# Short-term treatment with a 2-carba analog of cyclic phosphatidic acid induces lowering of plasma cholesterol levels in ApoE-deficient mice

Tamotsu Tsukahara[a*], Hisao Haniu[b], Yoshikazu Matsuda[c], and Kimiko Murakmi-Murofushi[d]

[a]Department of Molecular Pharmacology and Neuroscience, Nagasaki University Graduate School of Biomedical Sciences, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

[b]Institute for Biomedical Sciences, Shinshu University Interdisciplinary Cluster for Cutting Edge Research 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan

[c]Clinical Pharmacology Educational Center, Nihon Pharmaceutical University, Ina-machi, Saitama 362-0806, Japan

[d]Endowed Research Division of Human Welfare Sciences, Ochanomizu University, 2-1-1, Ohtsuka, Bunkyo-ku, Tokyo 112-8610, Japan

Running title: *cPA decreases plasma cholesterol*

To whom correspondence should be addressed: Tamotsu Tsukahara, Department of Molecular Pharmacology and Neuroscience, Nagasaki University Graduate School of Biomedical Sciences, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

E-mail: ttamotsu@nagasaki-u.ac.jp

Tel: +81-75-852-2473

Fax: +81-75-852-2420

**ABSTRACT**

Plasma cholesterol levels are associated with an increased risk of developing atherosclerosis. An elevated low-density lipoprotein cholesterol (LDL-C) level is a hallmark of hypercholesterolemia in metabolic syndrome. Our previous study suggested that when acetylated LDL (AC-LDL) was co-applied with a PPARγ agonist, rosiglitazone (ROSI), many oil red O-positive macrophages could be observed. However, addition of cyclic phosphatidic acid (cPA) to ROSI-stimulated macrophages completely abolished oil red O-stained cells, indicating that cPA inhibits PPARγ-regulated AC-LDL uptake. This study aimed to determine whether metabolically stabilized cPA, in the form of a carba-derivative of cPA (2ccPA), could reduce plasma cholesterol levels and affect the expression of genes related to atherosclerosis in apolipoprotein E-knockout ($apoE^{-/-}$) mice. 2ccPA reduced LDL-C levels in these mice (n = 3) from 460 to 330 mg/ml, from 420 to 350 mg/ml, and 420 to 281 mg/ml under a western-type diet. 2ccPA also reduced expression of lipid metabolism-related genes, cytokines, and chemokines in ApoE-deficient mice on a high-fat diet. Taken together, these results suggest that 2ccPA governs anti-atherogenic activities in the carotid arteries of apoE-deficient mice.

**Keywords:** atherosclerosis; cholesterol; cyclic phosphatidic acid; high-density lipoprotein cholesterol (HDL-C); low-density lipoprotein cholesterol (LDL-C)

## 1. Introduction

Atherosclerosis is a complex chronic vascular disease characterized by accumulation of plasma-derived lipids in arteries [1]. Modified lipoproteins, such as oxidized low-density lipoproteins (oxLDLs), which are generated in the vessel wall, up-regulate the expression of cytokines and chemokines [2, 3]. These molecules attract lymphocytes and monocytes. In the arterial intima, immune cells generate various cytokines and chemokines, leading to a cascade of events, involving recruitment of additional inflammatory cells, further lipid accumulation, and increased migration, differentiation, and proliferation of smooth muscle cells (SMCs). Low-density lipoprotein (LDL) is a transporter of phospholipids and cholesterol in the blood [4].

One such phospholipid, cyclic phosphatidic acid (cPA), with a unique cyclic phosphate ring at the sn-2 and sn-3 positions of its glycerol backbone, is an analog of the growth factor-like phospholipid lysophosphatidic acid (LPA), and is a therapeutic a target for many diseases, including atherosclerosis [5], obesity [6], cancer [7, 8], and inflammation [9] [10]. Our previous in vitro study suggested that the suppressive effects of cPA on the growth and migration of human coronary artery endothelial cells (HCAECs) obtained from donors with type 2 diabetes (D-HCAECs) require the inhibition of peroxisome proliferator-activated receptor gamma (PPARγ). cPA, along with a PPARγ antagonist, also prevented neointima formation, adipocyte differentiation, lipid accumulation, and downregulation of PPARγ-target gene transcription in mouse macrophages [5]. Furthermore, activation of the endothelium by alkyl-LPA (AGP) has been implicated in the development of atherosclerosis, while VEGF production was elicited by AGP in D-HCAECs that express high PPARγ levels [11]. AGP is present in blood plasma [12] and its levels increase after minimal oxidative modification of LDL. High levels of AGP are also found in the lipid-rich necrotic core of human carotid artherosclerotic plaques. Interestingly, AGP increases the expression and secretion of interleukin (IL)-6 in HCAECs [11], but this effect is inhibited when HCAECs are co-treated with cPA [13].

Although atherosclerosis is not a novel characteristic of ApoE-deficient humans, the ApoE-knockout mouse is a well-established genetic animal model of atherosclerosis and has been used

widely in studies of the effect of foods and drugs [14]. ApoE-deficient mice that are fed diets high in fat and cholesterol show markedly accelerated plaque development. In this study, we investigated the involvement of lipid metabolism-related as well as inflammation-related genes in carotid arteries of these mice, after treatment with or without a metabolically stabilized derivative of cPA, viz., 2-carba-cPA (2ccPA).

## 2. Materials and methods

### *2.1 Materials*

2ccPA (18:1) was a kind gift from Kimiko Murakami-Murofushi, from Ochanomizu University, Tokyo Japan. A Histone deacetylase (HDAC) antibody sampler kit (#9928S) was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA).

### *2.2 Animals and treatment*

All animal experiments were performed in collaboration with Unitech Co., Ltd. (Chiba, Japan). BALB/c. KOR/SteSlc-Apoe$^{shl}$ mice (6-week-old), weighing 20–25 g, were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were kept in a humidity-controlled room on a 12-h light–dark cycle with standard food and water available ad libitum for 1 week. The mice were then divided randomly into two groups, with four animals in each group, and were fed a high-fat diet (D12108C, 1.25% cholesterol), purchased from Research Diet Inc. (New Brunswick, NJ, USA), for 4 weeks. The ApoE$^{-/-}$ + 2ccPA group were administered 2ccPA (5 mL/kg bodyweight, 2 mg/mL in phosphate-buffered saline [PBS]) by subcutaneous injection, while the control group (ApoE$^{-/-}$ group) was given an equal volume of PBS, for 4 weeks.

### *2.3 Measurement of cholesterol and triglyceride levels*

*cPA decreases plasma cholesterol*

The plasma levels of triglycerides (TGs), LDL cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and total cholesterol (T-CHO) were measured in collaboration with Unitech Co., Ltd. (Chiba, Japan).

*2.4 Mouse carotid artery harvesting*

The carotid arteries of the mice were harvested 14 days after commencing the high fat diet in collaboration with Unitech Co., Ltd. (Chiba, Japan), and total RNA and protein was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA).

*2.5 Quantitative real-time PCR analysis*

The total RNA obtained from the carotid arteries was purified using a NucleoSpin RNA II kit (TaKaRa, Otsu, Japan), according to the manufacturer's protocol. Of this, 0.1 μg was used for the subsequent synthesis of cDNA using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan), as recommended by the manufacturer. The levels of mRNA were determined with an ECO Real-Time PCR system (Illumina, Inc., San Diego, CA, USA), using SYBR Green Real-Time PCR Master Mix-Plus (Toyobo) and the following primer pairs: mCD36, 5′-TCCAGCCAATGCCTTTGC-3′ (forward) and 5′-TGGAGATTACTTTTTCAGTGCAGAA-3′ (reverse); FABP4, 5′-GGGAACCTGGAAGCTTGTCT-3′ (forward) and 5′-ACTCTCTGACCG GATGGTGA-3′ (reverse); PPARγ, 5′-CCACCAACTTCGGAATCAGCT-3′ (forward) and 5′-TTTGTGGATCCGGCAGTTAAGA-3′ (reverse); IL-6, 5′-CTGCAAGAGACTTCCATCCAG-3′ (forward) and 5′-AGTGGTATAGACAGGTCTGTTGG-3′ (reverse); RANTES, 5′-ATATGGCTCGGACACCACTC-3′ (forward) and 5′-CCTTCGAGTGACAAACACGA-3′ (reverse); MCP-1, 5′-ATCCCAATGAGTAGGCTGGAGAGC-3′ (forward) and 5′-CAGAAGTGCTTGAGGTGGTTGTG-3′ (reverse); and β-actin, 5′-AGGCACCAGGGCGTGAT-3′ (forward) and 5′-GCCCACATAGGAATCCTTCTGAC-3′ (reverse). The specificity of the PCR amplifications was verified by melting curve analysis. Levels of expression were normalized to the

endogenous reference gene encoding β-actin using the relative quantitative method (ΔΔCt), as previously reported [15].

*2.6 Statistical analysis*

Student's *t*-test was used for statistical comparisons and $P < 0.05$ was considered statistically significant.

## 3. Results and discussion

During the progression of atherosclerosis, LDL accumulates within the macrophages and monocytes present in the intima [16]. In this study, plasma lipoprotein levels were determined after ApoE-deficient mice were fed a high-fat diet for 4 weeks (Fig. 1B). The average plasma T-CHO in the control high-fat diet group was 1756.7 mg/mL, and 2ccPA treatment caused an 11% reduction, to 1556.7 mg/mL (Fig. 1C). Plasma TG levels were not different between the control and 2ccPA-treated mice after feeding them a high-fat diet for 4 weeks (Fig. 1D). However, the average LDL-C levels were reduced from 433.3 mg/mL to 320.3 mg/mL (a 23% reduction; Fig. 1E) by 2ccPA treatment for 4 weeks, and HDL-C levels were increased in mice fed a high-fat diet and treated with 2ccPA, from 17.3 mg/mL to 19.7 mg/mL (Fig. 1F). These results suggested that treatment with 2ccPA at a dose of 2 mg/mL for 4 weeks was significantly more effective than vehicle control.

Based on our previous study [6], we hypothesized that exogenous application of 2ccPA *in vivo*, should mitigate atherogenic progression in carotid arteries. In the present study, we selected five atherogenic target genes (*Cd36*, *Pparg*, *Fabp4*, and *Vegf*) and four cytokine- and chemokine-related genes (*Il6*, *Rantes*, *Mcp1*, and *Tnfa*), and monitored their mRNA expression level in the carotid arteries of mice with or without 2ccPA treatment after 4 weeks on a high-fat diet. We previously identified these selected target genes from an *in vitro* experiment [5, 11].

As shown in Fig. 2B–D, we found that, in ApoE-deficient mice, the expression of the target genes was significantly decreased compared with the control group.

Macrophages are thought to play many roles in atherogenic progression, including scavenging of trapped lipoproteins and cytokines [17]. CD36 is a scavenger receptor that plays a role in cytokine-induced macrophage fusion [18].

Moreover, our previous study suggested that stimulation of macrophages with the TZD drug, ROSI, elicits direct activation of PPARγ-regulated genes and that cPA completely abolishes the transcriptional regulation of these genes by ROSI [5]. Furthermore, to examine the regulatory role of cPA on PPARγ-dependent pathophysiological responses, we utilized a rodent model of vascular remodeling [5]. An LPA analog, AGP, induced neointima formation when applied topically within the carotid artery [19]. PPARγ is a member of the nuclear receptor superfamily that is involved in the regulation of lipid metabolism, glucose homeostasis, and cell differentiation [20, 21]. Neointima formation requires PPARγ, because it is abolished by the PPARγ antagonist GW9662 and is absent in mice that are conditionally deficient in PPARγ in the cells of the vessel wall [5].

Fatty-acid-binding protein 3 (FABP3) is one of nine known cytosolic FABPs that range in size from 14 to 15 kDa. It is highly expressed in heart and skeletal muscle, as well as in other tissues (ref). Our previous study suggested that FABP3 is an LPA-binding protein expressed in HCAECs [22]. We have also demonstrated that FABP3 is translocated from the cytosol to the nucleus in response to LPA-mediated PPARγ activation. FABP appears to play a role in shuttling fatty acid ligands to the nucleus, where the ligands are released and bind to PPARγ [22].

VEGF is an important angiogenic factor reported to induce migration and proliferation of endothelial cells, and to enhance vascular permeability [23] [24]. A recent study has suggested that VEGF alters the rate of atherosclerotic plaque development. Celletti et al. reported that VEGF significantly increased macrophage levels in bone marrow and peripheral blood and increased the plaque area in cholesterol-fed rabbits [25]. As expected from our previous *in vitro* experiment [11], these results are consistent with the hypothesis that exogenously applied 2ccPA influences vascular wall responses *in vivo*.

Cytokines and chemokines have also been implicated in the pathogenesis of atherosclerosis, which is an inflammatory disease of the arterial wall [17]. Therefore, we also analyzed the expression of

inflammatory mediators in ApoE-deficient mice on a high-fat diet, with or without 2ccPA treatment. As shown in Fig. 3A, B, C, and D, our results were consistent with the hypothesis that exogenously applied 2ccPA can effectively modulate vascular wall responses and inflammation *in vivo*.

Cardiovascular disease has been shown to trigger chronic inflammatory processes, and pro-inflammatory cytokines, such as IL-6, are important mediators of endothelial dysfunction in coronary artery disease [26]. Furthermore, *in vivo* and *in vitro* investigations have shown that various chemokines activate endothelial cells and inflammatory cells [17]. One of these chemokines, monocyte chemo-attractant protein-1 (MCP-1), has been detected in atherosclerotic lesions [27].

Earlier studies have shown that PPARγ, which plays a key role in the cardiovascular system, is expressed in all cell types of the blood-vessel wall and in monocytes and macrophages [16]. Coronary artery disease is the most common type of heart disease, and the cardiovascular-related mortality typically involves atherosclerosis [26]. Our results thus raise the possibility of using cPA as a lead compound for the development of new treatments that act on PPARγ. The migration of endothelial cells contributes to diverse aspects of vascular physiology, such as the development of atherosclerosis. Currently, cPA is considered to play a role in the anti-neointima activity of carotid arteries [5]. A previous report has demonstrated that cPA inhibited AGP-induced expression of class I histone deacetylases (HDACs, namely HDAC1, HDAC2, HDAC3, and HDAC8), which may affect subsequent transcriptional activity of target genes. A recent study also reported that high levels of AGP accumulate in atherosclerotic lesions in rodents model [16]. Furthermore, increased AGP serum levels are an important risk factor for atherosclerosis, and AGP accumulation in the vessel wall has been suggested to trigger neointima formation [5].

Our previous study has shown that human coronary artery endothelial cells (HCAECs) express high levels of HDAC2 and low levels HDAC1, HDAC3, and HDAC8. HDACs, which are known to activate PPARγ and enhance the expression of its target genes [13], have well-known functions in the regulation of chromatin structure and gene transcription via interactions with nuclear receptor co-repressors, such as SMRT and nuclear receptor corepressor (NcoR) [5]. HDAC2 plays critical roles in

a wide range of biological events, mainly by means of its catalytic activity in removing acetyl groups from histones as well as non-histone proteins [28]. However, several independent investigations have pointed out that class I HDACs participate in chronic inflammation and vascular remodeling. HDAC2 is required for a major mechanism through which activated glucocorticoid receptors block transcription of inflammation related genes [29]. HDAC3 has been reported to inhibit PPARγ and nuclear transcription factor-κB (NF-κB) [30] and HDAC3 inhibition restores PPARγ function in obesity [31]. The inflammatory process involves activation of a variety of signaling pathways and expression of many target genes, which may be mediated by PPARγ [32]. As shown in Fig. 4A, reduction of HDAC2 is believed to result from AGP mediated PPARγ activation in coronary artery in mice. We found that *Hdac2* mRNA was expressed at high levels after treatment with 2ccPA for 28 days, while *Hdac3* was expressed at low levels. We therefore hypothesize that PPARγ inhibition prevents development of atherosclerosis and that anti-atherosclerotic effects of 2ccPA are dependent of serum cholesterol levels.

Taken together, the results of this *in vivo* study indicated a relationship between 2ccPA and blood cholesterol levels, and supported the conclusions drawn from our previous *in vitro* study.

Future studies employing HDAC2 transgenic animals will further clarify the role of the cPA–HDAC2 axis in atherogenesis *in vivo*, and should shed more light on to therapeutic strategies against atherosclerosis.

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**Author contributions**

T.T., Y.M., and H.H. conceived and designed the project; T.T. acquired the data; T.T. and H.H. analyzed and interpreted the data; and T.T. wrote the article. All authors read and approved the final version of the article.

**Author disclosure statement**

No competing financial interests exist.

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**Figure legends**

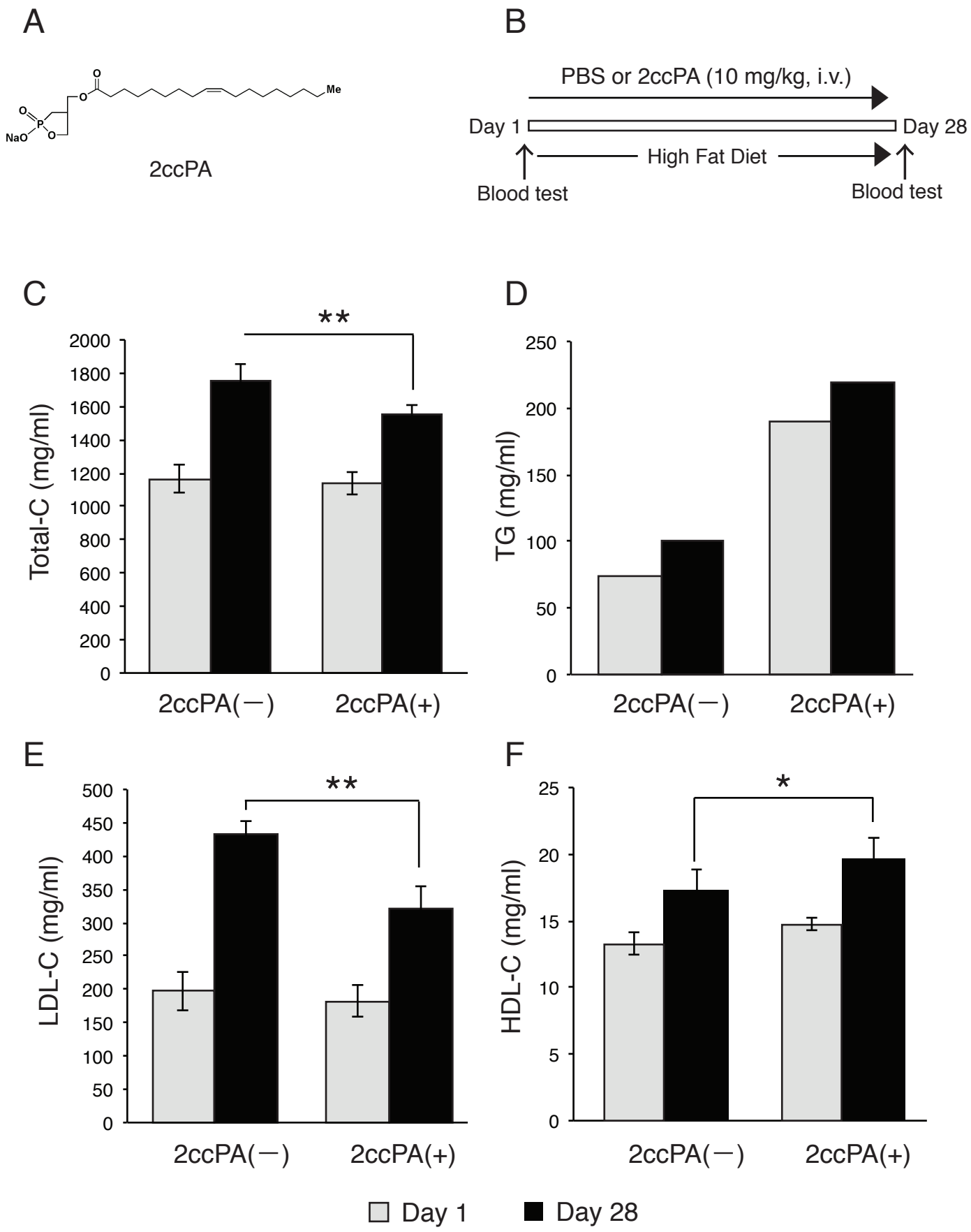
**Figure 1.** Effect of 2ccPA on plasma cholesterol and lipoprotein profiles in ApoE$^{-/-}$ mice. (A) Chemical structure of 2ccPA (18:1). (B) Schematic showing the protocol for dosing schedule in ApoE$^{-/-}$ mice. (C) 2ccPA significantly decreases total cholesterol levels (P = 0.01 versus non-treated mice). (D) Plasma triglyceride (TG) levels were not different between the control and 2ccPA-treated mice fed a high-fat diet for 4 weeks. (E) LDL-C (P = 0.01 versus non-treated mice) in ApoE$^{-/-}$ mice fed with high-fat diet. (F) 2ccPA treatment for 4 weeks increased high-density lipoprotein cholesterol (HDL-C) levels in mice fed a high-fat diet.

**Figure 2.** Expression of atherogenic target genes in carotid arteries of mice in response to 4 weeks of 2ccPA treatment. 2ccPA inhibited expression of atherogenic target genes in apolipoprotein E knockout mice. (A) Carotid artery lesions of 4-weeks old mice on high-fat diet after treatment with or without 2ccPA. (B) Real-time PCR measurement of mRNA of genes encoding CD36, PPARγ, FABP4, and VEGF in the carotid arteries of mice after treatment with or without 2ccPA for 4 weeks. Gene expression levels were normalized to that of the β-actin gene and are expressed as mean ± SEM (n = 3), **P < 0.01.

**Figure 3.** Expression of genes related to cytokines and chemokines in the carotid arteries of mice treated with 2ccPA for 4 weeks. (A) 2ccPA inhibited expression of atherogenic target genes in apolipoprotein E knockout mice. A) Real-time PCR measurement of mRNA of genes encoding IL-6, RANTES, MCP-1, and TNF-α in carotid arteries of mice after treatment with or without 2ccPA for 4 weeks. Gene expression levels were normalized to that of the β-actin gene and are expressed as mean ± SEM (n = 3), **P < 0.01.

**Figure 4.** Comparison of endogenous class I HDAC expression in carotid arteries in mice (A) Real-time PCR was used to measure *Hdac2,* and *Hdac3* mRNA expression in carotid arteries using specific primers for each *Hdac* gene. The relative expression of each *Hdac* gene isoform was calculated by normalization to the expression of the β-actin gene and was expressed as the mean ± SEM (n = 3). **P < 0.01.

**Figure**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25
20
15
10
5
0
2ccPA(−)
2ccPA(+)
Day 1
Day 28

Fig. 1

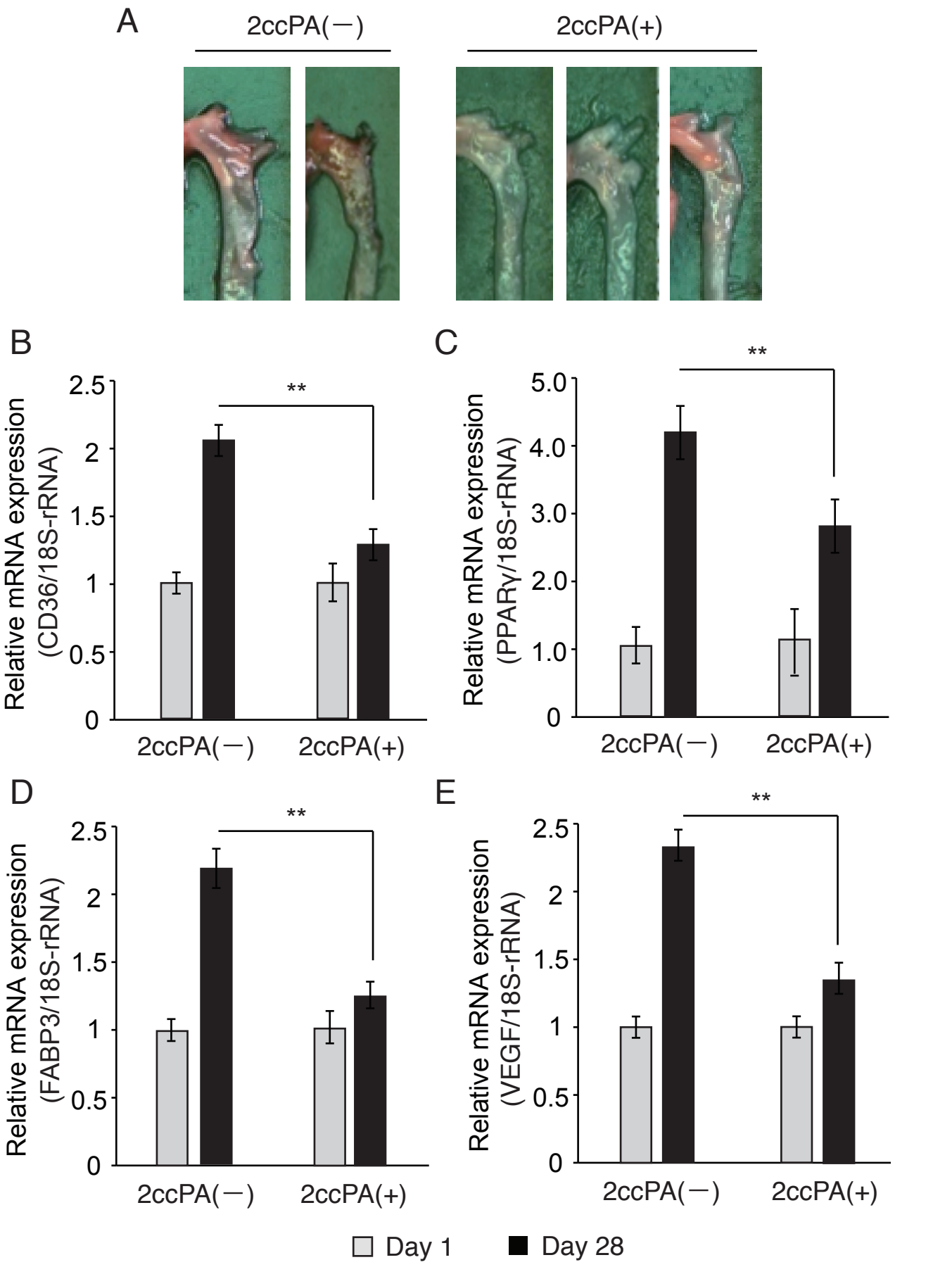

A
2ccPA(−)
2ccPA(+)
B
Relative mRNA expression
(CD36/18S-rRNA)
2.5
2
1.5
1
0.5
0
**
2ccPA(−)
2ccPA(+)
C
Relative mRNA expression
(PPARγ/18S-rRNA)
5.0
4.0
3.0
2.0
1.0
0
**
2ccPA(−)
2ccPA(+)
D
Relative mRNA expression
(FABP3/18S-rRNA)
2.5
2
1.5
1
0.5
0
**
2ccPA(−)
2ccPA(+)
E
Relative mRNA expression
(VEGF/18S-rRNA)
2.5
2
1.5
1
0.5
0
**
2ccPA(−)
2ccPA(+)
Day 1
Day 28

Fig. 2

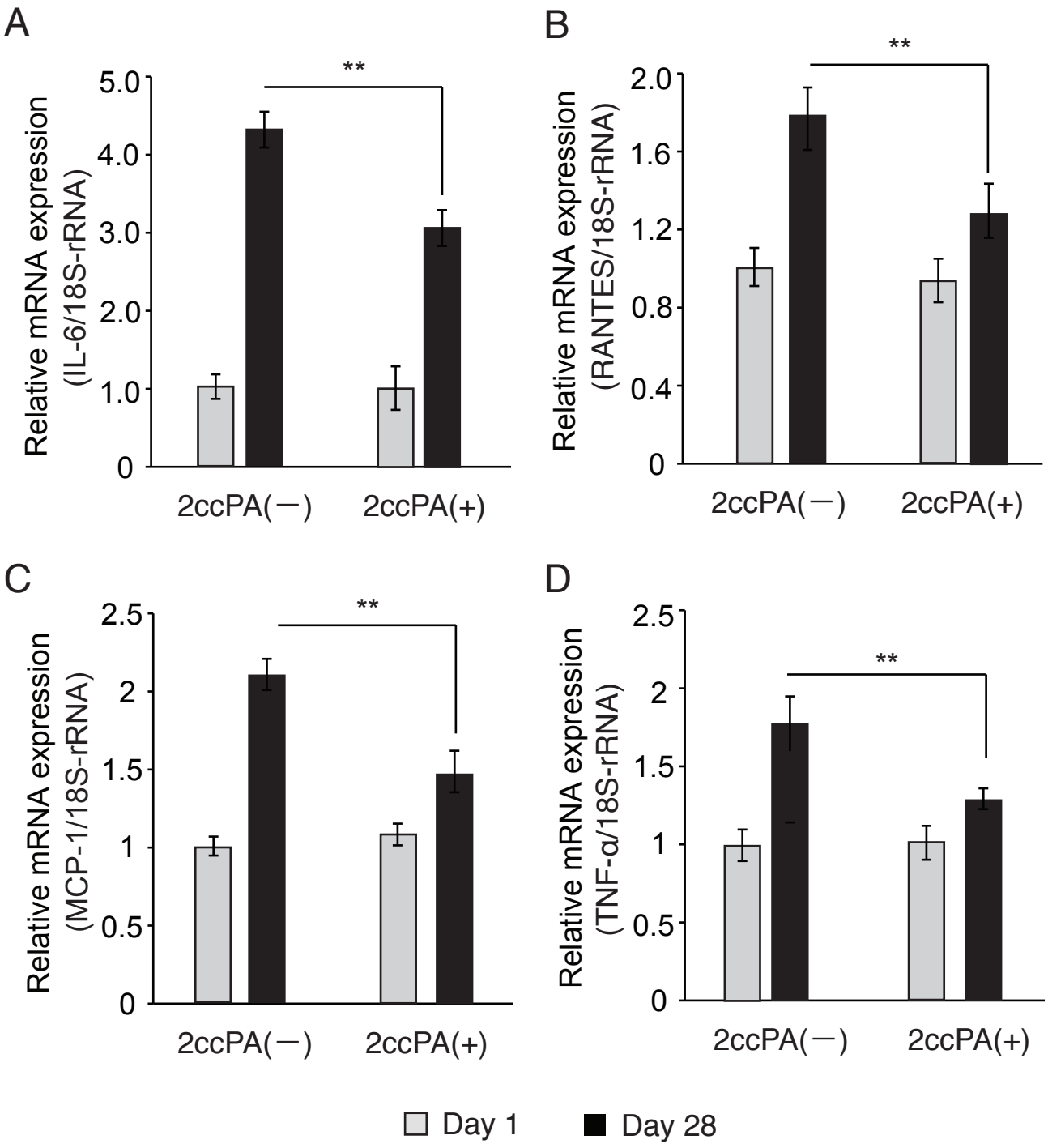
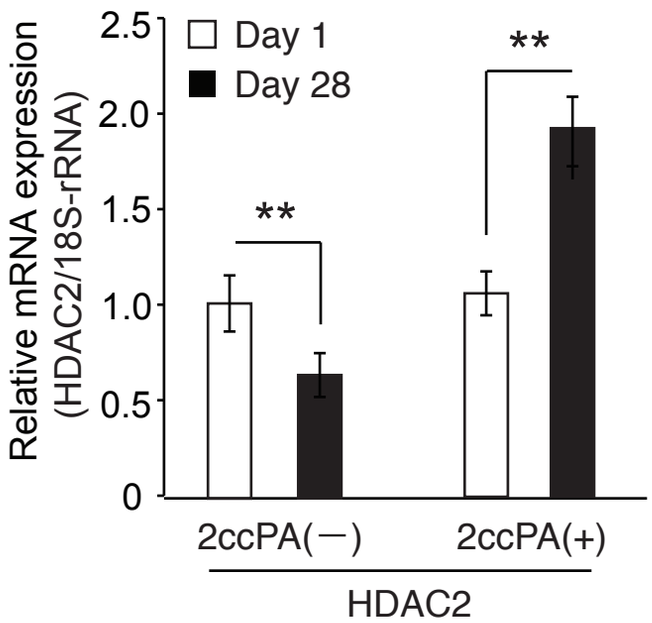
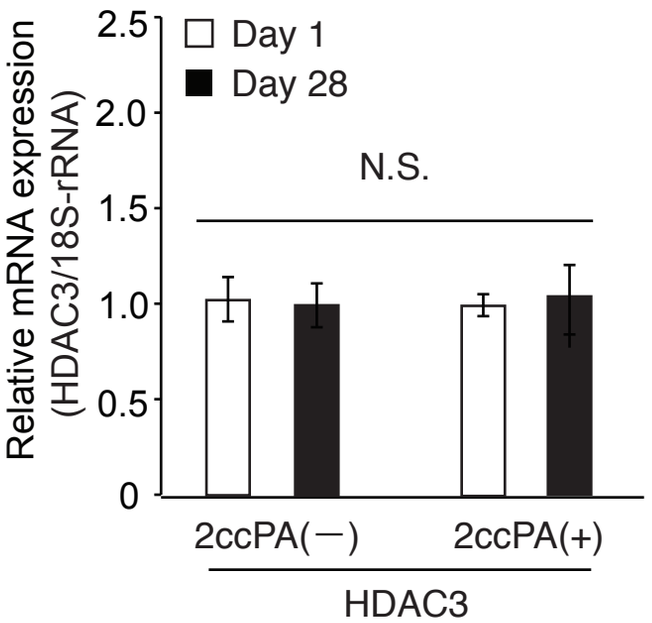

A
B
C
D
Relative mRNA expression
(IL-6/18S-rRNA)
(RANTES/18S-rRNA)
(MCP-1/18S-rRNA)
(TNF-α/18S-rRNA)
**
5.0
4.0
3.0
2.0
1.0
0
1.6
1.2
0.8
0.4
2.5
1.5
0.5
2ccPA(−)
2ccPA(+)
Day 1
Day 28

Fig. 3

A


Relative mRNA expression
(HDAC2/18S-rRNA)
2.5
2.0
1.5
1.0
0.5
0
Day 1
Day 28
**
**
2ccPA(−)
2ccPA(+)
HDAC2

B


Relative mRNA expression
(HDAC3/18S-rRNA)
2.5
2.0
1.5
1.0
0.5
0
Day 1
Day 28
N.S.
2ccPA(−)
2ccPA(+)
HDAC3