAPK inhibitor failed to block the suppression of lipin-1A and -1B mRNA by TNF-α.

To examine a role of ceramide signaling, the cells were exposed to C_2-Ceramide, a cell-permeable ceramide analogue, at a concentration of 50 or 100 μM for 8 h. C_2-Ceramide did not affect on both the lipin-1A and -1B mRNA expression (Fig. 3A and B). Because ceramide is synthesized from sphingomyelin by sphingomyelinase, we additionally examined the effect of desipramine, a sphingomyelinase inhibitor, on the reduced expression of lipin-1 mRNA by TNF-α. As shown in Fig. 3C and D, TNF-α significantly inhibited mRNA expression of lipin-1A and -1B in 3T3-L1 adipocytes that had been pretreated with desipramine. To test the involvement of a β-catenin/TCF pathway in the TNF-α-induced lipin-1 suppression, we used an inhibitor for glycogen synthase kinase-3β (GSK-3β), which inhibits β-catenin by phosphorylation and degradation. Fig. 3E and F showed that GSK-3β inhibitor VIII did not affect on both the lipin-1A and -1B gene expression either in the absence or presence of TNF-α.

Since TNF-α induces the tyrosine phosphorylation and activation of the intracellular Janus tyrosine kinase 2 (Jak2) in 3T3-L1 adipocytes [17], we next investigated whether Jak2 could be involved in the suppressive effect on lipin-1

expression by TNF-α. As seen in Fig. 4A and B, AG490 (50 μM), a Jak2 inhibitor [18], completely blocked the reduced expression of lipin-1A, while partly blocked that of lipin-1B.

Discussion

Lipid-regulating enzymes often affect whole body metabolism in addition to lipid metabolism. For examples, acyl CoA:diacylglycerol acyltransferase 1 or mitochondrial acyl-CoA:glycerol-sn-3-phosphate acyltransferase 1 knockout mice exhibited increased insulin sensitivity [19,20]. Moreover, we have recently reported that diacylglycerol kinase might be involved the glucose transport in muscle cells [21]. Here we focused on lipin-1, one of lipid-regulating enzymes, which works as phosphatidic acid phosphatase-1 to convert phosphatidic acid into diacylglycerol [2]. In obesity, adipose lipin-1 expression is decreased in agreement with several reports [9-11]. In contrast, adipose specific overexpression of lipin-1 in mice revealed obesity phenotype but amelioration of insulin resistance [12]. These results suggest that expression levels of lipin-1 in adipocytes closely and positively correlate with whole glucose metabolism. To elucidate the mechanism by which obesity-related lipin-1 suppression in adipose tissue may become a clue to a new target for treating obesity and insulin resistance.

Since TNF-α plays pivotal and exacerbating roles in adipocytes functions in obesity [14], we made to elucidate whether TNF-α may be implicated in decreased lipin-1 expression in adipocytes. We used fully differentiated 3T3-L1 adipocytes as

a model of adipocytes in the present study. Since there are functional differences between lipin-1A and -1B [3,8], we analyzed both lipin-1A and -1B isoforms throughout in this study. As clearly demonstrated in this study, TNF-α decreased lipin-1A and -1B expression in 3T3-L1 adipocytes in dose- and time- dependent manners. Very recently, Lu *et al.* [22] have reported in harmony with our result that TNF-α decreased lipin-1 expression in 3T3-L1 adipocytes. However, they have not mentioned the mechanism by which TNF-α decreased lipin-1 expression. Therefore we next tried to clarify the molecular mechanism by which TNF-α decreased lipin-1 expression in 3T3-L1 adipocytes.

Nuclear factor-kappa B (NF-κB) acts as a key transcription factor for many biological actions induced by TNF-α [14]. TNF-α suppresses various genes in 3T3-L1 adipocytes including insulin signal molecules. Ruan *et al.* [23] demonstrated that inactivation of NF-κB abolished the suppression of >98% of the genes normally suppressed by TNF-α through the expression of a non-degradable mutant of NF-κB inhibitor, IκBα-DN. We therefore tried to find the possibility that NF-κB could be involved in the suppressive effect of TNF-α on lipin-1 expression by using SN50, a cell-permeable inhibitory peptide for NF-κB. SN50 failed to block the inhibition of lipin-1 mRNA expression by TNF-α, indicating that NF-κB dose not play a role in the mechanism.

MAPKs are activated by TNF-α in phosphorylation cascades and will eventually phosphorylate and activate distinct sets of kinases and transcription factors [14]. Therefore, we tested the possibility that MAPKs influenced decreased lipin-1 expression by TNF-α. The possibility is unlikely because each MAPK inhibitor did not block the suppressive effect of TNF-α on lipin-1 mRNA expression.

Ceramide is an intracellular lipid and acts as an intermediate molecule linking

TNF-α to cellular insulin resistance by inhibiting insulin signalings [24]. Because TNF-α is known to increase intracellular ceramide, we hypothesized that ceramide may be involved in the decreased lipin-1 expression by TNF-α. We also used desipramine to evaluate an intrinsic sphingomyelin-ceramide signaling. The present results provided that C_2-Ceramide itself did not decrease both the lipin-1 expression and desipramine did not reverse the suppressive effect of TNF-α on the lipin-1 expression, suggesting ceramide pathway is not involved in the TNF-α action on lipin-1 expression.

Recently, canonical Wnt/β-catenin/TCF4 signaling has been involved in the inhibition of adipogenesis by TNF-α [25]. We applied the concept that TNF-α affects β-catenin signaling to make a hypothesis that this signaling might play a role in TNF-α-induced lipin-1 mRNA suppression. Because GSK-3β phosphorylates β-catenin and targets it for ubiquitin-mediated degradation, inhibition of GSK-3β enhances β-catenin/TCF4 pathway through accumulation of β-catenin [26]. If this pathway is involved in the inhibitory action of TNF-α on lipin-1 expression, inhibition of GSK-3β reduces lipin-1 expression similarly to the action of TNF-α, or may enhance the action of TNF-α. As demonstrated in this study, inhibition of GSK-3β alone or in combination with TNF-α did not affect the lipin-1 expression, suggesting that β-catenin pathway is not involved in the suppressive effect of TNF-α on lipin-1 expression.

Janus tyrosine kinase (Jak) family including Jak1, Jak2, Jak3 and Tyr2 is a receptor associated kinase and has been activated by various cytokines (e.g. erythropoietin, interleukin (IL) -3, and interferon (IFN) -γ). In 3T3-L1 adipocytes, Guo *et al.* [17] reported that murine TNF-α induces tyrosine phosphorylation and activation of the intracellular Jak1, Jak2 and Tyr2. Because TNF-α affects mostly on

Jak2 in the report, we investigated whether Jak2 could be involved in the lipin-1 suppression by TNF-α. The present study showed that AG490, a Jak2 inhibitor, clearly reversed the TNF-α-induced suppression of both the lipin-1A and -1B expression (Fig. 4A, B). Lu *et al.* [22] have reported that IL-1β and IFN-γ in addition to TNF-α suppressed lipin-1 expression in adipocytes. Because IFN-γ activates Jak2 signaling [27], involvement of Jak2 as showed in this study should be a pivotal role in the mechanism. The recovery level of lipin-1B was weaker than that of lipin-1A. There might be some additional signals involving in the suppression of lipin-1B. Because lipin-1A and -1B are produced from same lipin-1 gene by alternative splicing, such lipin-1B specific signals might affect lipin-1 gene expression including mRNA splicing mechanism.

The precise roles of Jak2 in glucose and lipid metabolism have not been elucidated, although Jak is known as the intracellular signaling molecule under stimulation of leptin [28] and growth hormone [29]. Thirone *et al.* [30] reported that siRNA-mediated gene knock-down for Jak2 in L6 skeletal muscle cells relieves insulin resistance induced by ceramide or knock-down for insulin receptor substrate-1. The report concluded that inhibition of Jak2 might be useful strategy to relieve insulin resistance of metabolic outcomes. Because amelioration of insulin resistance is thought to be obtained by the recovery of TNF-α-induced lipin-1 suppression by inhibiting Jak2 revealed in this study, there is a same metabolic direction between our and their results in the meaning of Jak2 to insulin resistance.

Activation of Jak2 by TNF-α is accompanied by the tyrosine phosphorylation of members of the STAT (signal transducers and activators of transcription) family of transcription factors and the induction of STAT DNA-binding activity [27]. This raises the possibility that STAT may be involved in the reduced lipin-1 expression by

TNF-α at transcriptional level. Further investigation is needed to be elucidated the precise involvement of Jak/STAT pathway in this mechanism.

Great majority of the suppressed genes by TNF-α have been mediated by NF-κB [23]. In this regard, mediation of Jak2 not NF-κB pathway for the suppression of lipin-1 gene expression by TNF-α is considered as a rare pathway among the overall gene suppressing effects of TNF-α. This rarity may take advantage of quest for specific means to recover lipin-1 expression in terms of not interfering with other common signalings of TNF-α.

In summary, our data showed that an obesity-related cytokine, TNF-α, reduces both the lipin-1A and lipin-1B mRNA expression. Our novel findings are Jak2 signaling could be involved in decreased lipin-1A and -1B mRNA expression induced by TNF-α. These findings suggest that Jak2 signaling could be a target for preventing adipose lipin-1 depletion, connecting to the new treatment for obesity.

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Figure legends

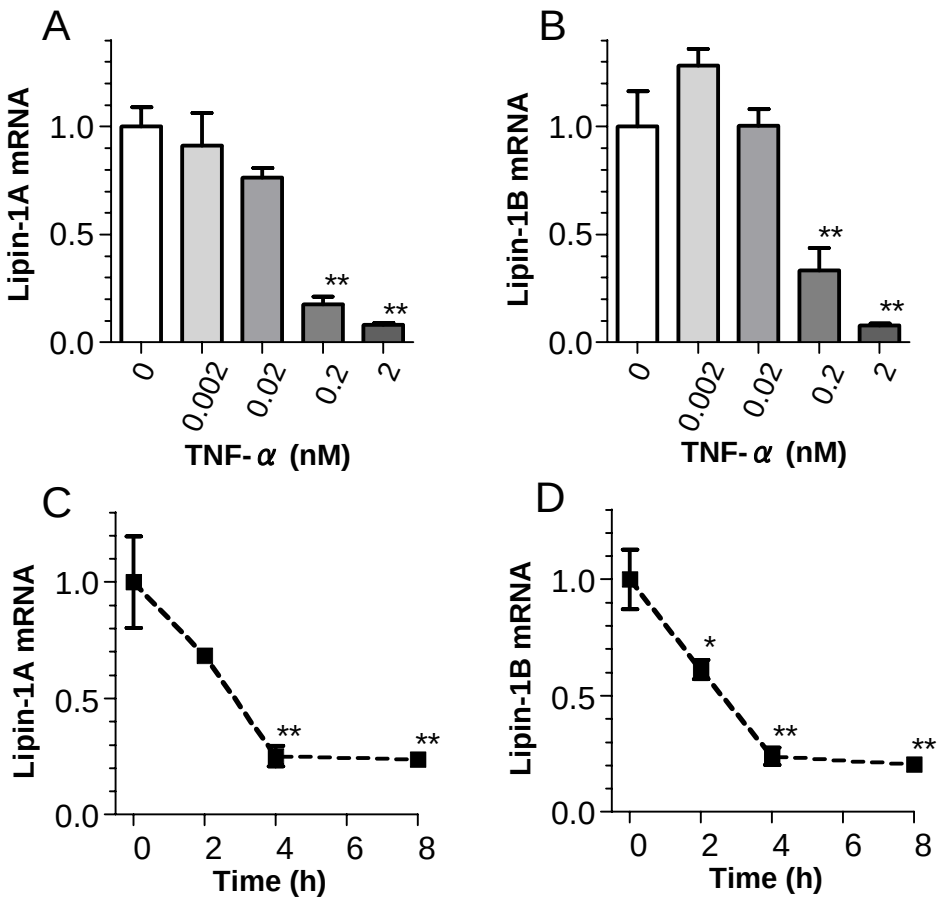
Fig. 1. Effect of TNF-α on mRNA expression of lipin-1A and lipin-1B in 3T3-L1 adipocytes. Serum-starved 3T3-L1 adipocytes (Day 14) were used. Dose-response effects of TNF-α (0.2 nM, 8h) on lipin-1A and -1B mRNA expression (A, B) and time-course change of lipin-1A and -1B mRNA expression by TNF-α in a dose of 0.2 nM were shown (C, D). The culture medium contained 0.1% DMSO in order to be same condition throughout the experiments. At the end of the treatment, specific mRNA was quantified by real-time RT-PCR. Data are normalized relative to the mRNA levels of 18S rRNA and expressed as the mean ± S.E. (n = 3). $*P < 0.05$, $**P < 0.01$ vs. vehicle alone (A, B) or a group of time 0 (C, D).

Fig. 2. Effects of NF-κB or MAPK inhibitor on TNF-α-induced lipin-1A and -1B gene suppression. Serum-starved 3T3-L1 adipocytes (Day 14) were cultured in the presence of SN50 (SN, 18 μM) (A, B), U0126 (U, 10 μM), SB202190 (SB, 10 μM), or SP600125 (SP, 20 μM) (C, D) for 1 h before TNF-α (TNF, 0.2 nM, 8 h) was added. At the end of the treatment, specific mRNA was quantified. Data are calculated by fold change versus DMSO control and expressed as the mean ± S.E. (n = 3-4). $**P < 0.01$ vs. DMSO; $†P < 0.05$, $††P < 0.01$ vs. SN alone.

Fig. 3. Effects of ceramide signaling and GSK-3β inhibitor on TNF-α-induced lipin-1A and -1B gene suppression. Serum-starved 3T3-L1 adipocytes (Day 14) were used. For evaluating ceramide signaling, cells were treated in the presence of C_2-Ceramide (C2, 50 or 100 μM) for 8 h or desipramine (DES, 20 μM) for 1 h before TNF-α (TNF, 0.2 nM, 8 h) was added (A-D). In another sets, cells were cultured in the presence of GSK-3β inhibitor VIII (GSK3βi, 0.5 or 1 μM) for 1 h before TNF-α

(TNF, 0.2 nM, 8 h) was added (E, F). At the end of the treatment, specific mRNA was quantified. Data are calculated by fold change versus DMSO control and expressed as the mean ± S.E. (n = 3-4). $^{**}P < 0.01$ vs. DMSO; $\dagger\dagger P < 0.01$ vs. each vehicle.

Fig. 4. Effects of AG490, a Jak2 inhibitor, on TNF-α-induced lipin-1A and -1B gene suppression. Serum-starved 3T3-L1 adipocytes (Day 14) were cultured in the presence or absence of AG490 (10-50 μM) for 1 h before TNF-α (TNF, 0.2 nM, 8 h) was added. At the end of the treatment, specific mRNA was quantified (A, B). Data are calculated by fold change versus DMSO control and expressed as the mean ± S.E. (n = 3-4). Similar results were reproduced independently three times. $^{**}P < 0.01$ vs. DMSO; $\dagger P < 0.05$, $\dagger\dagger P < 0.01$ vs. each TNF-α alone; $\ddagger P < 0.05$ vs. AG490 (50 μM) alone.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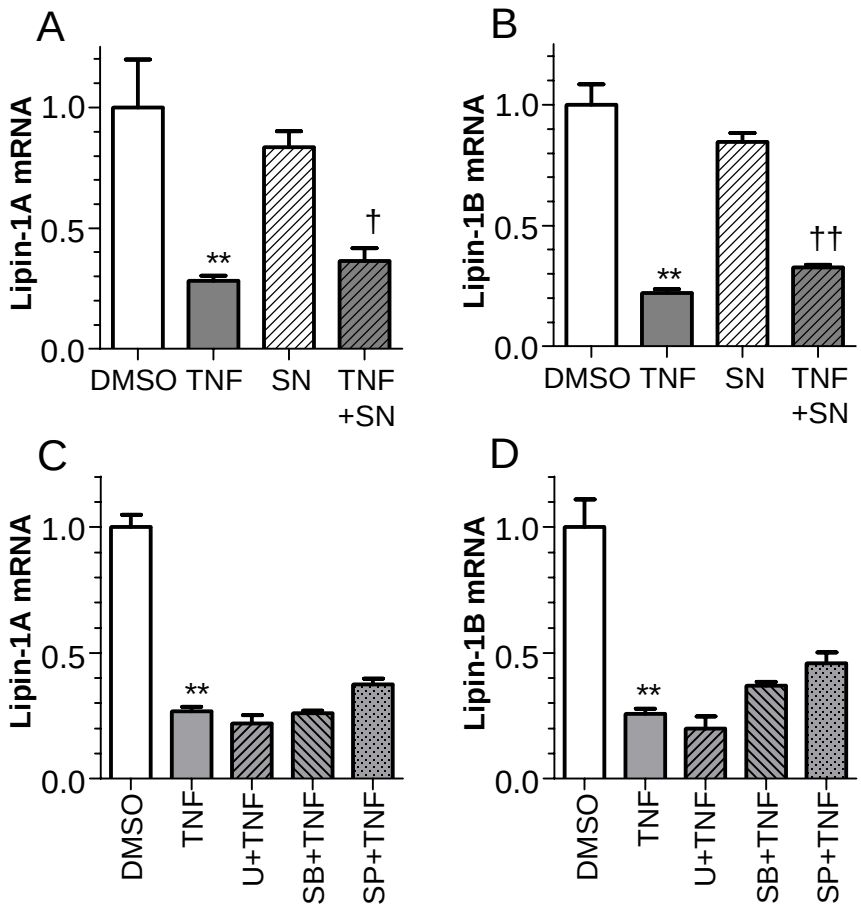
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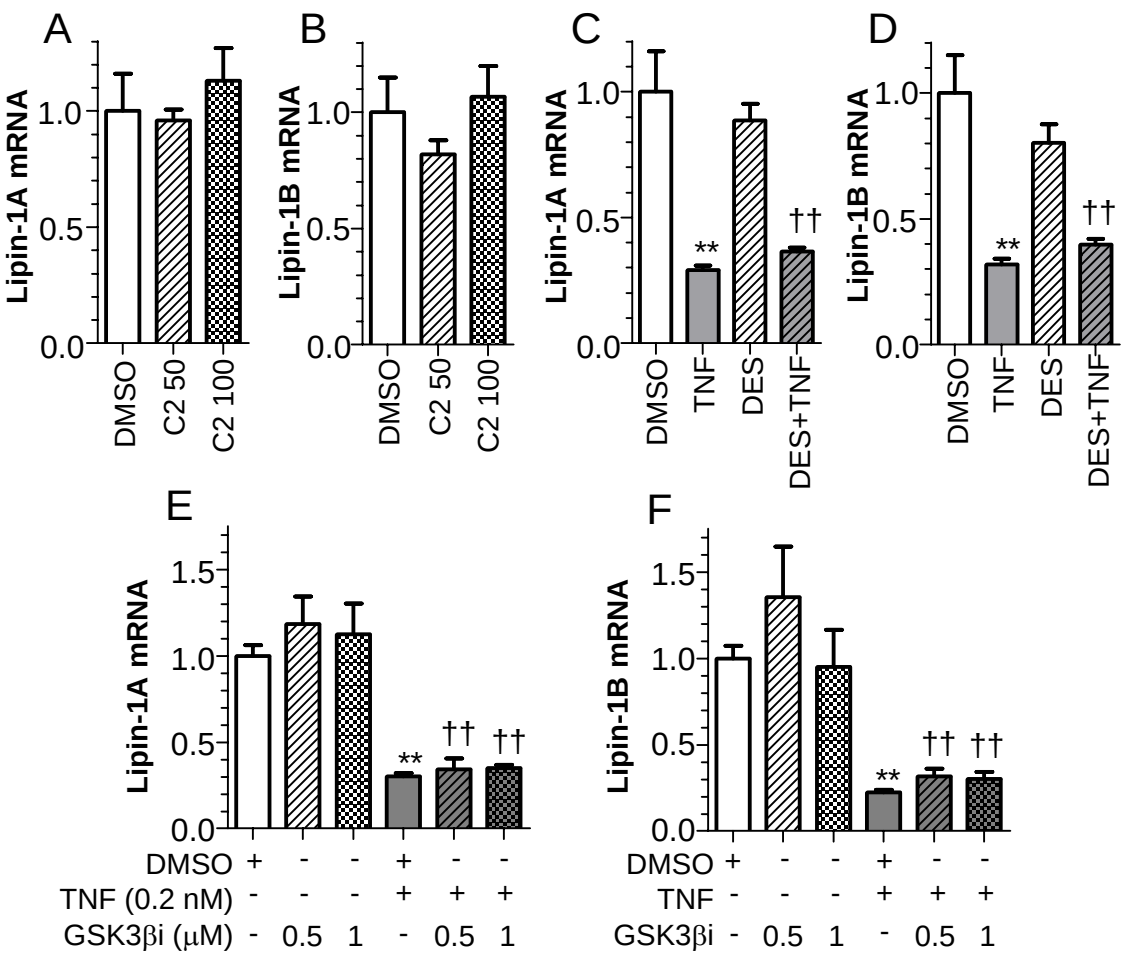
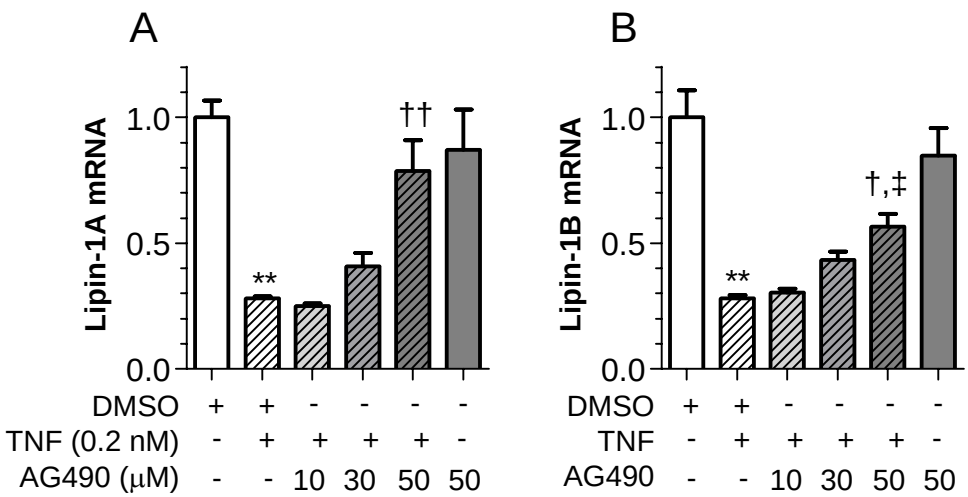
**
††
DMSO
TNF
SN
TNF
+SN
C
Lipin-1A mRNA
1.0
0.5
0.0
**
DMSO
TNF
U+TNF
SB+TNF
SP+TNF
D
Lipin-1B mRNA
1.0
0.5
0.0
**
DMSO
TNF
U+TNF
SB+TNF
SP+TNF


A
B
C
D
Lipin-1A mRNA
Lipin-1B mRNA
1.0
0.5
0.0
DMSO
C2 50
C2 100
TNF
DES
DES+TNF
**
††
E
F
1.5
DMSO + - - + - -
TNF (0.2 nM) - - - + + +
GSK3βi (μM) - 0.5 1 - 0.5 1
TNF -
GSK3βi -


A
Lipin-1A mRNA
1.0
0.5
0.0
**
††
DMSO + + - - - -
TNF (0.2 nM) - + + + + -
AG490 (μM) - - 10 30 50 50
B
Lipin-1B mRNA
1.0
0.5
0.0
**
†,‡
DMSO + + - - - -
TNF - + + + + -
AG490 - - 10 30 50 50