Cyclic Phosphatidic Acid Decreases Proliferation and Survival of Colon Cancer Cells by Inhibiting Peroxisome Proliferator-Activated Receptor γ

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Abbreviations: PPARγ, peroxisome proliferator-activated receptor gamma; PPRE, PPAR-response element; ACox, acyl-coenzyme (CoA) oxidase; TZD, thiazolidinedione; BADGE, bisphenol A diglycidyl ether; GW9662, 2-chloro-nitrobenzanilide;

1. Introduction

Globally, cancers of the colon and rectum are the third and fourth leading causes of cancer deaths in males and females, respectively [1]. Chemotherapeutic agents are the main tools for treating cancer. However, most of these drugs are nonspecific, or become less effective as tumor cells acquire multi-drug resistance. Therefore, numerous trials have been performed to enhance the therapeutic effectiveness and reduce the side effects of chemotherapeutic agents. Currently, the most common strategy for enhancing anti-cancer treatments is the use of sensitizers or drug combinations. If detected early, colon cancer is treatable; however, metastatic colon cancer is associated with high mortality. In combination with the first-choice chemotherapy agent 5-fluorouracil, new drugs, such as irinotecan, oxaliplatin, bevacizumab, and cetuximab, have improved the outcome of advanced colon cancer [2]. However, prognosis for metastatic colon cancer remains very poor. Therefore, novel therapeutic options are needed to reduce colon cancer mortality.

PPARγ is a nuclear receptor that plays an essential role in lipid and glucose homeostasis [3], cell proliferation [4], apoptosis [5], and inflammation [6]. Upon agonist binding, PPARγ becomes activated and forms a heterodimer with RXRα. The PPARγ-RXRα heterodimer translocates to the nucleus and binds to the peroxisome proliferator response element (PPRE; TGACCTnTGACCT) in the promoter region of target genes. Once bound, the heterodimer recruits the coactivators SRC-1, CBP, and TRAP220, and the corepressors NCoR and SMRT, to modulate gene transcription [7] [8] [9]. A variety of physiological and synthetic PPARγ agonists have been identified. Physiological agonists include 15d-PGJ_2 [10], selected forms of lysophospholipids, such as lysophosphatidic acid (LPA) [11] and alkyl glycerophosphate (AGP) [12], oxidized

phospholipids [13], and nitrated fatty acids [14]. Thiazolidinedione (TZD) agonists, including ROSI and troglitazone (TRO), are widely used to ameliorate insulin sensitivity in type II diabetes mellitus [15]. PPARγ is overexpressed in many types of cancer, including colon, lung, breast, and stomach cancer, suggesting that regulation of PPARγ might affect cancer pathogenesis [4]. Several studies indicate that PPARγ agonists inhibit cancer cell proliferation, survival, and invasion *in vitro* and *in vivo* [16]. However, clinical trials with drugs that alter PPARγ function have yielded limited success in the treatment of advanced cancer [16]. Nonetheless, recent reports suggest that PPARγ inhibition might be advantageous in cancer treatment [17]. Recently, we reported that cyclic phosphatidic acid (cPA) is a physiological antagonist of PPARγ [18]. cPA is a naturally occurring LPA analog, containing an *sn*-2 hydroxy group that forms a 5-membered ring with the *sn*-3 phosphate [19]. cPA is generated by phospholipase D (PLD) catalyzed transphosphatidylation of lysophosphatidyl choline (LPC) [19 20 21].

LPA is a PPARγ agonist [11] that induces cellular proliferation and invasion (24), but cPA exerts the opposite effects in tumor cells, inhibiting proliferation and cancer cell invasion and metastasis *in vitro* and *in vivo* [19 22 23]. These findings suggest that cPA-mediated PPARγ inhibition could provide a novel strategy for treating advanced cancers. Furthermore, circulating cPA levels are lower in patients with ovarian cancer than in normal subjects [19]. Modulation of PPARγ in colon cancer remains controversial, because experimental evidence implies that PPARγ can either inhibit or stimulate cancer progression and tumorigenesis [16]. Some studies demonstrate that PPARγ agonists inhibit colon cancer cell proliferation, survival, and invasion *in vitro* and *in vivo* [24 25]. However, others show that PPARγ agonists promote colon cancer progression [26], while PPARγ antagonists suppress it [27]. For example, PPARγ deficiency was shown to enhance colon tumorigenesis in $Apc^{Min/+}$ mice [28], while another study reports that ROSI induces colon tumors in normal mice [29]. In this manuscript, we demonstrate the effects of

cPA on cell growth inhibition in the human colon cancer cell line HT-29. We also provide the first evidence that PPARγ is required in cell growth inhibition induced by cPA. Thus, cPA and its analogs could serve as new drug candidates for the treatment of colon cancer.

2. Materials and methods

2-1. Reagents and antibodies

cPA (18:1 and 16:0) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). cPA purity was confirmed by negative ion liquid chromatography-mass spectrometry (data not shown). cPA was quantified by the molybdenum blue method [30], and prepared as a 10 mM stock in dimethylsulfoxide (DMSO). ROSI was purchased from ALEXIS Biochemicals (Lausen, Switzerland), and prepared as a 10 mM stock in ethanol. The cPA carba derivative (3ccPA 16:1) and cPA 17:0 were chemically synthesized as described previously (Fig. 1) [31 32]. T0070907 was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Anti-PPARγ rabbit polyclonal antibody (sc-7196), anti-β-actin mouse monoclonal antibody (sc-47778), PPARγ siRNA (sc-29455), and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-p53 mouse monoclonal antibody (K0181) was from Medical & Biological Laboratories Co. LTD. (Nagoya, Japan). Anti-rabbit IgG secondary antibodies were obtained from Promega Corp. (Madison, WI, USA) and GE Healthcare (Piscataway, NJ, USA). Anti-mouse IgG secondary antibody was from Calbiochem (San Diego, CA, USA).

2-2. Plasmids and vectors

The pSV40-β-galactosidase (Promega) and pcDNA3.1 plasmids were purchased from Promega and Invitrogen Corp. (Carlsbad, CA, USA), respectively. The pcDNA3.1-PPARγ and pGL3b-PPRE (ACO)-Fluc plasmids were constructed as described previously [12].

2-3. Cell culture and transfection

Human colon cancer HT-29, human cervical cancer HeLa, mouse melanoma B16F10, human histiocytic lymphoma U-937, and human promyelocytic leukemia HL-60 cell lines were all obtained from American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich Co., St Louis, MO, USA) containing 10% (v/v) fetal bovine serum (FBS), at 37°C in a humidified incubator with 5% CO_2. Transfection of plasmid DNA was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2-4. Growth curve studies

A dose-response growth curve was performed to determine the concentration of saturated or unsaturated cPA necessary to significantly inhibit cell growth. HT-29 cells (2×10^4 cells/well) were plated in a monolayer on 6-well tissue culture plates. The next day, DMEM containing 1–100 μM cPA 18:1 or cPA 16:0 was added. Five days after cPA treatment, the cells were trypsinized and counted using a hematocytometer. Cell numbers were expressed as percentage of untreated controls.

2-5. MTT and EdU incorporation assays

Cell proliferation was measured by MTT and EdU incorporation assays. Cells were plated onto 96-well plates (Iwaki, Tokyo, Japan) and treated with cPA and/or ROSI, with or without overnight serum starvation, as described in figure legends. Cell proliferation was then determined using the Cell Proliferation Kit I (MTT; Roche Applied Science, Indianapolis, IN, USA) or the Click-iT EdU Microplate Assay Kit (Invitrogen), following the manufacturers'

instructions.

2-6. Western blot analysis

Cells were seeded onto 6-well plates (Iwaki) at a density of 4×10^4 cells/well. After being treated as indicated, cells were lysed on ice for 30 min in cell lysis buffer containing 20 mM Tris-HCl (pH 7.4), 10% (v/v) glycerol, 100 mM NaCl, 1% (v/v) Triton X-100, 1:100 protease inhibitor cocktail (Sigma-Aldrich), and 1 mM dithiothreitol. After lysis, cells were centrifuged at 16,000 × *g* for 20 min at 4°C. The supernatants containing cell lysates were assayed for protein content by the Bradford method using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The lysates were separated on 10% SDS-polyacrylamide gels and transferred onto Immobilon-P membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% (w/v) non-fat dried milk in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.05% (v/v) Tween 20 (TBS-T) for 1 h, and incubated with primary antibody in TBS-T with 5% non-fat dried milk for 12 h at 4°C. Bands were visualized with the Amersham ECL-Plus Western Blot Detection Reagents (GE Healthcare).

2-7. Reporter gene assay

To determine endogenous PPARγ activation, HT-29 cells were co-transfected with pGL3b-PPRE (ACO)-Fluc and pSV40-β-galactosidase plasmids, and reporter gene expression was determined as previously described [12]. Twenty-four hours after transfection, cells were treated with the indicated compounds and incubated for an additional 20 h. Luciferase activity was measured with the Steady-Glo Luciferase Assay System (Promega) using the SpectraMax plate reader (Molecular Devices, Sunnyvale, CA, USA).

2-8. Preparation of subcellular fractions

Following serum starvation, HT-29 cells were treated with DMSO or 10 μM cPA 18:1, cPA 16:0, or 3ccPA 16:1 for 20 min or 1 h at 37°C, rinsed twice with phosphate-buffered saline (PBS), and harvested by scraping. The NE-PER^{TM} Cell Fractionation Kit (Pierce Biotechnology, Rockford, IL, USA) was used to isolate the nuclear fraction from the cells, according to the manufacturer's instructions. After the cytosolic fraction was separated, the nuclear fraction was subjected to a brief centrifugation, and the interface was removed to reduce cytoplasmic contamination. The cytoplasmic and nuclear fractions were tested for purity by immunoblotting for HSP90 (cytoplasmic marker) and p53 (nuclear marker), and stored at -80°C until use.

2-9. Quantitative determination of cPA in nuclear fractions by ESI-LC-MS

Lipids were extracted using the Bligh and Dyer method as previously described [33]. The extracted lipids were dissolved in 0.1 mL of methanol/chloroform/28% NH_4OH (90:10:0.1), and immediately analyzed by electrospray ionization, liquid chromatography-mass spectrometry (ESI-LC-MS). Liquid chromatography-mass spectrometry (LC-MS) was performed on an LCMS-2010A mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an Econosphere 3 μm, 50 × 4.6 mm silica column (Alltech Associates Inc., Deerfield, IL, USA). The sample was maintained at 250°C with drying gas flow of 1.5 L/min, and data were collected in the negative ion mode from 100 to 1000 *m/z*. cPA was quantified by comparing the $[M-H]^-$ signal intensity of cPA to that of the internal standard cPA 17:0.

2-10. Hoechst staining assay

Intact HT-29 cells were stained with Hoechst 33342 (Dojindo, Kumamoto, Japan, 5 μg/mL) for 10 min and analyzed on an Olympus IX71 fluorescence microscope (Tokyo, Japan) with

excitation at 360 nm. With Hoechst staining, viable cells were observed with intact nuclei, and apoptotic cells with fragmented or condensed nuclei.

2-11. Inhibition of PPARγ function using small interfering RNA

PPARγ expression was inhibited in HT-29 cells by transfection with a small interfering RNA (siRNA) targeting PPARγ , using Lipofectamine RNAiMAX (Invitrogen). siRNA not specific to any sequence was used as the negative control. Reduction of PPARγ levels was confirmed by western blot analysis as described above.

2-12. Statistical analysis

Student's *t*-test was used to make statistical comparisons. Differences were considered significant when the *p*-value was below 0.05.

3. Results

3-1. PPARγ activation does not inhibit HT-29 cell growth

We first examined the ability of ROSI, a PPARγ agonist, to inhibit proliferation of several cancer cell lines. Fig. 2A shows the effect of ROSI on cancer cell growth after a 72-h treatment. At concentrations below 20 μM, ROSI inhibited cell growth in HeLa cells, B16F10 cells, U-937 cells, and HL-60 cells, but had no effect on the growth of HT-29. We then investigated PPARγ protein levels in different cancer cell lines listed here (Fig. 2B). The only PPARγ isoform detected was PPARγ_1. Three isoforms of PPARγ have been identified in humans: PPARγ_1, PPARγ_2, and PPARγ_3. The biological significance of our finding only PPARγ_1 is unclear, since the functional differences among the PPARγ isoforms are unknown. Next, to test the functionality of the PPARγ receptor, we transfected the above cell lines with a luciferase

reporter plasmid. After ROSI treatment, we observed increased luciferase activity (Fig. 2B).

3-2. cPA inhibits HT-29 cell growth by inhibiting PPARγ

As shown in Figs. 3A and B, cPA inhibited cell growth in a dose- and time-dependent manner. cPA 18:1 (unsaturated form) and cPA 16:0 (saturated form) inhibited cell growth with IC_{50} of 10 μM and 4.5 μM, respectively. cPA 18:1 has 1 double bond in the carbon chain, and that double bond may react with oxygen. Hence, we decided to use the saturated form of cPA (16:0) and the metabolically stabilized cPA carba derivative (3ccPA 16:1), in which the phosphate oxygen is replaced with a methylene group, to define the role of cPA in the regulation of cell proliferation. As shown in Figs. 3C and D, cPA 16:0 and 3ccPA 16:1 inhibited the PPARγ activation elicited by ROSI, and PPARγ-mediated transcription negated the anti-proliferative effects of cPA 16:0 and 3ccPA 16:1. The synthetic PPARγ antagonist T0070907 also inhibited PPARγ activation elicited by ROSI when compared to the vehicle-treated control. Furthermore, decreasing PPARγ levels in HT-29 cells with siRNA reduced the cPA dose required to inhibit cell proliferation (Fig. 3E). These results suggest that inhibition of cell growth by cPA is mediated by its inhibition of the PPARγ pathway.

Our previous work showed that a wide range of cPA concentrations did not potentiate reporter gene expression mediated by either PPARα or PPARδ in B103 cells co-treated with WY-14643 (PPARα-specific agonist) or L-165041 (PPARδ-specific agonist). As shown in Fig. S1, treating cells with cPA in combination with either a PPARα or a PPARδ agonist did not inhibit HT-29 cell growth any more than did treatment with cPA alone.

3-3. cPA induces DNA condensation

Next, we confirmed nuclear distribution of exogenous cPA in HT-29 cells, using ESI-LC-MS.

Interestingly, the nuclear fraction of HT-29 cells was found to contain significant amount of AGP (Fig. 4A). AGP is a PPARγ agonist [12] in HT-29 cells, constitutively activating PPARγ. We then examined the amount cPA present in the cells. cPA 18:1 was added to cells at a concentration of 10 μM, and lipid uptake was determined by ESI-LC-MS. As shown in Fig. 4B, time-dependent accumulation of cPA 18:1 was detected in the nuclei. The same experiments were repeated with exogenous cPA 16:0 and ccPA 16:1 (Fig. 4B).

Recent studies have shown that inhibiting PPARγ with a synthetic antagonist (T0070907 or GW9662) caused apoptosis by preventing of cellular adhesion [17]. Light microscopy analysis of HT-29 cells revealed that treatment with cPA and its derivative induced cell detachment after 96 h (Fig. 4D). Hoechst 33342 staining demonstrated that treatment with cPA increased DNA condensation, and therefore apoptosis, by approximately 8-fold as compared to the vehicle control (Fig. 4E).

4. Discussion

We recently reported that cPA (Fig. 1) is a *bona fide* second messenger and a physiological inhibitor of PPARγ [18]. cPA has emerged as a potential anti-metastatic drug candidate [19], but the mechanisms of its action were not clear. In this study, we evaluated the effects of PPARγ inhibition by cPA in HT-29 cells. To determine whether PPARγ inhibition negatively affects HT-29 cells, we examined the effect of cPA on HT-29 cell proliferation. Studies of PPARγ function have been aided by the development of relatively high-affinity ligands, such as TZD drugs like ROSI. Numerous *in vitro* and *in vivo* reports indicate that these PPARγ agonists inhibit tumor cell growth. However, prior studies used comparatively high agonist doses (as high as 100 μM) that may saturate cellular responses. In this study, we examined the effect of PPARγ activation on the growth of multiple cancer cell lines using lower ROSI concentrations

(1–10 μM). While many *in vitro* and *in vivo* studies in cancer models used PPARγ agonists, relatively few have used antagonists. In the present study, we report for the first time that the PPARγ antagonist cPA inhibits cell growth of HT-29 cells.

A recent study shows that cPA and ccPA are poor activators of LPA_1/Edg-2, LPA_2/Edg-4, LPA_3/Edg-7, and LPA_4/p2y9 receptors [34]. These receptors are important in GPCR (G-protein coupled receptor)-mediated cell motility and invasion of normal and tumor cells [35]. Shida *et al*. reported the expression levels of the LPA receptor in HT-29 cells [36]. Among the 3 EDG-family LPA receptors ($LPA_{1–3}$), only LPA_2 mRNA was expressed in HT-29 cells, while no significant expression of LPA_1 and LPA_3 mRNA was detected. Because of the lack of reliable LPA receptor antibodies, we used real-time reverse transcription polymerase chain reaction (RT-PCR) analysis to determine the levels of LPA receptors gene expression. As shown in Fig. S2, the relative levels of $LPA_{1–7}$ receptor mRNA were $LPA_6 > LPA_2 > LPA_1$ in HT-29 cells. Our results suggest that LPA_6/p2y5 mRNA was significantly more abundant than the mRNA of the other LPA receptors. Lee *et al*. recently reported that LPA_6/p2y5 activation by LPA reduced intestinal cell adhesion [37]. However, 3ccPA had no significant effects on the morphology of LPA_6/p2y5-expressing cells (Mari Gotoh and Kimiko Murakami, Murofushi Ochanomizu University, personal communication). These results suggest that the growth inhibition observed in HT-29 cells treated with cPA is partly mediated by the PPARγ signaling pathway. Of note, it has been shown that cPA and ccPA activate the LPA_5 receptor strongly compared with the other LPA receptors [19]; however, we detected very low levels of LPA_5 mRNA in HT-29 cells (Fig. S2). Taken together, these data strongly suggest that cPA directly acts on PPARγ, rather than stimulating an LPA GPCR.

Recent studies indicate that PPARγ antagonists can reduce tumor cell growth in multiple cancer cell lines [38] [17]. Bisphenol A diglycidyl ether (BADGE) can act as both an agonist and an

antagonist toward PPARγ, and causes apoptosis in a colon cancer cell line [39]. Seargent *et al.* reported that GW9662 prevented PPARγ activation and proliferation of breast cancer cell lines. T0070907 was identified as a potent and selective PPARγ antagonist [40], and reduced colon cancer migration and invasion in a xenograft metastatic model [27]. These data indicated that the increase in floating cells upon cPA treatment (Fig. 4F) was due to the induction of apoptosis. PPARγ–mediated transcriptional repression may explain some of the effects of cPA and ccPA.

Our current proposed mechanism for cPA action is presented in Fig. 5. cPA suppresses PPARγ activation both by preventing binding of exogenous agonists to PPARγ, and by inducing a specific conformational change that suppresses PPARγ activation [18]. To date, TZD therapy is most successful in treating prostate cancer, a cancer type with reduced PPARγ levels and possible loss of PPARγ function [41]. In contrast, the use of TZDs in the treatment of cancers with moderate or high PPARγ levels, such as colon cancer, has shown no clinical benefit [42]. These data demonstrate that the effectiveness of using PPARγ as a target for chemotherapeutic cancer treatment varies in different types of cancer, depending on PPARγ expression. We expect that novel preventive and therapeutic cancer treatments can be developed that have a more favorable pharmacological impact than PPARγ agonists. Targeting specific molecules has become important for developing new cancer treatments. Members of the nuclear receptor superfamily are excellent examples of such targets. cPA binding to and inhibition of PPARγ might be involved in cPA-induced inhibition of HT-29 cell growth. It is not well known, however, if this *in vitro* finding can be extrapolated to *in vivo* systems, and further studies are necessary to verify our results. In summary, this study supports the potential use of cPA and its derivatives in the development of drugs targeting colon cancer and possibly other types of cancer.

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Figure Legends

Fig. 1. Structural formulas of the compounds used in this study.

Fig. 2. Peroxisome proliferator-activated receptor **(**PPARγ) activation suppresses growth in multiple cell types. A, ROSI inhibited cell proliferation in HeLa, B16, U937, and HL-60 cells, but not in HT-29 cells. Cells were seeded at a density of 3×10^4 cell/well in 6-well plates in

Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). After 12 h, the medium was replaced with fresh medium, and 10 or 20 μM ROSI was added to cells. The number of viable cells was determined after 3 d and expressed as percent of vehicle-treated control cells. Data are presented as mean (SEM); n = 3; $^{**}p < 0.01$. B, Whole cell lysates were prepared from the indicated cell lines, and 50 μg of protein were used to immunoblot for PPARγ$_1$ (50 kDa). *Trace amount of PPARγ$_2$ (65 kDa) expressed in HL-60 cells. C, Cells express functional PPARγ. Indicated cell lines were transfected with the PPRE-luc and CMV-β-galactosidase plasmids, and treated with vehicle or the indicated PPARγ ligands for 20 h. The level of luciferase activity was measured in lysates of treated cells and normalized to β-galactosidase activity. Data are presented as mean (SEM); n = 5; $^{**}p < 0.01$.

Fig. 3. Inhibition of cell growth by cPA was mediated by inhibition of the PPARγ pathway. A and B, cPA inhibited growth of HT-29 cells in a dose- and time-dependent manner. Cells were plated on 6-well plates (2×10^4 cell/well) and treated with vehicle or various concentrations of cPA for 96 h. Following treatment, the cells were detached using trypsin-EDTA, and the number of cells in each well was counted. $^{**}p < 0.01$ compared with vehicle-treated control. C, ROSI treatment alleviated the cPA-induced inhibition of cell growth and PPARγ activation. HT-29 cells were plated on 6-well plates (2×10^4 cell/well) and treated with vehicle or various concentrations of cPA, ROSI, or both for 96 h. Following the treatment, the cells were detached using trypsin-EDTA, and the number of cells in each well was counted. LPA18:1 and T0070907 were used as the negative and positive controls, respectively. Data are presented as mean (SEM); n = 3; $^{**}p < 0.01$. D, HT-29 cells were transfected with the PPRE-luc and CMV-β-galactosidase plasmids, and treated with vehicle or the indicated PPARγ ligand for 20 h. The level of luciferase activity was measured in lysates of treated cells and normalized to

β-galactosidase activity. Data are presented as mean (SEM); n = 5; $^{**}p < 0.01$. E, HT-29 cells were transfected with control siRNA or siRNA against PPARγ. Twenty-four hours later, cells were treated with vehicle or cPA for 72 h. Following treatment, the cells were detached using trypsin-EDTA and the number of cells in each well was counted and expressed as a percent of the vehicle-treated control cells. Data are presented as mean (SEM); n = 3; $^{**}p < 0.01$. Right panel: siRNA-mediated PPARγ knockdown. Levels of PPARγ in cells transfected with siRNA against PPARγ or non-specific siRNA for 6, 12, and 24 h were determined by western blot.

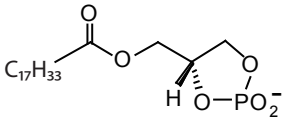
Fig. 4. cPA induced DNA condensation and cellular detachment**.** A and B, Electrospray ionization, liquid chromatography-mass spectrometry (ESI-LC-MS) quantification of cPA in the nuclear fraction of HT-29 cells. Total lipids were extracted using the Bligh & Dyer method, with 100 pmol cPA 17:0 as the internal standard. The lipids were dissolved in chloroform:methanol (1:9), and infused onto the electrospray source. Significant amounts of cPA were detected in the nuclei after a 20 min exposure. Data are expressed in pmol as mean (SEM); n = 3. C, 20 μg of nuclear extract was analyzed by western blotting using specific antibodies against p53 (nuclear marker) and heat shock protein 90 (HSP-90; cytoplasmic marker). D, cPA induced DNA condensation in HT-29 cells**.** Cells were seeded at a density of 4 × 10^4 cells/well in 6-well plates in DMEM with 10% FBS. After 12 h, cells were treated with vehicle or 20 μM cPA for 72 h. Thereafter, cells were stained with Hoechst 33342 and analyzed by fluorescence microscopy. Apoptotic nuclei (indicated by arrows) are brightly stained and condensed when compared to nuclei in vehicle-treated cells. The scale *bar* indicates 200 μm. E, At least 5 fields of cells per sample were counted and tabulated; values are expressed as mean (SEM); n = 5; $^{**}p < 0.05$. F, Number of floating cells as a proportion of the total cell number (attached and floating) in control cells and cells treated with cPA. Data are expressed as mean

(SEM); n = 3.

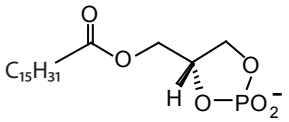
Fig. 5. Schematic representation of the proposed mechanism of cPA action in HT-29 cells. A, cPA enters the nucleus and bind to the PPARγ complex. B, cPA suppresses PPARγ activation both by preventing binding by exogenous agonists (e.g., ROSI), and by inducing a specific conformational change that actively suppresses PPARγ activity. C, PPARγ can repress gene transcription by negatively interfering with other signal-transduction pathways. cPA enhances nuclear condensation. These combined effects may contribute to growth inhibition in HT-29 cells

Figure(s)

cPA 18:1



cPA 16:0



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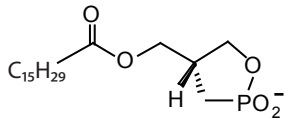

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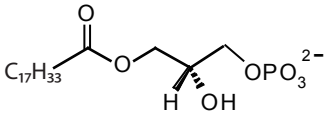

Rosiglitazone(ROSI)

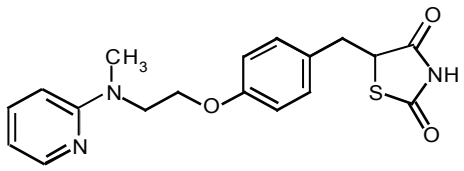

Fig.1

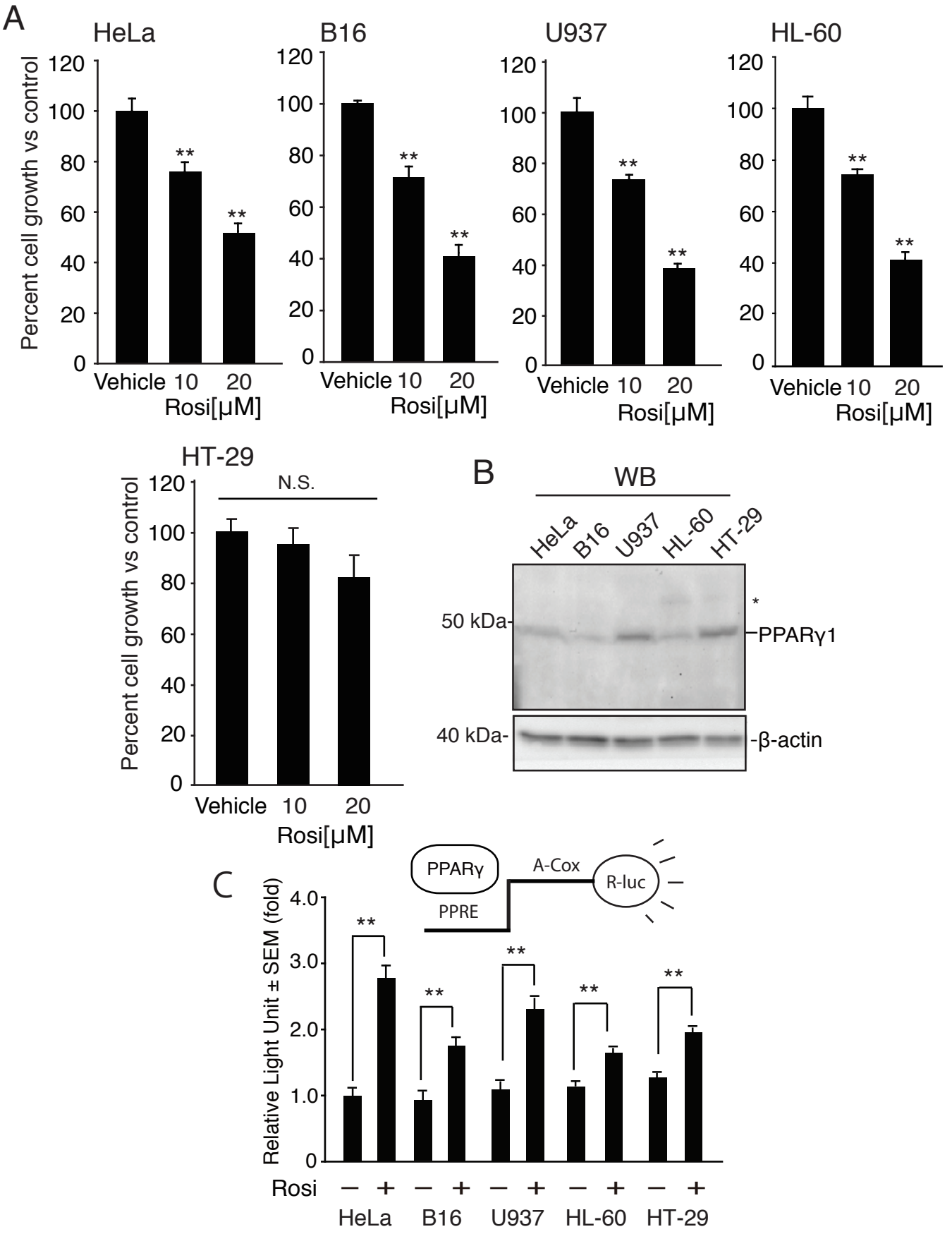
A
HeLa
B16
U937
HL-60
HT-29
Percent cell growth vs control
120
100
80
60
40
20
0
**
N.S.
Vehicle
10
20
Rosi[μM]
B
WB
HeLa
B16
U937
HL-60
HT-29
50 kDa-
PPARγ1
*
40 kDa-
-β-actin
C
PPARγ
A-Cox
R-luc
PPRE
Relative Light Unit ± SEM (fold)
4.0
3.0
2.0
1.0
0
Rosi
−
+
HeLa
B16
U937
HL-60
HT-29

Fig.2

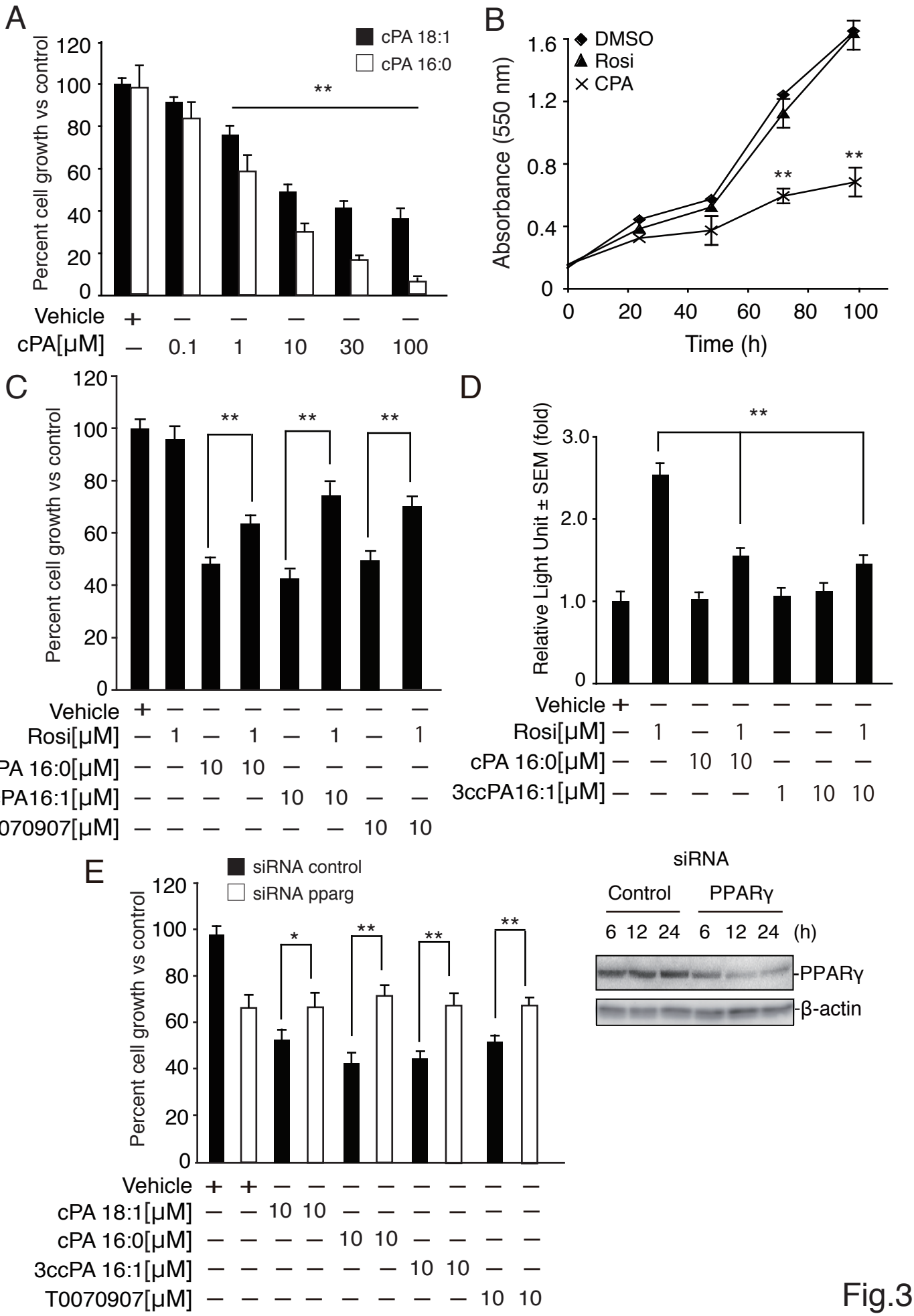
A
cPA 18:1
cPA 16:0
Percent cell growth vs control
120
100
80
60
40
20
0
**
Vehicle + — — — — —
cPA[μM] — 0.1 1 10 30 100
B
DMSO
Rosi
CPA
Absorbance (550 nm)
1.6
1.2
0.8
0.4
0
**
**
0 20 40 60 80 100
Time (h)
C
Percent cell growth vs control
120
100
80
60
40
20
0
** ** **
Vehicle + — — — — — — —
Rosi[μM] — 1 — 1 — 1 — 1
cPA 16:0[μM] — — 10 10 — — — —
3ccPA16:1[μM] — — — — 10 10 — —
T0070907[μM] — — — — — — 10 10
D
Relative Light Unit ± SEM (fold)
3.0
2.0
1.0
0
**
Vehicle + — — — — — —
Rosi[μM] — 1 — 1 — — 1
cPA 16:0[μM] — — 10 10 — — —
3ccPA16:1[μM] — — — — 1 10 10
E
siRNA control
siRNA pparg
Percent cell growth vs control
120
100
80
60
40
20
0
* ** ** **
Vehicle + + — — — — — — — —
cPA 18:1[μM] — — 10 10 — — — — — —
cPA 16:0[μM] — — — — 10 10 — — — —
3ccPA 16:1[μM] — — — — — — 10 10 — —
T0070907[μM] — — — — — — — — 10 10
siRNA
Control
PPARγ
6 12 24 6 12 24 (h)
-PPARγ
-β-actin

Fig.3

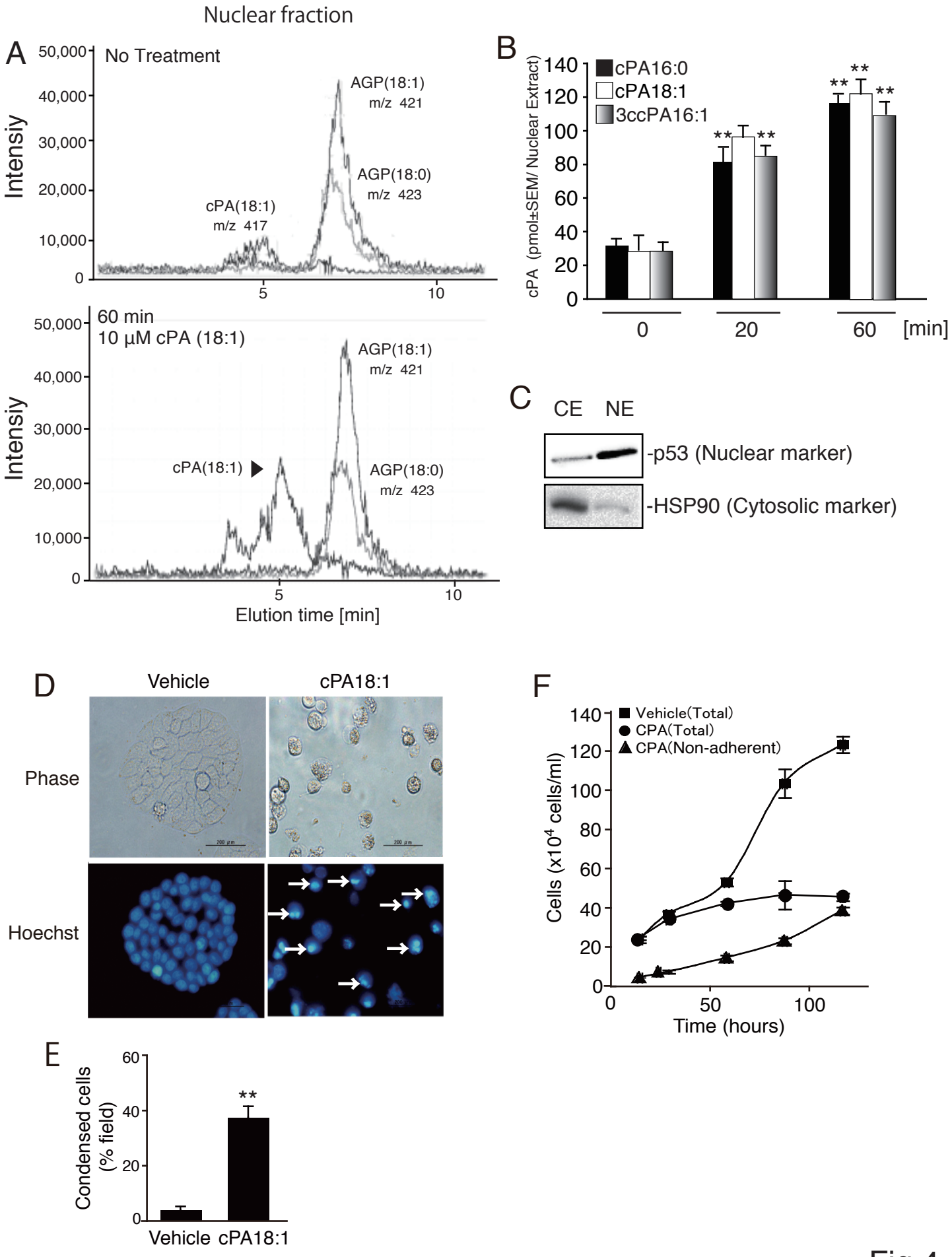
Nuclear fraction
A
No Treatment
Intensiy
AGP(18:1)
m/z 421
AGP(18:0)
m/z 423
cPA(18:1)
m/z 417
60 min
10 μM cPA (18:1)
cPA(18:1)
Elution time [min]
B
cPA16:0
cPA18:1
3ccPA16:1
cPA (pmol±SEM/ Nuclear Extract)
[min]
C
CE NE
-p53 (Nuclear marker)
-HSP90 (Cytosolic marker)
D
Vehicle
cPA18:1
Phase
Hoechst
E
Condensed cells (% field)
Vehicle cPA18:1
F
Vehicle(Total)
CPA(Total)
CPA(Non-adherent)
Cells (x10⁴ cells/ml)
Time (hours)

Fig.4

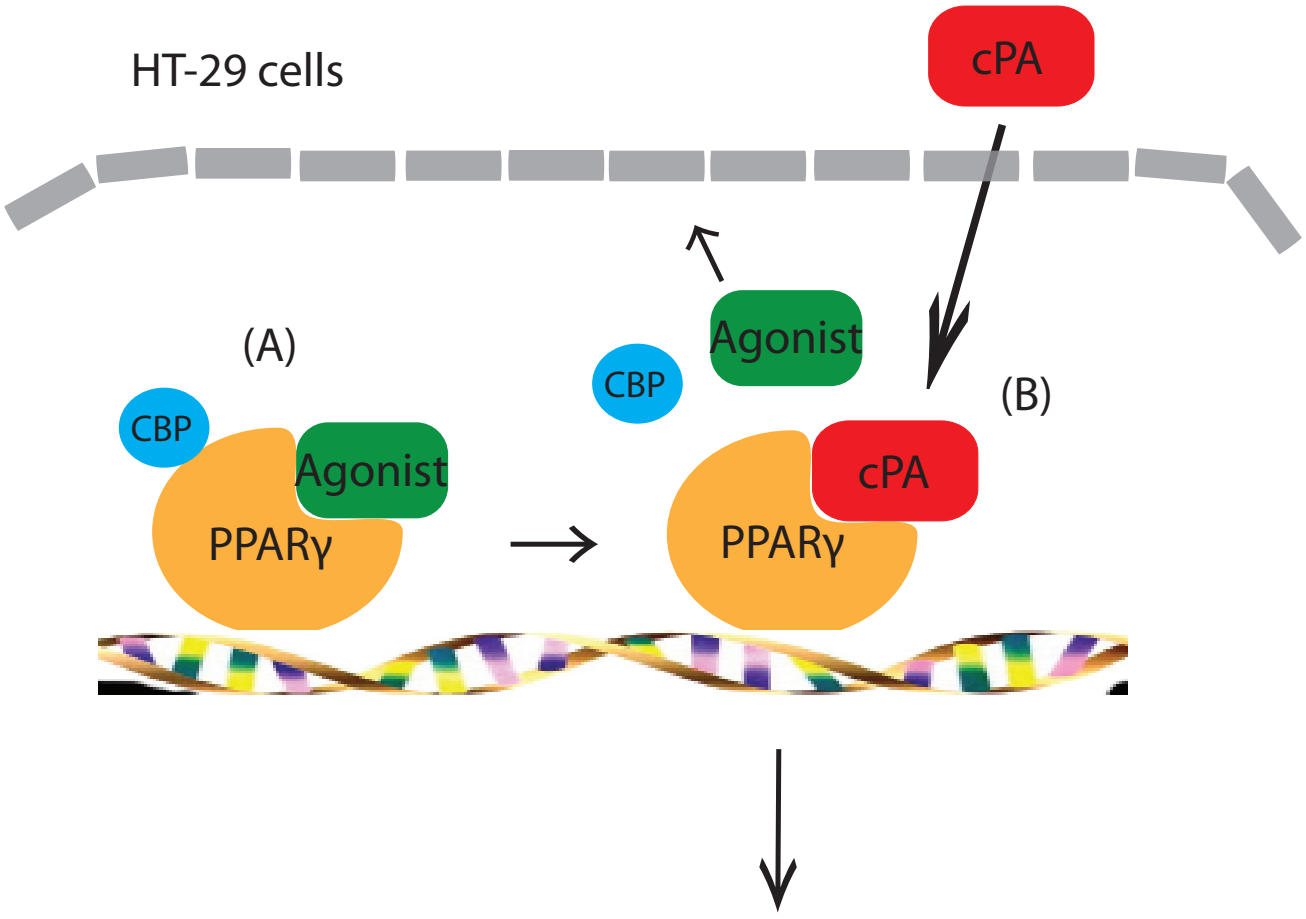
HT-29 cells
cPA
(A)
CBP
Agonist
PPARγ
Agonist
CBP
(B)
cPA
PPARγ
(C) Post ligand signal transduction

Fig.5

Supplemental files

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