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JNK AND CERAMIDE KINASE GOVERN THE BIOGENESIS OF LIPID DROPLETS THROUGH ACTIVATION OF GROUP IVA PHOSPHOLIPASE A_2

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Running head: JNK and CERK govern LD biogenesis

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The biogenesis of lipid droplets (LD) induced by serum depends on Group IVA phospholipase A_2 (cPLA$_2\alpha$). This work dissects the pathway leading to cPLA$_2\alpha$ activation and LD biogenesis. Both processes were Ca^{2+}-independent, as they took place after pharmacological blockade of Ca^{2+} transients elicited by serum or chelation with BAPTA-AM. The single mutation D43N in cPLA$_2\alpha$, which abrogates its Ca^{2+}-binding capacity and translocation to membranes, did not affect enzyme activation and formation of LD. In contrast, the mutation S505A did not affect membrane relocation of the enzyme in response to Ca^{2+}, but prevented its phosphorylation, activation, and the appearance of LD. Expression of specific activators of different mitogen-activated protein kinases showed that phosphorylation of cPLA$_2\alpha$ at Ser505 is due to JNK. This was confirmed by pharmacological inhibition and expression of a dominant-negative form of the upstream activator MEKK1. LD biogenesis was accompanied by increased synthesis of ceramide-1-phosphate (C1P). Overexpression of its synthesizing enzyme CERK increased phosphorylation of cPLA$_2\alpha$ at Ser505 and formation of LD, while its downregulation blocked the phosphorylation of cPLA$_2\alpha$ and LD biogenesis. These results demonstrate that LD biogenesis induced by serum is regulated by JNK and CERK.

Intracellular lipid droplets (LD) are cytosolic inclusions present in most eukaryotic cells, containing a core of triacylglycerols (TAG) and cholesteryl esters, surrounded by a phospholipid monolayer and by specific proteins, among which the best characterized belong to the perilipin family (1-3). In the last few years, the biology of LD has received increasing interest, due to the close relationship between excess of lipid storage in certain tissues and pathologies such as obesity, diabetes, or atherosclerosis (3-5). Cellular stress has been related with the generation of LD, which might play a cytoprotective role (6, 7). Therefore, dissecting the signaling pathways leading to LD formation may have important clinical applications in metabolic and also in neurodegenerative diseases like Parkinson's and Alzheimer's (8, 9).

Cells generate LD from exogenous sources -free fatty acids or lipoproteins- but LD also appear in the absence of external lipids when cells are under stress, apparently after the recycling of membrane phospholipids into TAG (7). We have shown recently that Group IVA PLA_2 (also termed cPLA$_2\alpha$) is required for LD biogenesis either after exogenous lipid loading (10) or during stress (7). Regardless of the LD-inducing situation, the enzyme is not involved in the synthesis of neutral lipids; rather, it allows the formation of LD from TAG-containing membranes. cPLA$_2\alpha$ can be activated by several mechanisms (reviewed in 11 and 12). It contains a Ca^{2+} binding domain (C2) that allows interaction with membrane phospholipids in response to increased intracellular Ca^{2+} concentrations, and the single substitution D43N in this domain abrogates the

Ca^{2+}-dependent translocation of $cPLA_2\alpha$ to membranes (13-15). However, the increase of intracellular Ca^{2+} is not universally required, as several reports have shown $cPLA_2\alpha$ activation *in vivo* under resting Ca^{2+} conditions (13, 16-19). In this regard, anionic phospholipids like phosphatidylinositol (4,5)-bisphosphate (PtdIns $4,5P_2$) (20, 21) and ceramide-1-phosphate (C1P) (22-25) bind specific regions of the enzyme and decrease its Ca^{2+} requirement to interact with membranes. Also, phosphorylation of $cPLA_2\alpha$ at Ser residues 505, 515, and 727 appears to play a role in enzyme activation (11, 12). Among these sites, it is generally accepted that Ser505 is the one that leads to increased catalytic activity (26), and phosphorylation of Ser505 depends on mitogen-activated protein kinases (MAPK), among which most reports implicate ERK and p38 (11, 12).

Recently, we have shown that phosphorylation of $cPLA_2\alpha$ at Ser505 is key for LD biogenesis, as evidenced by the inability of the S505A mutant to recapitulate the effect of the endogenous enzyme (10). The present work was undertaken to delineate the mechanism of $cPLA_2\alpha$ activation that generates LD in the cell after serum addition. In essence, we show that the activation of $cPLA_2\alpha$ is independent of increases of intracellular Ca^{2+}; further, we identify c-Jun N-terminal kinase (JNK) as the MAPK implicated in $cPLA_2\alpha$ activation. Finally, we show that ceramide kinase (CERK), the enzyme responsible for the synthesis of C1P, mediates LD biogenesis by inducing the phosphorylation of $cPLA_2\alpha$ by JNK.

Experimental procedures

Materials—Nile red, primuline, trypsin, U73122 and C1P were from Sigma-Aldrich. SP600125, SB20358, PD98059 and N-acetyl-D-sphingosine (C_2-ceramide) were from Calbiochem, and methylarachidonyl fluorophosphonate (MAFP) from Cayman Chemical Co. [5,6,8,9,11,12,14,15 - ^{3}H]Arachidonic acid ([^{3}H]AA) (200 Ci/mmol) was purchased from American Radiolabeled Chemicals, and [9,10(n)-^{3}H]palmitic acid (49 Ci/mmol) from Amersham Pharmacia Biotech. OPTIMEM was from Gibco. Rabbit anti-$cPLA_2\alpha$, anti-phospho-Ser505-$cPLA_2\alpha$, anti-JNK, anti-phospho-Thr183/Tyr185-JNK, anti-p38, anti-phospho-Thr180/ Tyr182-p38, anti-p44/42 and anti-phospho-Thr202/Tyr204-p44/p42 antibodies were from Cell Signaling, chicken anti-ADRP from GenWay Biotech, rabbit anti-GAPDH from Ambion, and rabbit anti-CERK from Abcam.

Cells— CHO-K1 cells were cultured in Ham's F-12 medium (Sigma-Aldrich), containing 7.5% fetal bovine serum (FBS, from Sigma-Aldrich), 100 units/ml penicillin, and 100 μg/ml streptomycin (both from Invitrogen). Cell passages were made once a week by trypsinization. For the experiments, cells were seeded at a density of 30,000 cells/ml in 24- (0.5 ml) or 6-well (2 ml) plates and maintained in FBS-containing medium during 48 hours. Before LD induction, cells were switched to serum-free culture medium for 24 hours to set control conditions with minimal occurrence of LD (7). When indicated, cells (40–70% confluence) were transfected with 1 μg plasmid/ml using Lipofectamine Plus^{TM}, following the manufacturer's instructions.

Nile red staining and fluorescence microscopy— Cells cultured on glass bottom culture dishes were washed with phosphate-buffered saline (PBS), fixed with 3% paraformaldehyde for 10 minutes, and washed twice with PBS. Cells were overlaid with 0.5 ml PBS, to which 2.5 μl of a stock solution of Nile red in acetone (0.2 mg/ml) were added, so that the final concentrations of Nile red and acetone were 1 μg/ml and 0.5%, respectively. Samples were kept in the dark until photographed in a Leica Qwin 500 microscope with a Leica DC200 camera, using the Leica DCviewer 3.2.0.0 software.

Flow cytometry— Indirect quantification of LD by flow cytometry in Nile red-stained cells was performed as described previously (7, 10). Briefly, paraformaldehyde-fixed cells were stained with 1 μg/ml Nile red during 45 min, and analyzed with a Cytomics FC 500 (Beckman Coulter) equipped with an argon laser (488 nm), in the FL1 channel (505-545 nm). After gating out cellular debris, 30,000 events were acquired in all the assays, in linear scale. Fluorescence intensities were quantified as the median value of each distribution of events.

[^{3}H]Arachidonic release—Stimulated [^{3}H]AA release from cells, which is an estimate of cPLA$_2\alpha$ activity, was measured as described (7, 10, 27).

Ca^{2+} *imaging*— Cells grown onto poly-lysine coated coverslips were incubated with the Ca^{2+} indicator Fura-2/AM at 4 μM in Krebs buffer of the following composition (in mM): (119 NaCl, 4.75 KCl, 5 $NaHCO_3$, 1.2 $MgSO_4$, 1.18 KH_2PO_4, 1.3 $CaCl_2$, 20 Hepes, and 5 glucose, pH 7.4). After 1 hour, cells were washed and coverslips mounted in a static chamber on an inverted Nikon TE2000U microscope with a conventional epifluorescence system. Cells were excited alternatively at 340 and 380 nm, and emission light collected at 510 nm every 4-10s using a 12 bit-CCD ERG ORCA Hamamatsu camera. Ratio image of cells was analyzed using the Metafluor software (Universal Imaging). 14-20 cells were analyzed in each experiment.

Western blots— Cells were lysed with 62.5 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 10% glycerol, 50 mM dithiotreitol, and 0.01% bromophenol blue, and around 20 μg of protein were separated by standard 10% SDS-PAGE and transferred to nitrocellulose membranes. Primary (1:1,000) and secondary antibodies (1:5,000) were diluted in 25 mM Tris-HCl buffer, pH 7.4, containing 140 mM NaCl, 10% defatted dry milk, 0.1% bovine serum albummin and 0.1% Tween 20. Antibody binding was detected using the enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences) and visualized using a GeneGenome HR chemiluminiscence detection system coupled to a CCD camera or with High Performance Chemiluminiscence Films (Amersham Biosciences).

siRNA transfection— We used three short interfering RNA (siRNA) duplexes from Gene Link, targeting accession number NM_022766 (human CERK mRNA): CERK1-[537], CERK2-[1377], and CERK3-[1679], with the following sequences: 5'-GGACAAGGCAAGCGGATA UTT (sense), and 5'-TTCCUGUUCCGUUCG CCUAUA (AS) for siRNA CERK1; 5'-CGGAAAUGCUCCAGGUUCATT (sense), and 5'TTGCCUUUACGAGGUCCAAGU (AS) for siRNA CERK2; and 5'-ACGAGGAAU UGAAGAGAAUTT (sense), and 5'-TTUGCU CCUUAACUUCUCUUA (AS) for siRNA CERK3. Cells were transfected at 60% confluence with 30 nM siRNA, by adding to each 35 mm culture well 1 ml of Optimem (GIBCO) containing 1.5 μl of the stock siRNA solution (20 μM) and 5 μl of Lipofectamine Plus^{TM} (1 mg/ml, from Invitrogen). After 5 hours, 1 ml of Ham's F-12 medium containing 7.5% FBS was added, and the cells were incubated for 72 hours, then changed to serum-free medium during 24 hours prior to treatments. When appropriate, labeling with [^{3}H]palmitate to monitor the formation of C1P was done in the last 24 hours.

Formation of ^{3}H-C1P— [^{3}H]palmitate-prelabeled cells were stimulated with 7.5% FBS during 15 min, harvested on ice, washed with 1 ml PBS and centrifuged prior to extraction of lipids (28). To separate C1P, 0.2 ml aliquots of the chloroform phases were evaporated under vacuum, dissolved in 15 μl of chloroform / methanol (3:1, v/v), and spotted onto silica gel G thin layer chromatography plates (Merck), which were developed once in chloroform / methanol / water (67.5: 28.4: 4, v/v/v) and three times in hexane / diethyl ether / formic acid (55:45:1, v/v/v). After staining with primuline spray (5 mg primuline in 100 ml of acetone/water (80/20, v/v), identification of ceramide and ceramide-1-phosphate was made by co-migration with authentic standards. To quantify [^{3}H]C1P, silica in regions of the TLC plates co-migrating with standard C1P were scraped into vials, which after addition of scintillation cocktail were counted in a Perkin-Elmer Tri-Carb 2810TR at a 40% efficiency.

Confocal microscopy— Transiently transfected CHO-K1 cells were treated for 10 minutes with 5 μM ionomycin. To monitor re-location of EGFP-cPLA$_2\alpha$, EGFP-D43N-cPLA$_2\alpha$ or EGFP-S505A-cPLA$_2\alpha$, images were acquired every 60 seconds with a Leica TCS SP2 AOBS confocal microscope.

Constructs— The construct encoding for the expression of a fusion protein containing N-terminal enhanced green fluorescent protein (EGFP) followed by the entire sequence of the

human cPLA$_2\alpha$ (EGFP-cPLA$_2\alpha$) was described elsewhere (21, 27). Those for EGFP-D43N-cPLA$_2\alpha$ and EGFP-S505A-cPLA$_2\alpha$ were described in ref. 27 and 10, respectively. Transfection of pGFP-C3 (Clontech) was used as control. Constructs pCMV5-ΔMEKK1 and pSRα-K432M-MEKK1, encoding constitutively active and dominant negative forms of MEKK1, respectively, were kindly provided by Dr. Pura Muñoz-Cánoves (Centre de Regulació Genòmica, Barcelona). Constructs pCMV5-myc-MEKK3-EE and pcDNA3-MEK1-EE, encoding constitutively active MEKK3 and MEK1, were gifts from Dr. Ana Cuenda (Centro Nacional de Biotecnología, CSIC-Universidad Autónoma de Madrid, Madrid) and Dr. Piero Crespo (Instituto de Investigaciones Biomédicas, CSIC-Universidad de Cantabria, Santander), respectively. The construct for CERK expression (pcDNA3.1 HisTOPO-hCERK) is described elsewhere (29).

Statistical analysis— Data analysis was carried out with Prism software (GraphPad). Responses among different treatments were analyzed with one-way analysis of variance followed by Bonferroni's multiple comparison test. All experiments were carried out with determinations in triplicate. Most results are presented as means ± SEM of three independent experiments, except those in Figure 8B, which are means ± range of two experiments. Results shown as fluorescence Ca^{2+} signals, western blots or microphotographs are representative of at least three independent experiments with essentially the same outcome.

Results

The activation of cPLA$_2\alpha$ required for LD biogenesis is independent of Ca^{2+}—Serum-starved cells challenged with 7.5% FBS produced transient Ca^{2+} increases (Figure 1A). As expected, this Ca^{2+} signal disappeared when cells were pretreated with 45 μM BAPTA-AM during 30 minutes prior to stimulation with FBS (Figure 1A). The inhibitory effect of BAPTA-AM was confirmed after the inability of ionomycin to induce a Ca^{2+} response (not shown). cPLA$_2\alpha$ was phosphorylated upon FBS stimulation when intracellular Ca^{2+} increases were abrogated by BAPTA-AM (Figure 1B), and in fact cPLA$_2\alpha$ activity, measured as [^{3}H]AA release to the medium, was unaffected by intracellular Ca^{2+} chelation (Figure 1D). This Ca^{2+}-chelating strategy was toxic over 6 hours, however, and did not allow us to measure LD levels, which we usually monitor after a 6-hour treatment (7, 10). Instead, we used the phosphoinositide phospholipase C inhibitor U73122 to block the Ca^{2+} signals induced by FBS, which are due to the generation of inositol 1,4,5-trisphosphate (30). As shown in Figure 1A, pretreatment with 10 μM U73122 blocked the Ca^{2+} response but it did not affect phosphorylation of cPLA$_2\alpha$ at Ser505 (Figure 1C) or [^{3}H]AA releasing activity (Figure 1D). Importantly, in the presence of the phospholipase C inhibitor, FBS still promoted ADRP expression (Figure 1C) and LD biogenesis that was sensitive to the cPLA$_2\alpha$ inhibitor MAFP (Figure 1E).

Additional evidence for a Ca^{2+}-independent activation of cPLA$_2\alpha$ during LD biogenesis came from the transfection of a D43N-cPLA$_2\alpha$ mutant. Asp43 resides in the Ca^{2+}-binding loop 1 at the membrane binding face of the C2 domain of the enzyme, and participates in the coordination of the two Ca^{2+} ions that drive membrane binding (11). Substitution of this residue for Asn increases 100-fold the Ca^{2+} requirement for activity in vitro (31), and prevents translocation of the enzyme to membranes in response to Ca^{2+} ionophores (15, 27). In agreement with this, EGFP-D43N-cPLA$_2\alpha$ did not relocate in CHO-K1 cells stimulated with 5 μM ionomycin, unlike the wild type enzyme, which translocated to perinuclear membranes and structures resembling the Golgi apparatus (Figure 2A-D). In contrast, EGFP-S505A-cPLA$_2\alpha$, which lacks the Ser505 phosphorylation site but retains its Ca^{2+} binding capacity, translocated to membranes in response to ionomycin just as the wild type enzyme (Figure 2E-F). Importantly, FBS treatment induced phosphorylation of wild type and D43N cPLA$_2\alpha$, at Ser-505 (Figure 2G). Further, transfection of EGFP-D43N-cPLA$_2\alpha$ resulted in increased release of [^{3}H]AA (Figure 2H), both under basal conditions and upon FBS stimulation, to the same levels achieved by transfection of EGFP-cPLA$_2\alpha$. Regarding LD occurrence, the effect of the D43N mutant was identical to the wild type enzyme: there was a 2.5-fold increase of LD upon FBS stimulation, monitored either by flow cytometry (Figure 2I),

ADRP content (not shown) or by fluorescence microscopy (Figure 2J, K). Taken together, these results demonstrate that the activation of cPLA$_2$α and biogenesis of LD induced by FBS are independent of Ca^{2+} and do not require massive relocation of the enzyme. Further, the results confirm our previous finding that phosphorylation of cPLA$_2$α at Ser505 is essential for its activation by FBS, while there is no apparent relocation in response to the faint Ca^{2+} signal elicited by the stimulus (10).

JNK, but not ERK or p38 MAP kinases, is responsible for cPLA$_2$α phosphorylation and LD biogenesis—cPLA$_2$α is phosphorylated at Ser505 by MAPKs (11, 12), and to dissect the pathway during LD biogenesis, we monitored JNK, p38 and ERK MAPKs at different times after FBS replacement. As shown in Figure 3, all three MAPKs became activated after stimulation with 7.5% FBS. We therefore activated each MAPK individually by transfecting constitutively active upstream activators of each pathway. As shown in Figure 4A, transfection of the catalytic domain of MEKK1, which encodes a constitutively active kinase (ΔMEKK1) induced phosphorylation of JNK in the absence of FBS stimulus, but not of ERK and only a slight increase of p38. Likewise, transfection of MKK3EE and MEK1EE resulted in the specific phosphorylation of p38 and ERK, respectively, in the absence of FBS. Importantly, only the activation of JNK increased phosphorylation of cPLA$_2$α at Ser505 (Figure 4A), and this was inhibited in the presence of the JNK inhibitor SP600125 at 10 μM (not shown). ΔMEKK1-JNK-mediated phosphorylation of cPLA$_2$α was mirrored by an increased [^{3}H]AA-releasing activity in the absence of FBS stimulus, which again was inhibited by SP600125 and also by MAFP (Figure 4B). In contrast, [^{3}H]AA released from cells transfected either with MKK3EE or MEK1EE was not different from that in control cells (Figure 4B). In close agreement with data on cPLA$_2$α phosphorylation and activity, Figure 4C shows that cells transfected with ΔMEKK1 had increased LD content as monitored by flow cytometry. In contrast, stimulation of p38 or ERK did not induce the formation of LD. Again, LD biogenesis induced by expression of ΔMEKK1 in the absence of FBS stimulus was sensitive to inhibition by SP600125 and MAFP.

The preceding results show that the JNK inhibitor SP600126 antagonizes the activation of cPLA$_2$α and the biogenesis of LD in the absence of a FBS stimulus. In order to validate this pharmacological evidence in a more physiological setting, we compared the ability of MAPK inhibitors to block cPLA$_2$α activation and LD biogenesis induced by FBS. As shown in Figure 5A, phosphorylation of cPLA$_2$α induced by 7.5% FBS was inhibited in the presence of 10 μM SP600125, but was not affected by the same concentration of the p38 inhibitor SB20358 or the MEK inhibitor PD98059. In close agreement with these results, [^{3}H]AA release stimulated by FBS was only decreased in the presence of the JNK inhibitor but was unaffected by SB20358 or PD98959 (Figure 5B). Further, the occurrence of LD induced by FBS, monitored either by flow cytometry (Figure 5C) or after the expression of ADRP (Figure 5A) was sensitive only to the JNK inhibitor.

We also inhibited JNK by transfecting a dominant-negative form of MEKK1, the upstream activator of JNK. Figure 6A shows that phosphorylation of JNK in response to FBS stimulation was partially inhibited in cells transfected with K432M-MEKK1, as compared to cells transfected with empty vector. The partial inhibition of JNK was enough to fully inhibit cPLA$_2$α phosphorylation induced by FBS (Figure 6A). In close agreement with this, cPLA$_2$α activity monitored after the release of [^{3}H]AA (Figure 6B) and LD biogenesis measured by flow cytometry (Figure 6C) were clearly inhibited by transfection of K432M-MEKK1. Results on the inhibition of LD were confirmed by ADRP expression levels (not shown) and by fluorescence microscopy. Panels D and E of Figure 6 show that, unlike cells transfected with empty vector, which generate LD after FBS replacement, those transfected with K432M-MEKK1 do not contain LD. Taken together, these results demonstrate that the MAP kinase that activates cPLA$_2$α during LD biogenesis is JNK.

Ceramide-1-phosphate induces LD biogenesis in the absence of FBS— Next, we assessed the possible implication of C1P in cPLA$_2$α activation in our system. C1P drives the translocation of cPLA$_2$α to membranes and is absolutely required for its activation in response to inflammatory

agonists (25), but it also might contribute to the phosphorylation state of the enzyme (32). We treated serum-starved cells with 2.5 μM C1P in ethanol:dodecane (98:2) at a final vehicle dilution of 1:1000 (33). C1P induced both JNK and cPLA$_2$α phosphorylation (figure 7A). Also, C1P stimulated the release of [^{3}H]AA in a SP600125- and MAFP-sensitive fashion, to levels that were similar to those attained with FBS stimulation (Figure 7B). In contrast to C1P, C$_2$-ceramide at the same concentration did not affect JNK and cPLA$_2$α phosphorylation or cPLA$_2$α activity. In agreement with these results, C1P induced LD biogenesis that was inhibited by SP600125 and MAFP (Figure 7C). LD quantification with flow cytometry agreed with ADRP expression levels and epifluorescence microscopy (not shown). To confirm results on exogenously supplied C1P, we overexpressed CERK, the enzyme responsible for C1P synthesis. Expression of CERK in CHO-K1 cells induced the phosphorylation of JNK and cPLA$_2$α (Figure 7D). Further, the synthesis of C1P was enhanced, and this was accompanied by the stimulation of cPLA$_2$α activity and the biogenesis of LD (Figure 7E-G). These results indicate that an increase of C1P can induce LD formation through JNK activation and subsequent cPLA$_2$α phosphorylation at Ser505.

FBS increases C1P, and silenced expression of CERK inhibits the formation of LD induced by FBS— To tackle the relevance of the latter set of results, we knocked down the expression of CERK with a siRNA approach. Among the three different siRNA sequences used, two of them (siRNA CERK1 and siRNA CERK3) effectively downregulated the enzyme, whereas siRNA CERK2 had no effect (figure 8A). Figure 8B shows that FBS stimulation increased the synthesis of [^{3}H]C1P in [^{3}H]palmitate-prelabeled cells, and this was blocked by siRNA CERK1 and siRNA CERK3 but not by siRNA CERK2. We did not observe changes in [^{3}H]ceramide content under any condition (not shown). CERK downregulation also inhibited the phosphorylation of JNK and cPLA$_2$α induced by FBS (Figure 8A). Further, knocked-down expression of CERK inhibited [^{3}H]AA release (Figure 8C) and LD biogenesis monitored by flow cytometry (Figure 8D), ADRP expression (Figure 8A), or by fluorescence microscopy (not shown). In conclusion, the above results show that serum-induced LD biogenesis is mediated by CERK and formation of C1P.

Discussion

The present work addresses the mechanism of cPLA$_2$α activation needed for the biogenesis of LD induced by FBS. Our findings can be summarized in three major points: First, cPLA$_2$α activation and LD biogenesis are independent of intracellular Ca^{2+} rises, with no apparent relocation of the enzyme. Second, JNK is the MAPK that activates cPLA$_2$α in our experimental model. And third, the C1P-generating enzyme CERK is required for the activation of JNK, and hence of cPLA$_2$α.

This study shows that the pathway leading to cPLA$_2$α activation and LD biogenesis is Ca^{2+} independent. Intracellular chelation with BAPTA-AM or blocking of the Ins1,4,5P$_3$-induced Ca^{2+} transient elicited by FBS does not alter cPLA$_2$α activation and LD biogenesis. Moreover, the single mutation D43N in the C2 domain of the enzyme, which abrogates its Ca^{2+}-binding capacity, does not affect activity in response to FBS. Several reports have established the dispensability of Ca^{2+} rises in terms of full enzyme activation *in vivo* (13, 16-19) while putting forward the requirement of the signaling lipids PtdIns4,5P$_2$ (17, 20, 21, 34) and C1P to allow interaction with membranes (22-25). The D43N mutation in cPLA$_2$α renders the enzyme refractory to physiological Ca^{2+} concentrations (31) but it is fully active on PtdIns4,5P$_2$-containing micelles (34). We have not addressed the possible role of PtdIns4,5P$_2$ in LD biogenesis, but in light of the present results this is certainly a goal in the near future. It is now clear that cPLA$_2$α activity is essential for the generation of LD after TAG present in the ER (7, 10), however we have consistently found no enzyme translocation to the nuclear envelope and nearby membranes in response to FBS (10). Bearing in mind that the D43N mutation does not suppress interfacial activity of the C2 domain (34), in our experimental model the enzyme could interact with the diffuse RE membrane system and/or nascent LD. This would probably escape detection in our studies, which were carried out within minutes following FBS stimulation (10).

Our results show that JNK regulates cPLA$_2$α activation and LD biogenesis by inducing phosphorylation at Ser505. Although phosphorylation at this site increases activity *in vitro* only 2-fold (11), it confers increased membrane affinity at low micromolar Ca^{2+} concentrations (11, 12), and this tighter binding to the lipid surface could become critical for enzyme activation under our resting Ca^{2+} conditions. FBS activates ERK, p38 and JNK, and we identify the JNK cascade as the one governing cPLA$_2$α phosphorylation in our system. This also stands probably for oleate-induced LD[1]. A literature survey shows that the identity of the MAPK cascade leading to the phosphorylation of cPLA$_2$α at Ser505 is a matter of cell type and triggering stimulus, often involving ERK or/and p38 MAPK. However, to our knowledge only two recent reports have uncovered a key role of JNK in cPLA$_2$α-activation, both related to the microbicidal activity of phagocytes: Lee et al. (35) showed that secretion of monocyte chemoattractant protein-1 induced by stimulation of macrophages with synthetic oligodeoxynucleotides containing CpG motifs depends on cPLA$_2$α activity, which can be blocked by pharmacological inhibition or downregulation of JNK. Casas et al. (36), on the other hand, showed that translocation of cPLA$_2$α to phagosomal membranes of macrophages, an event required for eicosanoid generation and killing of the ingested microbe, requires enzyme phosphorylation at Ser505 and is blocked after pharmacological inhibition of JNK. The present paper, therefore, describes for the first time the crucial role of the JNK cascade in the activation of cPLA$_2$α for LD biogenesis. We showed previously that the implication of cPLA$_2$α in LD biogenesis after serum lipoproteins is not unique to the CHO cell model (10). It remains to be established whether this generalization also stands for JNK, as it may hold promise for new pharmacological approaches in the treatment of metabolic diseases. In this regard, LDs have been shown to interfere with membrane translocation of the insulin-sensitive glucose transporter, an observation that might account for insulin resistance in type 2 diabetes (5). It is tempting to speculate, therefore, that the observation that animal models of obesity have abnormally elevated JNK activity, and that JNK1 knock-out mice on obesity-inducing diets are protected against adiposity and insulin resistance (37) are related to cPLA$_2$α-mediated formation of LD.

This report also presents CERK and its product C1P as new key players in the biogenesis of LD. This is somehow not surprising, as C1P is a firmly established activator of cPLA$_2$α (32, 38). C1P also has pro-survival effects (39), in line with the protective role that LD play during nutrient starvation and other stress conditions (6, 7). C1P interacts with the cationic β-groove (Arg57, Lys58, Arg59) of the C2 domain of cPLA$_2$α. This enhances interaction with membranes and lowers the EC_{50} for Ca^{2+} down to 31 nM, resulting in increased and catalytic activity *in vitro* (22, 24). Recently, it has been described that CERK is required for cPLA$_2$α activation and translocation to the Golgi in response to Ca^{2+} increases in the cell (25). Our results clearly show the requirement of CERK for cPLA$_2$α activation and LD biogenesis. However, they do not quite fit with a direct interaction of C1P and cPLA$_2$α: first, cPLA$_2$α activation for LD biogenesis is independent of Ca^{2+}, and it does not require enzyme translocation to the nuclear envelope and the Golgi; second, our data show that CERK activates JNK and downstream phosphorylation of cPLA$_2$α at Ser505. This suggests that C1P does not solely promote association of the enzyme with membranes. Our data fully support a formerly proposed mechanism (32, 40) whereby C1P activates the JNK cascade, either directly or after the inhibition of protein phosphatases 1 and 2A. This mechanism is in keeping with our observation that okadaic acid induces LD biogenesis in a JNK- and cPLA$_2$α-dependent fashion[1]. Regardless of the mechanism, our results clearly point CERK as a potential target for the treatment of metabolic diseases.

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Footnotes

1- Gubern, A., and Claro E., unpublished results.

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Abbreviations

AA, arachidonic acid; ADRP, adipophilin (adipose differentiation-related protein); CERK, ceramide kinase; C1P, ceramide-1-phosphate; C_2-ceramide, N-acetyl-D-sphingosine; FBS, fetal bovine serum; GFP, green fluorescent protein; JNK, c-Jun N-terminal kinase; LD, lipid droplets; MAFP, methylarachidonyl fluorophosphonate; MAPK, mitogen-activated protein kinase; PLA_2, phospholipase A_2; PMA, phorbol 12-myristate 13-acetate; ; siRNA, short interfering RNA; TAG, triacylglycerols.

Figure legends

Figure 1: FBS activates cPLA2α and induces LD biogenesis in the presence of BAPTA-AM or U73122
A: Ca^{2+} responses of serum-starved, fura-2/AM-loaded CHO-K1 cells under control conditions (continuous line) or after pre-treatment with 45 μM BAPTA-AM (circles) or 10 μM U73122 (triangles) for 30 minutes before fluorescence measurements were started. At the time indicated, 7.5% FBS was added. **B, C:** Western blots of serum-starved CHO-K1 pre-treated with 45 μM BAPTA-AM or 10 μM U73122 during 30 minutes, then stimulated with 7.5% FBS for 6 hours. **D:** $cPLA_2$ activity, measured after the radioactivity released to the medium of serum-starved CHO-K1 cells that had been prelabeled during 24 hours with 0.5 μCi/ml [^{3}H]arachidonic acid, then washed and treated with 45 μM BAPTA-AM, 10 μM U73122 and/or 10 μM MAFP for 30 minutes before a 15-min stimulation with 7.5% FBS. **E:** Indirect quantification of LD in serum-starved CHO-K1 cells that had been pretreated for 30 minutes with 10 μM U73122 and/or MAFP, then stimulated with 7.5% FBS for 6 hours. Cells were fixed and stained with Nile red to quantify LD by flow cytometry. Fluorescence intensities in FL1 were quantified as the median values of each event distribution. Results in panels C and D are means ± SEM of three independent experiments. *Significantly different ($p < 0.01$) from controls.

Figure 2: EGFP-D43N-cPLA$_2$α is as effective as EGFP-cPLA$_2$α to induce LD biogenesis in response to FBS
A-F: CHO-K1 cells were transiently transfected with plasmids encoding EGFP-$cPLA_2$α (A, B), EGFP-D43N-$cPLA_2$α (C, D) or EGFP-S505A-$cPLA_2$α (E, F). Confocal images were obtained before (A, C, E) or 10 min after addition of 5 μM ionomycin. **G:** Western blots of total $cPLA_2$α and phospho-Ser^{505}-$cPLA_2$α of transiently transfected cells. **H:** [^{3}H]AA release from serum starved CHO-K1 cells transfected with EGFP-$cPLA_2$α, EGFP-D43N-$cPLA_2$α or EGFP-S505A-PLA_2α, and kept unstimulated (open bars) or stimulated with 7.5% FBS for 15 minutes (filled bars). **I:** Indirect quantification of LD by flow cytometry in cells after 6 hours under control conditions (open bars) or with 7.5% FBS (filled bars). **J, K:** Serum-starved CHO-K1 cells transiently transfected with EGFP-D43N-$cPLA_2$α (J) or with EGFP-S505A-PLA_2α (K) were stained with Nile red for epifluorescence microscopy. Results in panels H and I are means ± SEM of 3 independent experiments. *Significantly different ($p < 0.01$) from GFP-transfected cells. $^{\#}$Significantly different ($p < 0.01$) from FBS-stimulated, GFP-transfected cells.

Figure 3: FBS induces the phosphorylation of JNK, p38 and ERK in CHO-K1 cells
Serum-starved CHO-K1 cells were treated with 7.5% FBS for the indicated times. Total and phosphorylated JNK, p38, and ERK were monitored by western blot.

Figure 4: The activation of JNK, but not of p38 or ERK, is sufficient to phosphorylate cPLA$_2$α and induce LD biogenesis in the absence of FBS
CHO-K1 cells were transiently transfected with plasmids encoding ΔMEKK1, MEKK3EE or MEK1EE and switched to culture medium without FBS. **A:** Phosphorylation of JNK, p38, ERK and $cPLA_2$α in the absence of FBS. **B:** Cells were prelabeled with 0.5 μCi/ml [^{3}H]AA during 24 hours, then washed, and the release of radioactivity to the medium was quantified after 15 min. **C:** indirect quantification of LD by flow cytometry. Results in panels B and C are means ± SEM of three independent experiments. *Significantly different ($p < 0.01$) from control.

Figure 5: The JNK inhibitor SP600125 prevents cPLA$_2$α activation and LD biogenesis induced by FBS.
Serum-deprived CHO-K1 cells were pretreated for 30 minutes with 10 μM concentrations of SP600125, SB20358, or PD98059, and then challenged with 7.5% FBS for 15 minutes (B) or 6 hours (A, C). **A:**

Western blots of total cPLA2α, phospho-Ser505-cPLA2α, and ADRP. **B:** Radioactivity released from serum-deprived CHO-K1 cells that were prelabeled during 24 hours with 0.5 μCi/ml [^{3}H]AA, washed, and pretreated with drugs for 30 minutes before a 15-min stimulation with 7.5% FBS. **C:** Serum-starved cells were treated for 30 min with drugs, then stimulated with FBS during 6 hours, fixed and stained with Nile red to quantify LD by flow cytometry. Results in panels B and C are means ± SEM of three independent experiments. *$p < 0.01$ compared to control in the absence of FBS. #$p < 0.01$ compared to FBS-treated cells.

Figure 6: Transfection of a dominant negative form of MEKK1 (K432M-MEKK1) inhibits JNK phosphorylation, activation of cPLA$_2$α and LD biogenesis

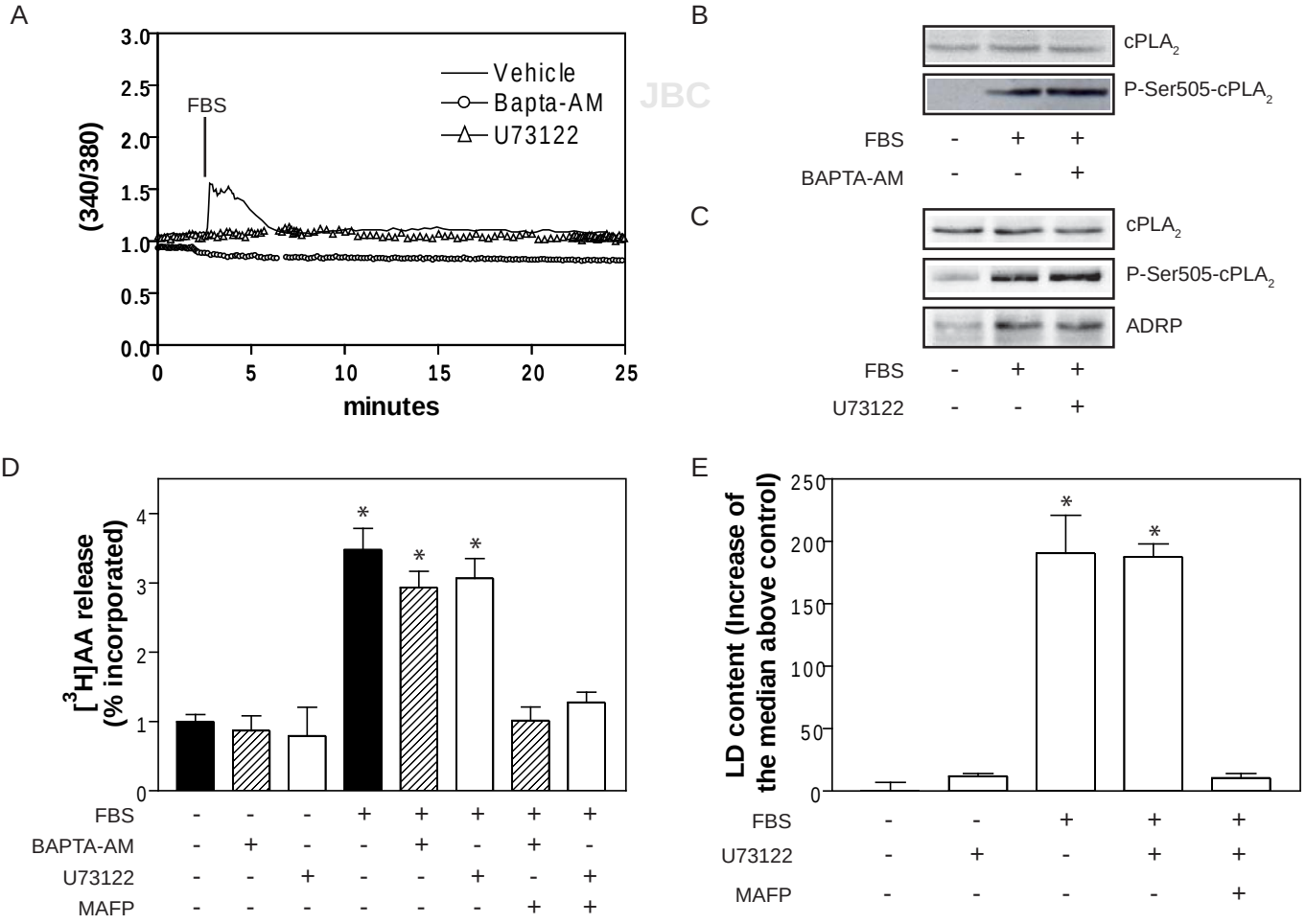
CHO-K1 cells were transfected with empty vector or vector encoding K432M-MEKK1. Cells were serum-starved for 24 hours and then challenged for 15 minutes (A, B) or 6 hours (C) with 7.5% FBS. **A:** Phosphorylation levels of JNK and cPLA$_2$α. **B:** radioactivity released from cells that had been prelabeled during 24 hours with 0.5 μCi/ml [^{3}H]AA. **C:** Indirect quantification of LD by flow cytometry. **D, E:** Cells were transfected with empty vector (D) or with vector encoding K432M-MEKK1 (E), stimulated for 6 hours with 7.5% FBS, and stained with Nile red for epifluorescence microscopy. Results in panels B and C are means ± SEM of three independent experiments. *Significantly different ($p < 0.01$) from control.

Figure 7: C1P and CERK stimulate cPLA$_2$α and induce LD biogenesis

A: Western blots of phospho-JNK and phospho-cPLA$_2$α from serum-starved CHO-K1 cells that were treated with vehicle, 2.5 μM C$_2$-ceramide, 2.5 μM C1P or 7.5 % FBS for 15 minutes. **B:** Radioactivity released from [^{3}H]AA prelabeled, serum-starved cells that were pretreated for 30 min with MAFP or SP600125, then stimulated for 15 min with C$_2$-Cer, C1P or FBS. **C:** Quantification of LD by flow cytometry after a 6-hour treatment with C$_2$-Cer, C1P of FBS. **D-G:** Cells were transiently transfected with a plasmid encoding CERK, then maintained in the absence of FBS. Overexpression of CERK increased the phosphorylation of JNK and cPLA$_2$α (D). CERK-transfected cells were prelabeled for 24 hours with [^{3}H]palmitate or with [^{3}H]AA to monitor synthesis of C1P (E) and cPLA$_2$α activity (F), respectively. CERK also increased basal levels of LD in the absence of FBS (G). *Significantly different ($p < 0.01$) from control.

Figure 8: CERK is required for the activation of JNK and cPLA$_2$α, and for LD biogenesis

CHO-K1 cells were transfected with three siRNA-CERK duplexes as described in Methods, and maintained in 7.5% FBS-containing medium during 72 hours, then in medium without FBS for 24 hours. During these last 24 hours, cells were prelabeled with [^{3}H]palmitate or with [^{3}H]AA to monitor synthesis of C1P and cPLA$_2$α activity, respectively (B, C). **A:** Western blots showing that siRNA-CERK1 and siRNA-CERK3 knocked down the expression of CERK, precluded phosphorylation of JNK and cPLA$_2$α induced by a 6-hour treatment with FBS, and decreased ADRP content. FBS treatment was **B:** [^{3}H]palmitate-prelabeled cells were stimulated with FBS during 15 min. Lipid extracts were separated by TLC, and areas co-migrating with C1P standard were scraped onto vials and counted. **C:** [^{3}H]AA-prelabeled cells were stimulated with FBS during 15 min, and radioactivity in the medium was counted. **D:** Cells were stimulated with FBS during 6 hours, fixed, stained with Nile red, and LD were quantified by flow cytometry. Results in panels C and D are means ± SEM of three experiments. Results in B are means ± range of two experiments. *Significantly different ($p < 0.01$) from controls.



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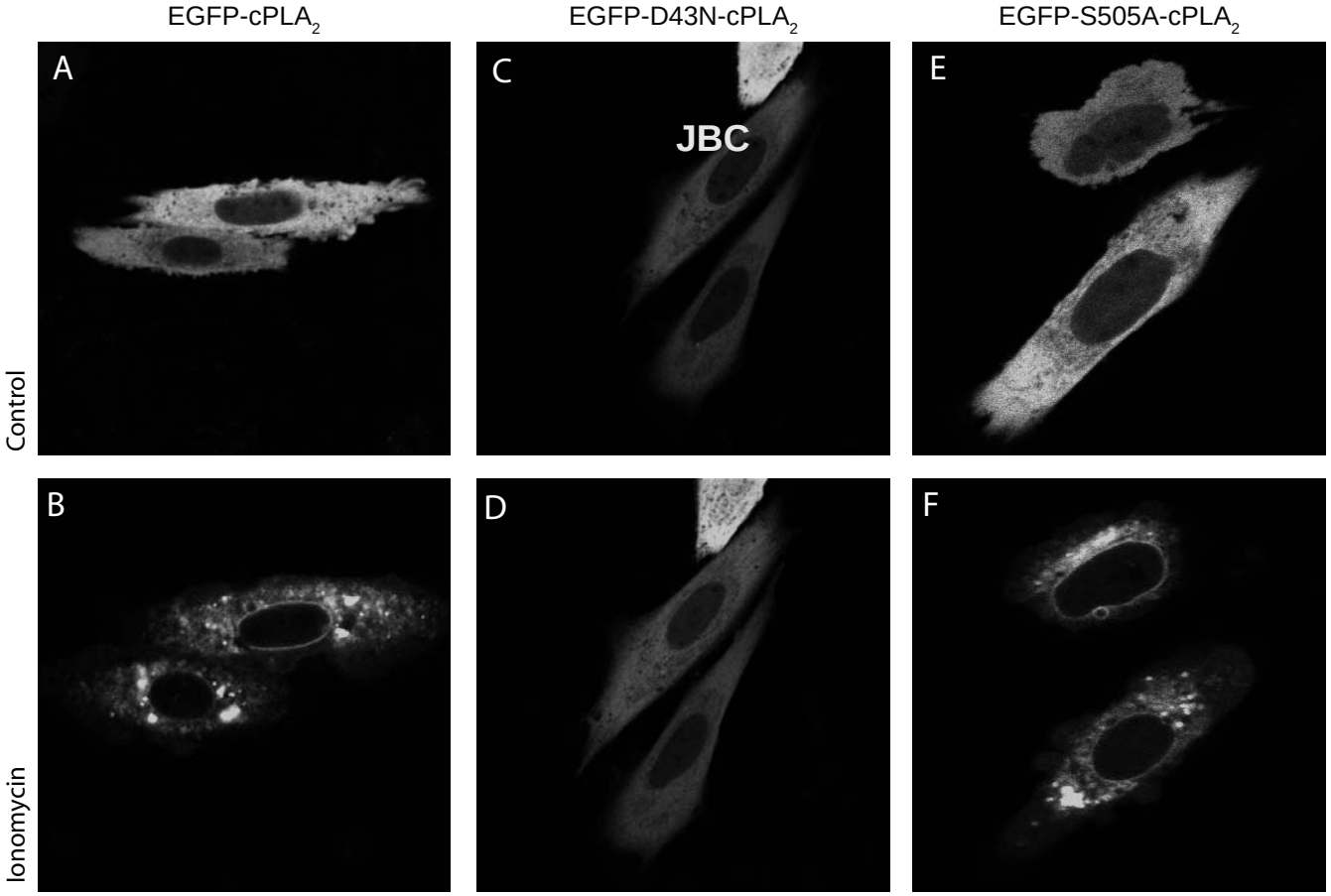

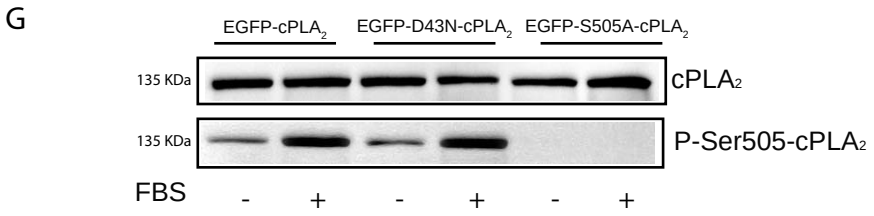

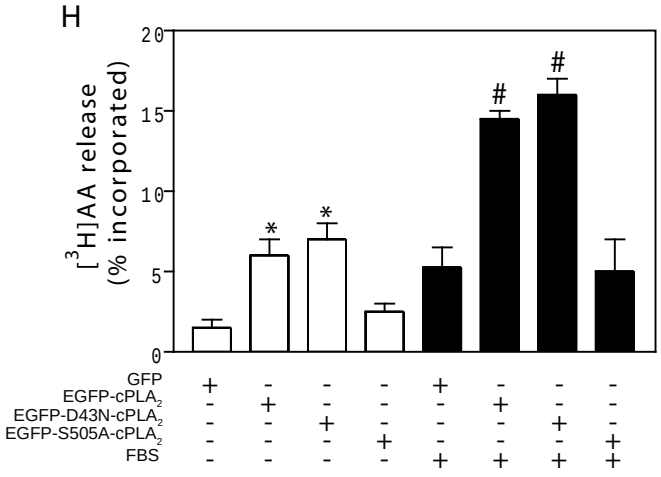

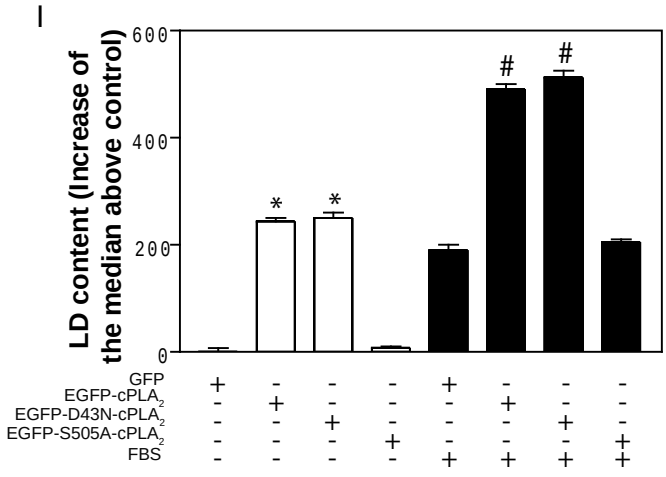

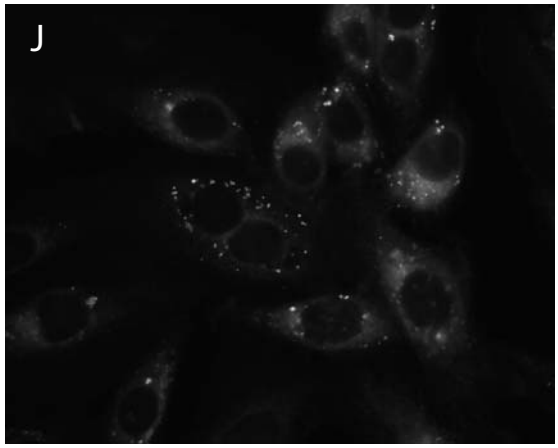

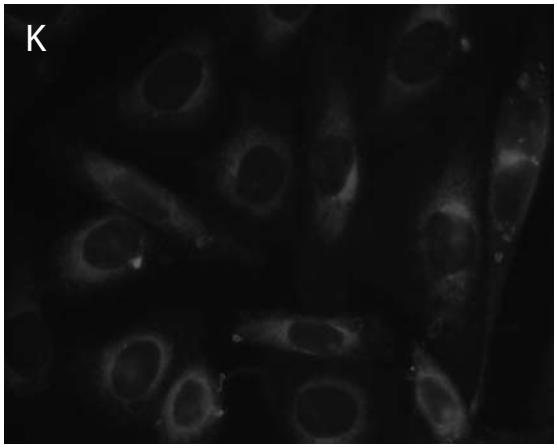

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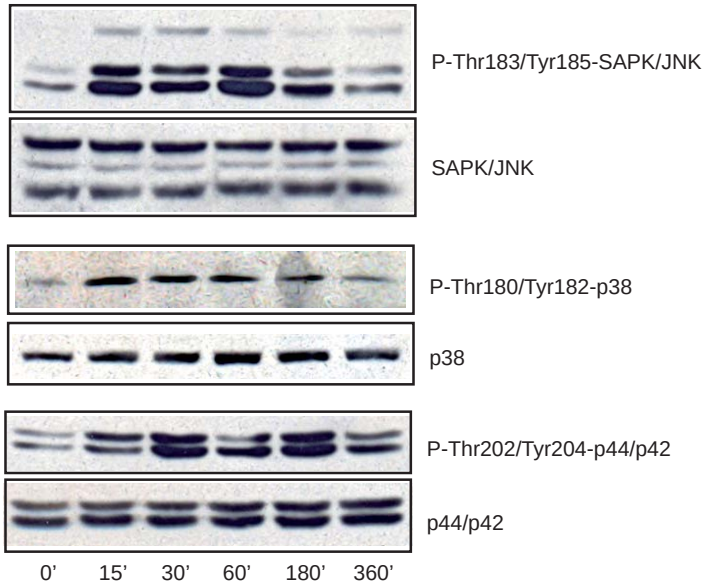

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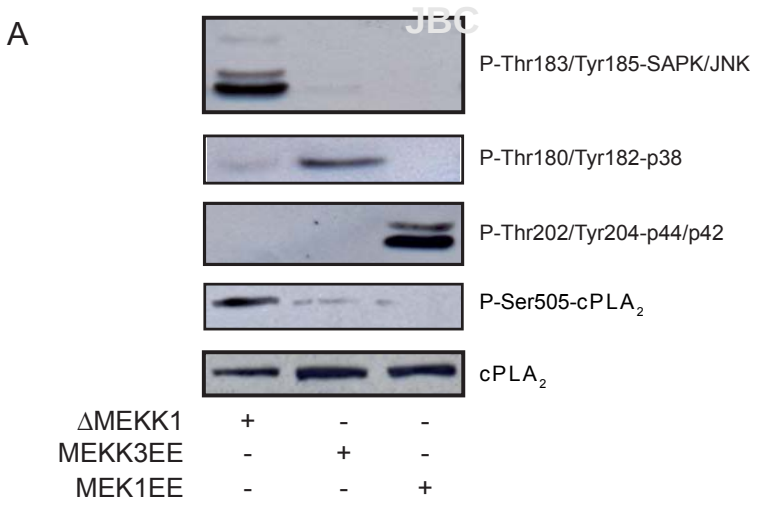
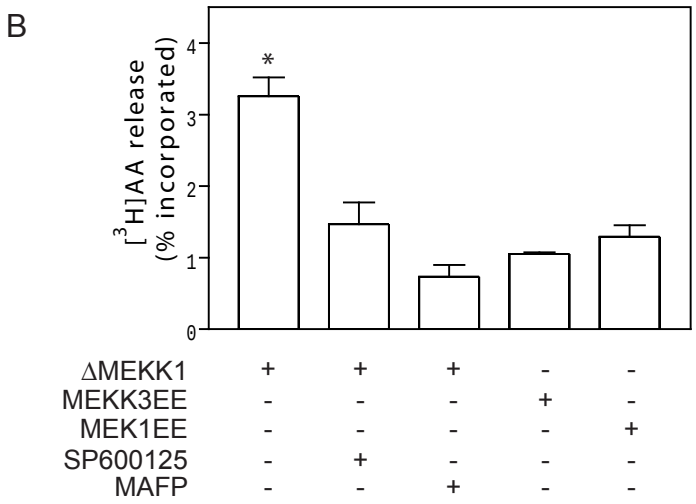




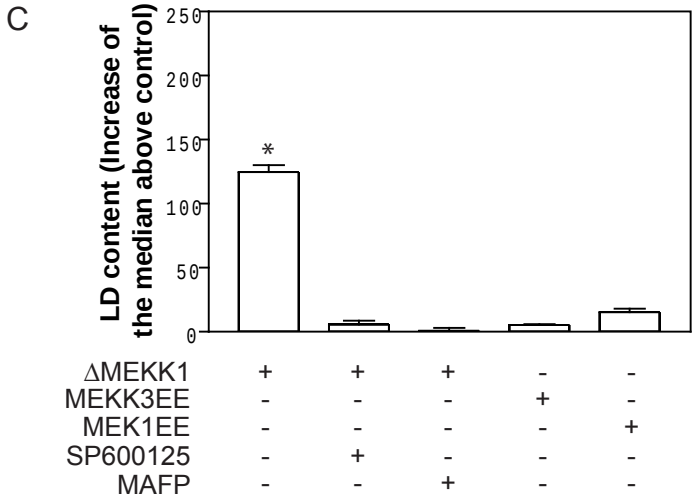


figure 4

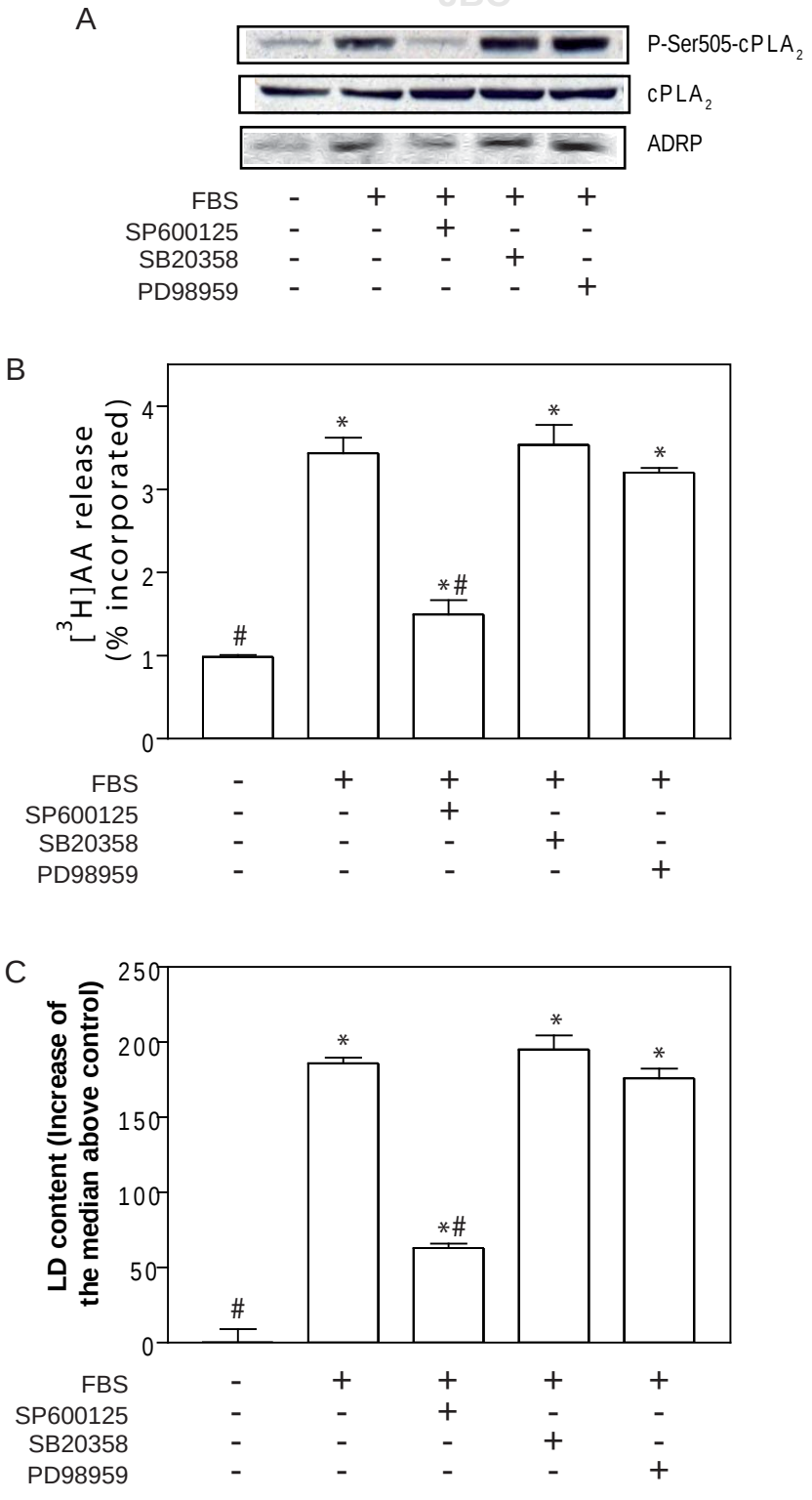
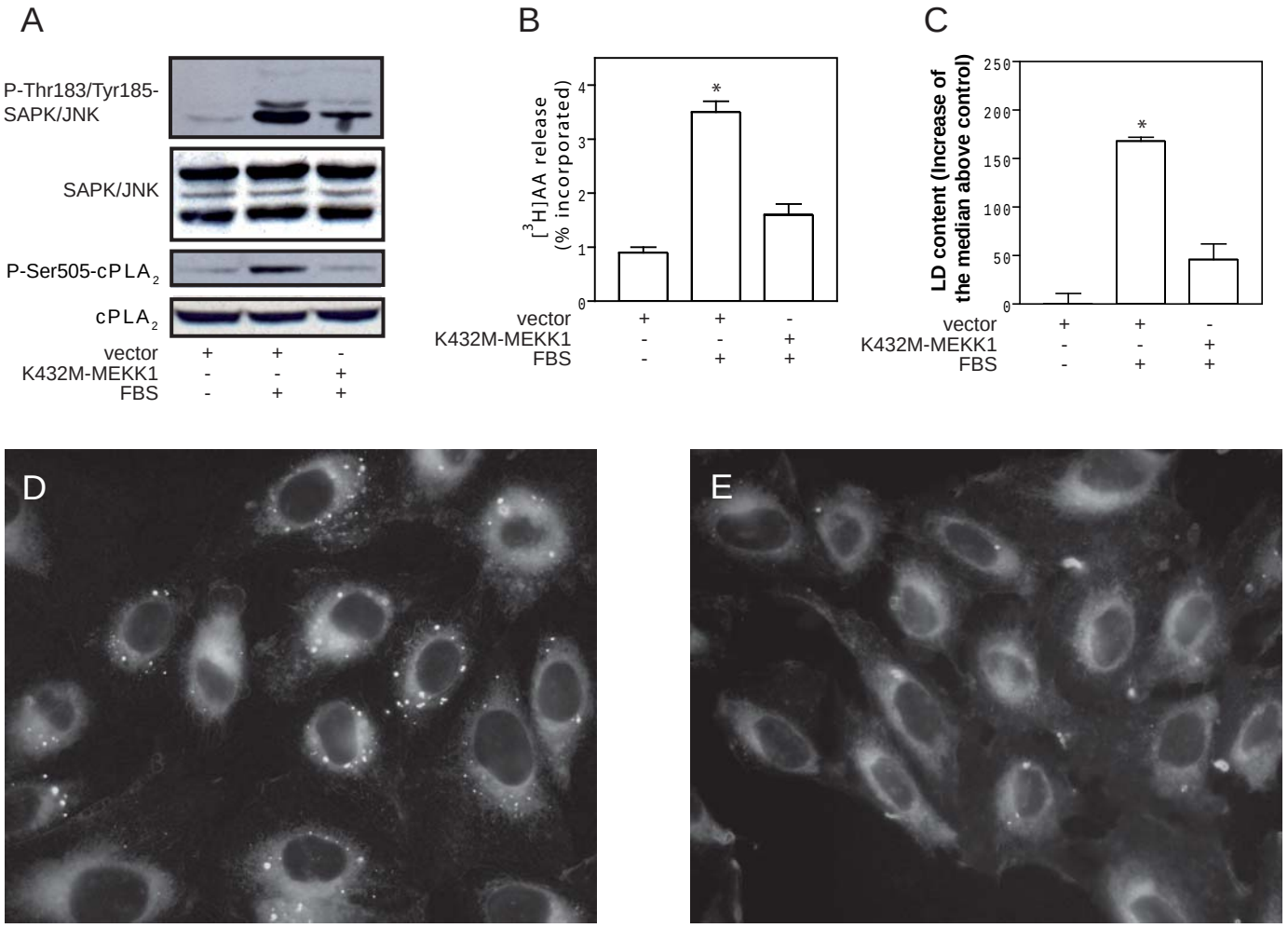


figure 5



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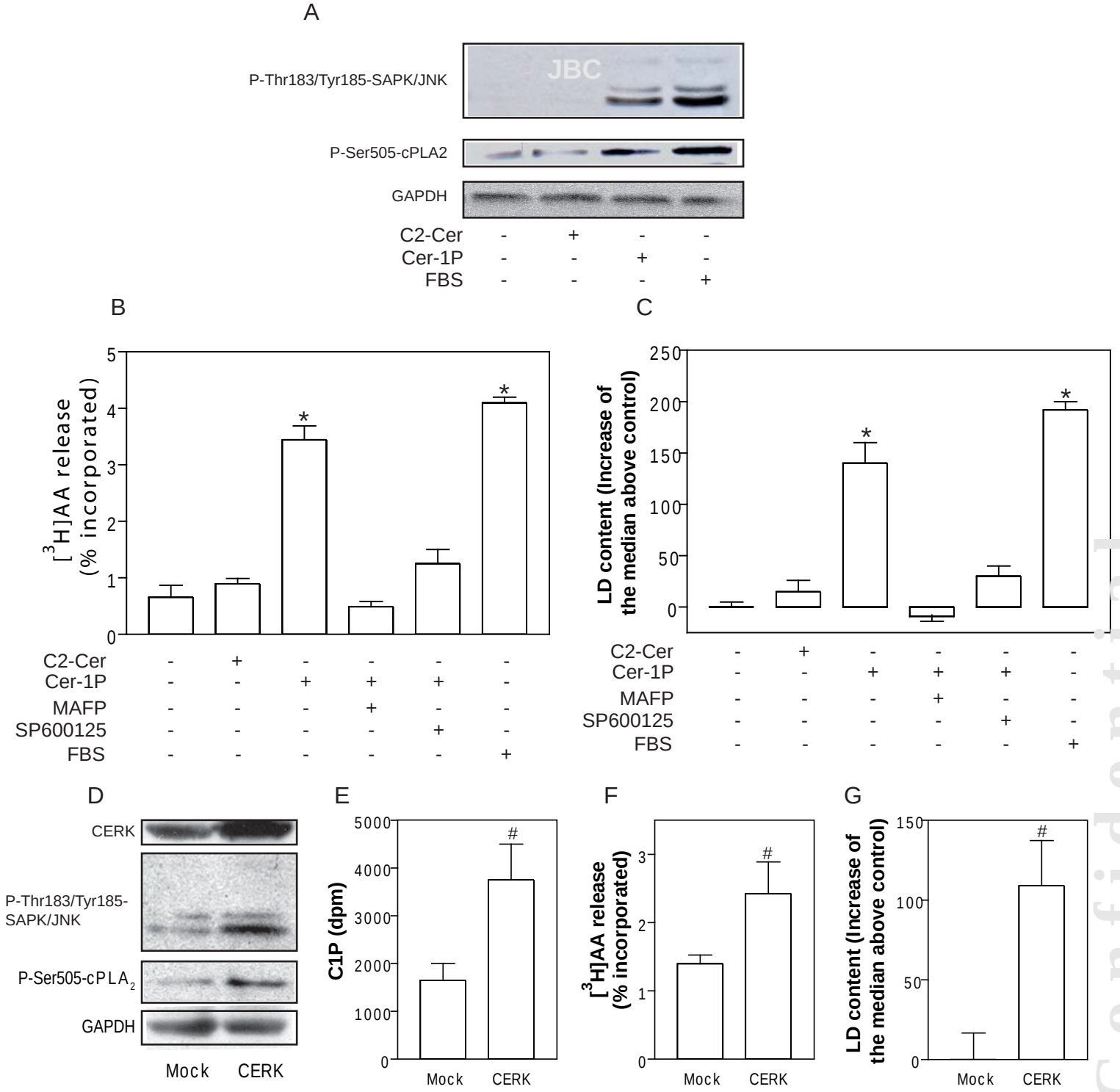

figure 7

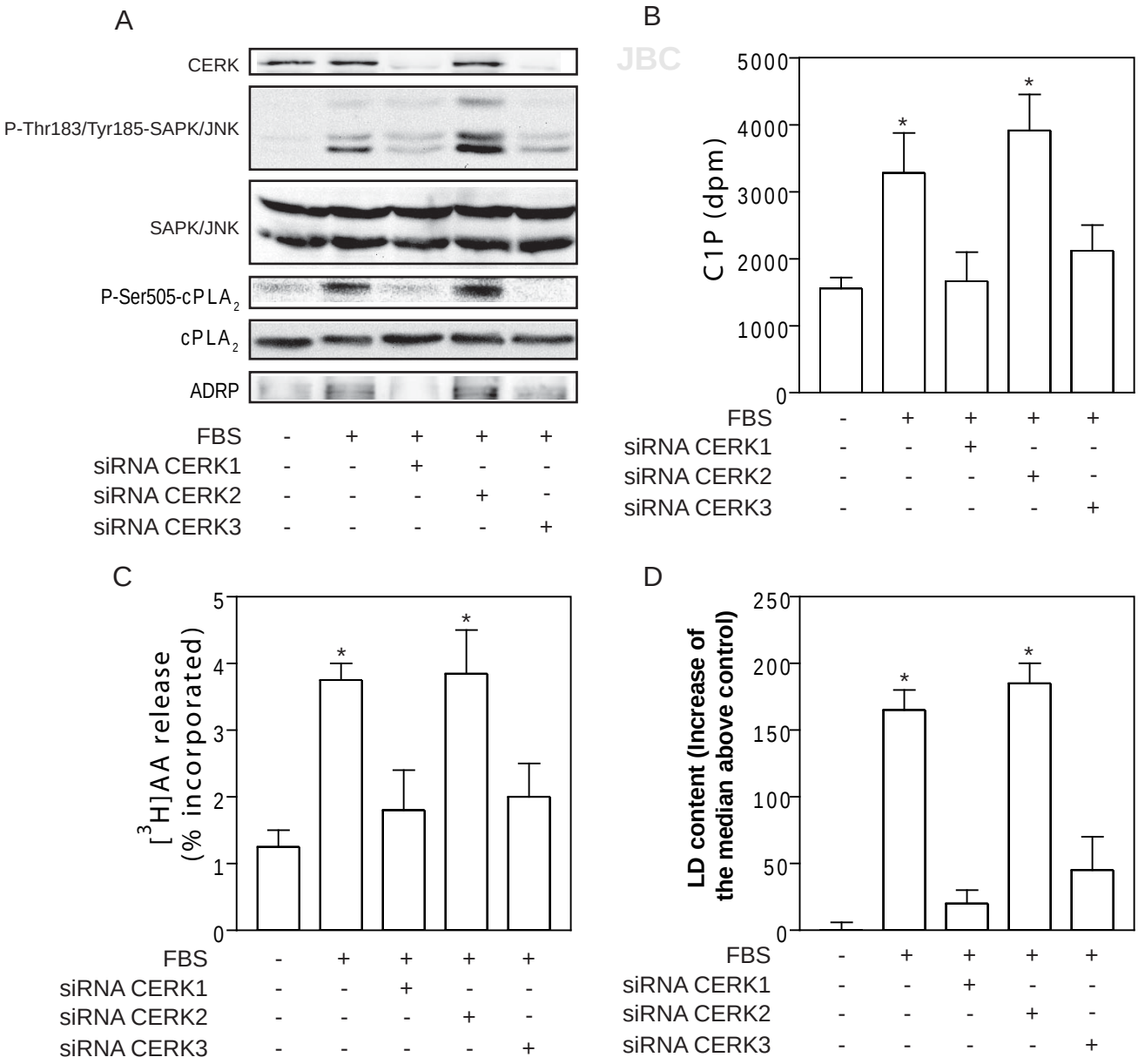

figure 8