Title: Pitavastatin Induces PON1 Expression through p44/42 Mitogen-Activated Protein Kinase Signaling Cascade in Huh7 cells

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ABSTRACT

It has been shown that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors have pleiotropic effects and that human serum paraoxonase (PON1) inhibits the oxidative modification of low-density lipoprotein. We investigated the effects of pitavastatin on PON1 gene promoter activity and PON1 protein expression through the activation of mitogen-activated protein (MAP) kinase signaling cascades in cultured Huh7 cells. Both PON1 gene promoter activity and PON1 protein expression were elevated by pitavastatin stimulation. Pitavastatin phosphorylated p44/42 MAP kinase. The effects of pitavastatin on PON1 promoter activity and PON1 protein expression were attenuated by PD98059. The cotransfection of Sp1 expression vector increased PON1 promoter activity, and mithramycin suppressed pitavastatin-enhanced PON1 promoter activity. The latter activity was attenuated by cotransfection with the expression vector of sterol regulatory element-binding protein-2 (SREBP-2) with mutated p44/42 MAP kinase specific phosphorylation sites. Pitavastatin increased the Sp1-PON1 DNA complex and this effect was attenuated by

PD98059. These observations suggest that pitavastatin phosphorylates p44/42 MAP kinase and then activates the transcription of PON1 gene and increases the PON1 protein expression in Huh7 cells. Furthermore, we speculate that pitavastatin affects both the phosphorylation of SREBP-2 and the Sp1 binding to PON1 DNA through the activation of p44/42 MAP kinase signaling cascade.

Keywords: paraoxonase, HMG-CoA reductase inhibitor, ERK, SREBP-2, Sp1

INTRODUCTION

Human serum paraoxonase (PON1) is an esterase that hydrolyzes aromatic carboxylic acid esters, organophosphates and carbonates. It is noted that PON1 associates with high-density lipoprotein (HDL) [1] and inhibits the oxidation of low-density lipoprotein (LDL) and HDL [2, 3]. PON1 knockout mice were unable to protect against the progression of atherosclerosis by feeding on a high fat and high cholesterol diet [4], and atherosclerotic lesion formation was decreased in PON1 transgenic mice [5]. We are included among researchers who have reported that PON1 activity is related not only to macroangiopathy, but also to microangiopathy, such as retinopathy and nephropathy in diabetic patients [6–8]. These reports indicate that PON1 has effects against oxidative disorders *in vivo* and also that it plays an important role in the suppression of the development and progression of atherosclerosis [9].

A clinical trail has shown that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) have various pleiotropic effects against atherosclerosis apart from their cholesterol-lowering action [10]. Anti-oxidation

has been suggested as one of the pleiotropic effects of statins [11]. Also, simvastatin has been reported to inhibit macrophage-dependent LDL oxidization [12], and atorvastatin to inhibit copper-derived LDL oxidization [13].

Clinical studies showed that simvastatin normalized the lower enzyme activity of PON1 in patients with familial hypercholesterolemia [14] and that the statin increased the serum concentrations and activities of PON1 in patients with hypercholesterolemia [15]. We previously reported using a reporter gene assay method showing that pitavastatin enhanced the promoter activity of the PON1 gene in HepG2 cells of a human hepatocellular carcinoma cell line and in human embryonic kidney 293 (HEK293) cells through a depletion of mevalonic acid-derived farnesyl pyrophosphate [16]. Deakin et al. reported that simvastatin increased sterol regulatory element-binding protein-2 (SREBP-2) binding to PON1 DNA, and that SREBP-2 up-regulated PON1 gene promoter activity in HepG2 cells [15]. SREBP-2 is a transcription factor that is a major regulator of cholesterol homeostasis and that is activated by cellular cholesterol depletion. It was recently demonstrated that SREBP-2 was linked to p44/42

mitogen-activated protein (MAP) kinase signaling cascade [17, 18]. On the other hand, statins regulate the induction of various proteins or genes through an activation of MAP kinase signaling cascades [19-21]. However, there have been no reports in related literature that MAP kinase signaling cascades are involved in PON1 protein expression or its promoter activation in human hepatocytes.

In the present study, we investigated whether pitavastatin stimulated PON1 gene promoter activity and affected its protein expression through MAP kinase signaling cascades in cultured human hepatoma Huh7 cells *in vitro*. We showed that pitavastatin increased PON1 gene promoter activity and PON1 protein expression and that these effects were regulated by p44/42 MAP kinase signaling cascade.

MATERIALS and METHODS

Cell culture and maintenance

Huh7 cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Life Technologies, Rockville, MD), 100 U/ml penicillin (Life Technologies) and 20 μg/ml streptomycin (Life Technologies) in 90-mm plastic plates (Nunc, Roskilde, Denmark), and incubated at 37°C in 5% CO_2. The cells were seeded into 90-mm tissue culture plates and routinely passaged every 3–4 days. These cells were seeded into 24-well plastic plates (Corning, Corning, NY) for luciferase assays and 6-well plastic plates (Nunc) for Western blotting.

Treatment and reagents

Pitavastatin was gifted from Kowa Ltd. (Tokyo, Japan). PD98059 and U0126 were purchased from Promega (Madison, WI), and SP600125 and SB203580 from Sigma. All of the above reagents were dissolved in dimethyl

sulfoxide (DMSO) (Nakarai Tesque, Kyoto, Japan) adjusted with DMEM to a final concentration of 0.1%. The medium of control wells was adjusted to 0.1% DMSO. Plasmid constructs were transfected 120 min before treatment with each reagent for luciferase assay.

Plasmid constructs and transfection

We used plasmid constructs with PON1 gene 5'-flanking regions for luciferase assay, as reported previously [22]. pGL3 luciferase reporter vectors (Promega) introduced DNA fragments of PON1 genes (–587/–6) (pGL3-PON1[587/-6]) were used in the present study. The number of DNA fragments is shown from the ATG start codon because of multi-transcription sites of the PON1 genes.

We used an expression vector of the specificity protein 1 (Sp1) for mammalian cells, as reported previously [16]. We also constructed an expression vector of SREBB-2 and a mutated form at the positions of major p44/42 MAP-kinase specific phosphorylation sites, whose Ser-432 and Ser-455

were substituted with Ala (pCL-SREBP-2 S432/455A) according to previous reports [18]. The mutated construct was made by using a mutagenesis kit (QuikChange Multi Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA) with specific oligos: 5'-AGAATGTCCTTCTGAT**GCA**CCCCCAGCCTCTG-3' and 5'-GACTCTGAGCCAGGAG**CCC**CTCTATTGGATGATGC-3'. (The underlined capitals indicate the substituted sequences.)

Transient transfection into Huh7 cells was performed using a cationic lipid method employing Tfx-20 (Promega), according to the manufacturer's instructions. PON1 plasmid DNA (0.4 μg per well) was cotransfected with the pRL-TK vector (Promega) (0.2 μg per well), which expressed Renilla luciferase as an internal control. Cell extracts were prepared at 24 h for the luciferase activity assay. Both firefly and Renilla luciferase activities in the cell lysates were measured using the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's instructions. Promoter activities were expressed as firefly luciferase activity divided by Renilla luciferase activity. Six wells were

used for each transfection condition. Each examination was repeated at least three times, and representative results are shown.

Cell lysis and Western blotting

Huh7 cells were grown to confluence, harvested, and lysed as described previously [23]. The protein concentration was adjusted (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA). Western blotting was performed as described previously [23]. Antibodies for PON1 [8], α-tubulin (Sigma), p44/42 MAP kinase, phospho-p44/42 MAP kinase, SAPK/JNK, phospho-SPAK/JNK, p38 MAP kinase and phospho-p38 MAP kinase (all from Cell Signaling, Beverly, MA) were used. The bands were made visible by enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Arlington Heights, IL). Each experiment was repeated at least three times, and representative results are shown.

Preparation of nuclear extracts and electrophoretic mobility shift assay

(EMSA)

Huh7 cells were grown to confluence and harvested, and the nuclear fraction was isolated and extracted as described previously [16, 23]. EMSA was performed as described previously [16, 23]. The synthetic sense and antisense strands of oligonucleotides (-187/-159) were 5'-GGTGGGGGCTGACCGCAAGCCGCGC-3' and 5'-GGCGCGGCTTGCGGTCAGCCCCCAC-3', respectively. For a supershift study, Sp1-specific polyclonal antibody (PEP2) (Santa Cruz Biotechnology, Santa Cruz, CA) was used. The dried gel was analyzed by a computerized system for radioluminography (BAS2500, Fuji Photo Film, Kanagawa, Japan) and for analyzing software (MacBAS version 2.3, Fuji Photo Film). The intensities of bands were compared by using the software. Each experiment was repeated at least three times, and representative results are shown.

Statistical analysis

Comparisons for two groups were performed using the Tukey–Kramer test.

Statistical differences among three groups or more were determined by analysis of variance (ANOVA). *P* values <0.05 were considered statistically significant.

RESULTS

Effects of pitavastatin on PON1 promoter activity and PON1 protein expression

We previously reported that pitavastatin enhanced the promoter activity of PON1 gene in HepG2 cells using a reporter gene assay method and that we could detect PON1 mRNA but not PON1 protein in HepG2 cells [22]. Huh7 is another human hepatoma cell line and expresses PON1 protein. Consequently, in the present study we examined the effects of pitavastatin on PON1 promoter activity and PON1 protein expression in Huh7 cells. PON1 promoter activity was significantly increased dose-dependently by pitavastatin from 5 to 50 μM (relative promoter activity; 1.0 ± 0.1 at pitavastatin 0 μM, 1.6 ± 0.1 at 5 μM, 1.6 ± 0.1 at 10 μM, 1.7 ± 0.1 at 20 μM and 1.9 ± 0.1 at 50 μM). PON1 protein expression was increased dose-dependently by pitavastatin from 0.1 to 10 μM in Huh7 cells after 24 h.

Effects of pitavastatin on MAP kinase signaling cascades

We examined the effects of pitavastatin on the activation of p44/42 MAP kinase signaling cascade, using immunoblotting in Huh7 cells through 120 min (Fig 1A). Pitavastatin 10 μM phosphorylated p44/42 MAP kinase after 5 min, and this effect was bolstered for 60 min. Pitavastatin 0.1–20 μM dose-dependently effected on the phosphorylation of p44/42 MAP kinase in 60 min incubation (Fig 1B). On the other hand, 10 μM pitavastatin affected neither the phosphorylation of SAPK/JNK nor p38 MAP kinase (Fig 1C and 1D).

Role of MAP kinase signaling cascades in pitavastatin-enhanced PON1 promoter activation

We examined which pathway in the MAP kinase signaling cascades was involved in pitavastatin-induced PON1 promoter activation in Huh7 cells. Each specific inhibitor of MAP kinase signaling cascades was added to cell cultures 2 h after plasmid transfection. PON1 promoter activity was measured at 24 h after treatment. Both 10 μM PD98059 (MAP kinase kinase I inhibitor) and 10 μM U0126 (MAP kinase kinase inhibitor) attenuated the pitavastatin-induced

increase in promoter activity (Fig 2A and 2B). Neither SP600125 (SAPK/JNK inhibitor) nor SB203580 (p38 MAP kinase inhibitor) abolish the pitavastatin-induced increase in promoter activity (Fig 2C and 2D).

Role of p44/42 MAP kinase signaling cascade in pitavastatin-enhanced PON1 protein expression

Because the phosphorylation of p44/42 MAP kinase was related to the pitavastatin-induced activation of PON1 promoter, we studied whether the pitavastatin-induced phosphorylation of p44/42 MAP kinase was an influence on the PON1 protein expression in Huh7 cells (Fig 3). The PON1 protein expression in Huh7 cells was significantly increased by 10 μM pitavastatin (p<0.05), though 5μM PD98059 abolished this effect after 24 h (Fig 3A and 3B).

Role of Sp1 in pitavastatin-enhanced PON1 promoter activation

We examined the relationship of Sp1 to PON1 promoter activity in Huh7 cells. An overexpression of Sp1 significantly increased the PON1 promoter

activity in Huh7 cells (relative promoter activity; 17.8 ± 0.5 (control 1.0 ± 0.4, $p<0.0001$)). However, 50 nM mithramycin, an Sp1 inhibitor, almost attenuated pitavastatin-enhanced PON1 promoter activity.

Role of SREBP-2 phosphorylation in pitavastatin-enhanced PON1 promoter activation

Next we studied the relationship between SREPB-2 phosphorylation and pitavastatin-enhanced PON1 promoter activation in Huh7 cells. The construct with PON1 promoter was transfected, and the mutated SREBP-2 expression plasmid (pCL-SREBP-2 S432/455A) or an empty vector was simultaneously cotransfected. Pitavastatin-enhanced PON1 promoter activity was attenuated by cotransfection with PCL-SREBP-2 S432/455A (Fig 4).

Role of p44/42 MAP kinase signaling cascade in Sp1 binding to PON1 DNA in Huh7 cells

Finally, we investigated whether pitavastatin-induced phosphorylation of

p44/42 MAP kinase was associated with the binding of Sp1 to DNA fragments of the PON1 gene promoter (-187/-159), since pitavastatin enhanced PON1 promoter activation through Sp1. An EMSA showed Sp1-DNA complex bands, which were attenuated following an addition of the competitors and supershifted with the addition of the anti-Sp1 antibodies (Fig 5A). Treatment with 50 μM pitavastatin increased the band intensity of Sp1-DNA complex ($p < 0.01$). Pretreatment with 10 μM PD98059 abolished the pitavastatin-increased band intensity (Fig. 5B).

DISCUSSION

In the present study, pitavastatin affected PON1 gene promoter activity and PON1 protein expression through the activation of the MAP kinase signaling cascades in Huh7 cells. To the best of our knowledge, our result of p44/42 MAP kinase activation by pitavastatin is the first report. Further, we showed that pitavastatin induced phosphorylation of p44/42 MAP kinase without affecting either phosphorylation of SAPK/JNK or p38 MAP kinase among the MAP signaling cascades.

Three major MAP kinases, which were p44/42 MAP kinase, SAPK/JNK, and p38 MAP kinase, are generally recognized as being the central elements of intracellular signaling in mammalian cells [24, 25]. Many recent reports have shown that statins cause not only activation, but also an inhibition of various MAP kinase signaling cascades [19-21, 26]. The present study shows that pitavastatin activates p44/42 MAP kinase cascade, a classical MAP kinase pathway. Some recent studies showed that statins stimulated to release vascular endothelial factor in vascular smooth muscle cells (VSMC), induced

cycloxygenase-2 gene expression in macrophages, or promoted HDL-induced prostacyclin release in VSMC, all of which were carried through the activation of p44/42 MAP kinase cascade [19-21]. On the other hand, another study of different cell systems showed that pitavastatin inhibited the activation of major MAP kinases induced by C-reactive protein [26]. The differences of both the cell types and stimulation conditions may determine the outcome of MAP kinase signaling cascades and may be responsible for incompatible function on the stimulating cells.

It has been in no reports that PON1 was controlled through MAP kinase signaling cascades. Our results showed that specific inhibitors of the p44/42 MAP kinase signaling cascade had a suppressive effect on pitavastatin-stimulated PON1 promoter activity and PON1 protein expression. On the other hand, inhibitors of SAPK/JNK or p38 MAP kinase signaling cascade did not affect pitavastatin-stimulated PON1 promoter activity. These results suggest that pitavastatin enhances PON1 promoter activity and PON1 protein expression through the phosphorylation of p44/42 MAP kinase in Huh7

cells. PON1 may be controlled through specific phospholylation of p44/42 MAP kinase in response not only to statin, but also to other extracellular stimulations that were yet detected.

SREBP-2 has constitution of the cytosolic N-terminal segment including the basic helix-loop-helix leucine zipper (bHLHZ) domain and the C-terminal regulatory segment interacting with the SREBP cleavage-activating protein. The N-terminal domain of SREBP-2 can be cleaved and translocated into the nucleus, followed by activating transcription factors that bind to sterol-responsive elements not only in the LDL receptor gene but also in many other genes coding for enzymes involved in cholesterol metabolism. Moreover, SREBP-2 has p44/42 MAP kinase specific phosphorylation sites that did not influence protein-DNA interaction [17, 18]. Consequently, we constructed two expression vectors of the mutated form of the SREBP-2 to investigate whether pitavastatin accelerated PON1 gene promoter transcription through SREBP-2 activation in Huh7 cells. One is a construct with mutated bHLHZ in the N-terminal domain of SREBP-2 (pCL-SREBP-2 L380/387/394A), and the other is a construct with

mutated p44/42 MAP kinase specific phosphorylation sites in the N-terminal domain of SREBP-2 (pCL-SREBP-2 S432/455A) [18]. Pitavastatin could not enhance PON1 promoter activity simultaneously cotransfected with pCL-SREBP-2 L380/387/394A in Huh7 cells (data not shown). Therefore we considered that pitavastatin increased PON1 promoter activity through the acceleration of SREBP-2-DNA binding. This result was consistent with a previous report [15]. On the other hand, pCL-SREBP-2 S432/455A inhibited pitavastatin-enhanced PON1 promoter activity in Huh7 cells (Fig 4). The result supposed pitavastatin influenced PON1 promoter activity through SREBP-2 which is phosphorylated by p44/42 MAP kinase. The phosphorylations at S432/455A of SREBP-2 were already reported to have no influence on DNA interaction, but they increased its transactivity [18]. Therefore we suggested that pitavastatin-phosphorylated SREBP-2 through p44/42 MAP kinase might influence other transcription factors or cofactors that are important for the function of SREBP-2, for example Sp1, which follows by pitavastatin-increased PON1 promoter activity.

Sp1 is a ubiquitous transcription factor and also reportedly activate PON1 gene transcriptions [23, 27]. We also showed that PON1 gene promoter transcription was associated with Sp1 in this study. Moreover, EMSA results showed that the binding intensity of Sp1 to DNA fragments of PON1 promoter was increased by treatment with pitavastatin and that PD98059 attenuated pitavastatin-increased band intensity of the Sp1-DNA complex (Fig 5). These results suggest that pitavastatin activates PON1 transcription through phosphorylation of p44/42 MAP kinase, which increases Sp1-DNA binding.

In conclusion, pitavastatin may phosphorylate SREBP-2 and/or increase the binding of Sp1 to the PON1 gene promoter region through the phosphorylation of p44/42 MAP kinase, and pitavastatin increases PON1 gene transactivation and PON1 protein expression through those mechanisms.

ACKNOWLEDGMENTS

We would like to thank Kowa Corporation (Tokyo, Japan) for the provision of pitavastatin.

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FIGURE LEGENDS

Figure 1. The effect of pitavastatin on the phosphorylation of p44/42 MAP kinase, SAPK/JNK, or p38 MAP kinase in Huh7 cells.

Cultured cells were stimulated with 10 μM pitavastatin for time course studies (A, C, and D) and with various concentrations of pitavastatin at 60 min in a dose-dependent study (B).

Figure 2. Effect of specific inhibitors of MAP kinase signaling cascades on pitavastatin-enhanced PON1 promoter activation in Huh7 cells.

pGL3-PON1 (–587/–6) plasmid was transfected into Huh7 cells and treated with 50 μM pitavastatin and 10μM various specific inhibitors of MAP kinase signaling cascades PD98059 (A), U0126 (B), SP600126 (C) and SB203580 (D).

Figure 3. Effects of PD98059 on pitavastatin-increased PON1 protein expression in Huh7 cells.

Cultured cells were incubated with or without 5 μM PD98059 at 60 min after

stimulation with 10 μM pitavastatin. Immunoblotting was performed with antibody to PON1 or α-tubulin (A). Relative PON1 protein expressions (PON1/α-tubulin) were calculated (B).

Figure 4. The relationship of SREBP-2 phosphorylation to pitavastatin-enhanced PON1 promoter activation in Huh7 cells.

pGL3-PON1 (–587/–6) plasmid was transfected into Huh7 cells, and the mutated SREBP-2 expression plasmid (PCL-SREBP-2 S432/455A) or an empty vector was simultaneously cotransfected, and then each were treated with or without 50 μM pitavastatin.

Figure 5. Effect of p44/42 MAP kinase signaling cascade on the binding of Sp1 to the PON1 gene upstream (-187/-159) in Huh7 cells.

The Sp1-DNA complex band (∗) was supershifted after the addition of the anti-Sp1 antibodies (∗∗) and disappeared following an addition of competitor (A). Relative band intensities of the complexes with DNA fragments and Sp1 were

calculated (B).

Figure. 1

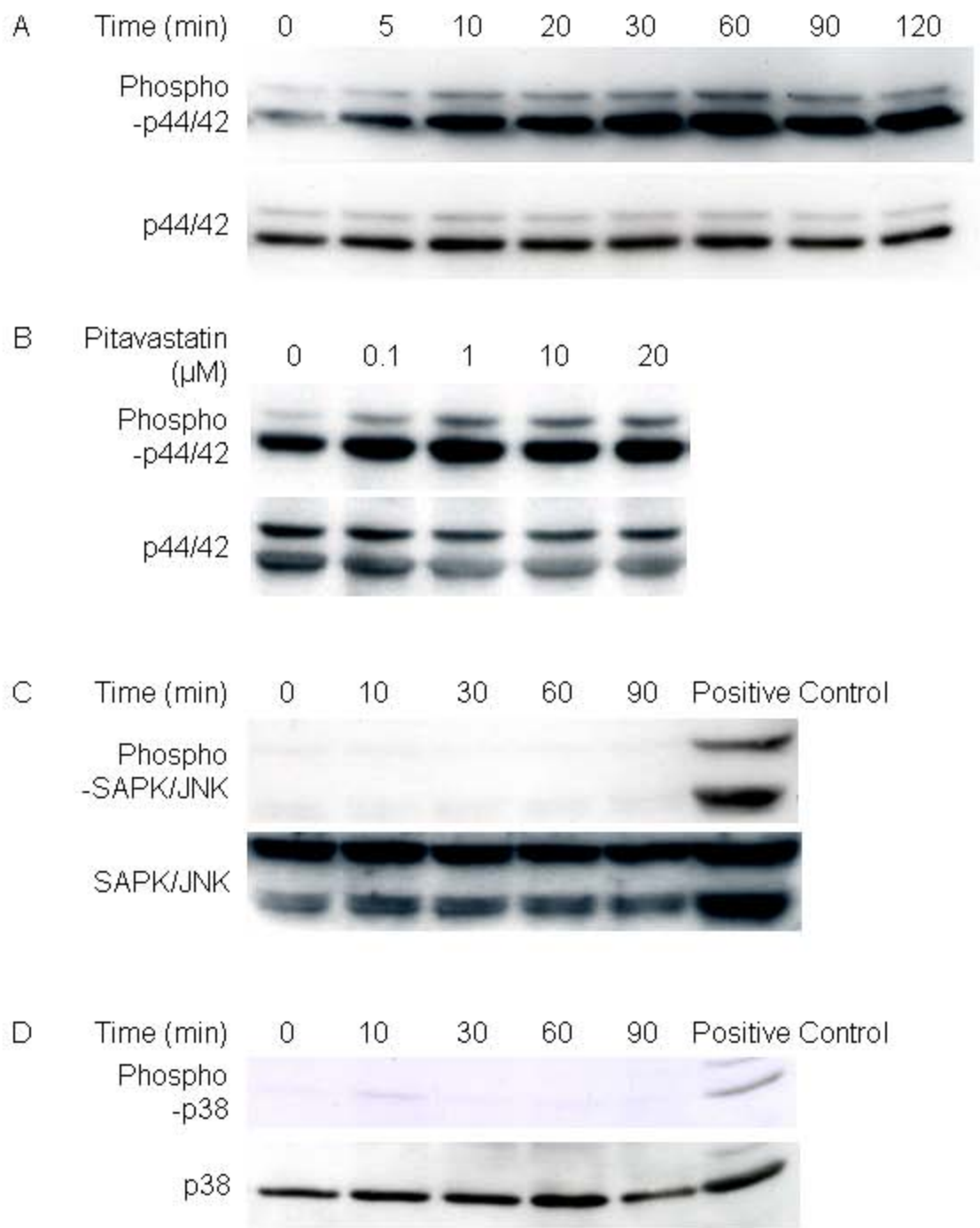

A
Time (min)
0
5
10
20
30
60
90
120
Phospho
-p44/42
p44/42
B
Pitavastatin
(μM)
0
0.1
1
10
20
Phospho
-p44/42
p44/42
C
Time (min)
0
10
30
60
90
Positive Control
Phospho
-SAPK/JNK
SAPK/JNK
D
Time (min)
0
10
30
60
90
Positive Control
Phospho
-p38
p38

Figure. 2

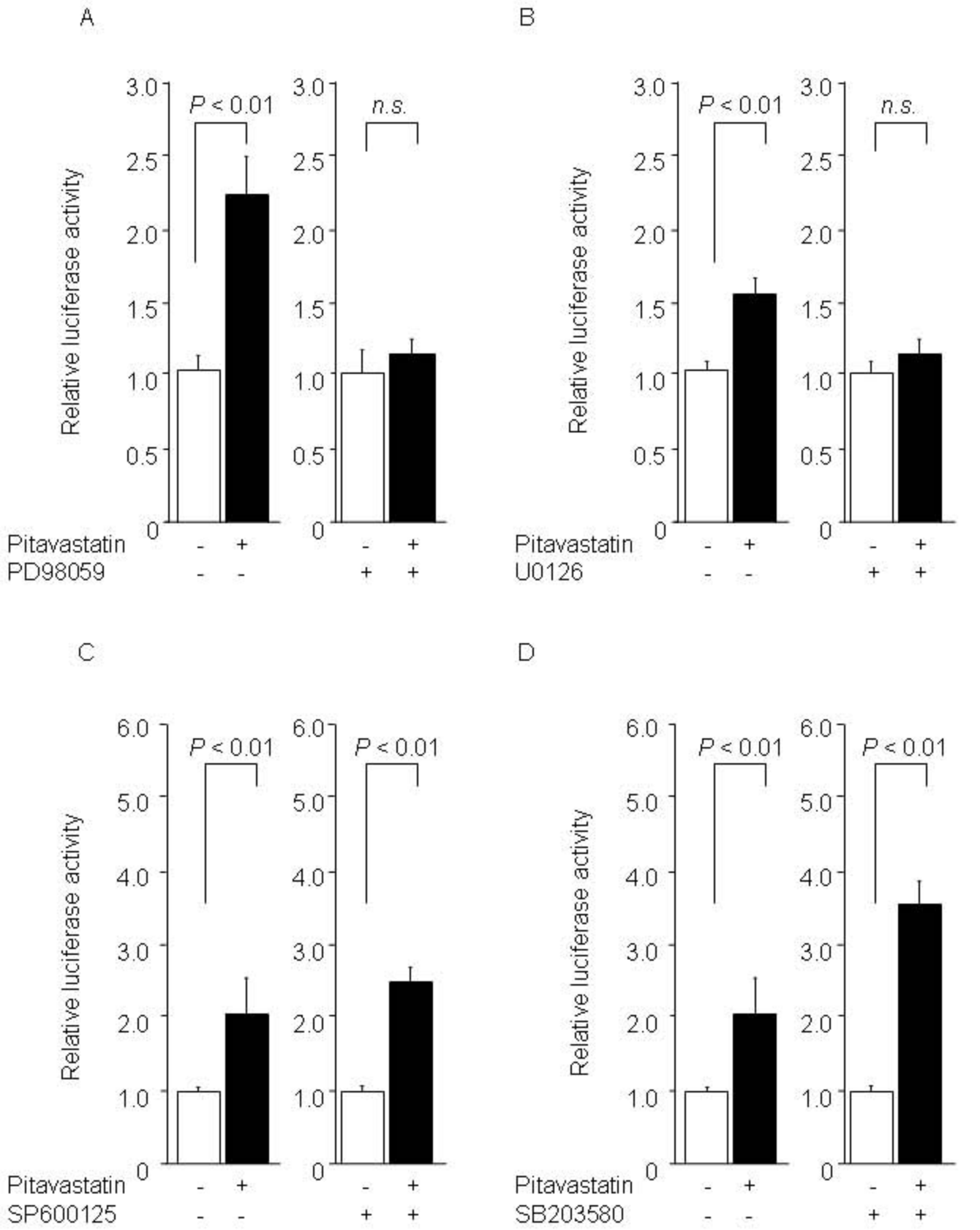
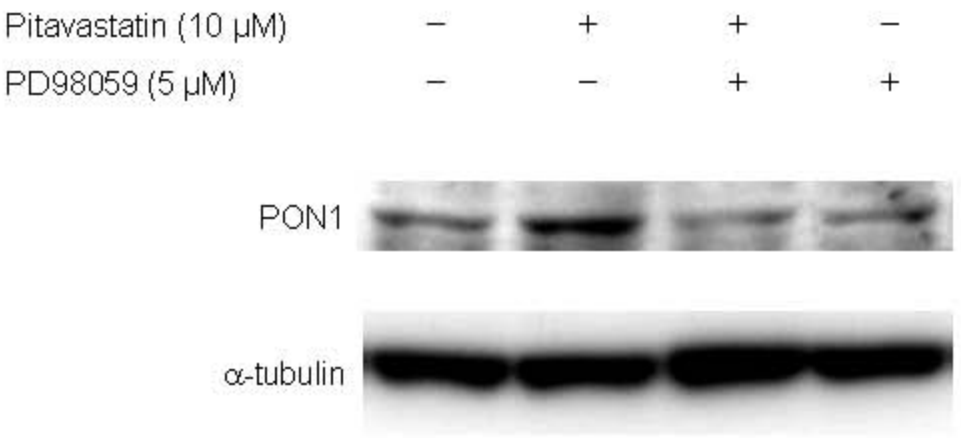

A
B
C
D
Relative luciferase activity
P < 0.01
n.s.
Pitavastatin
PD98059
U0126
SP600125
SB203580

Figure. 3

A


Pitavastatin (10 μM) − + + −
PD98059 (5 μM) − − + +
PON1
α-tubulin

B

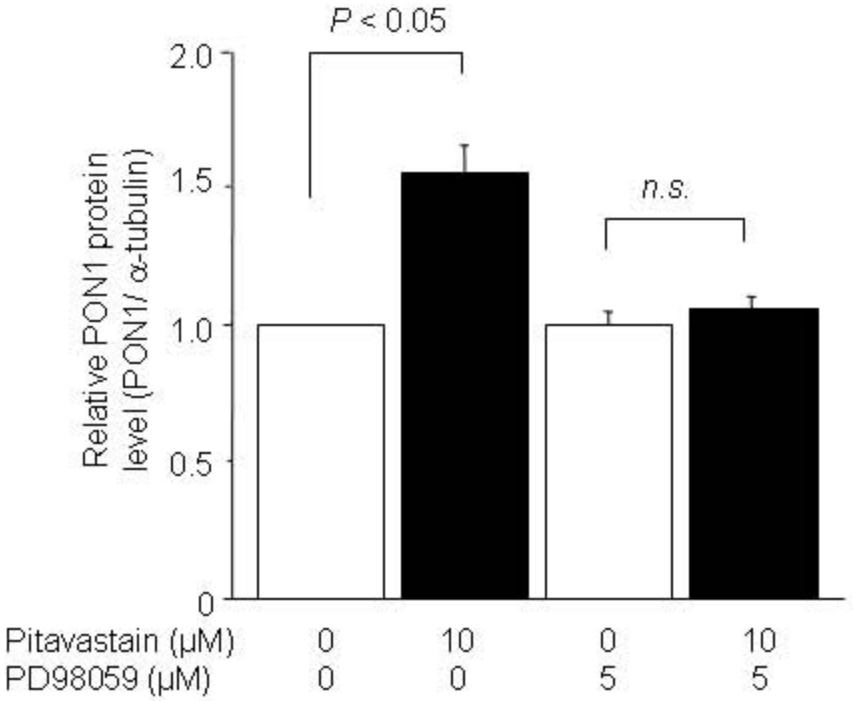

P < 0.05
n.s.
Relative PON1 protein level (PON1/ α-tubulin)
2.0
1.5
1.0
0.5
0
Pitavastain (μM) 0 10 0 10
PD98059 (μM) 0 0 5 5

Figure. 4

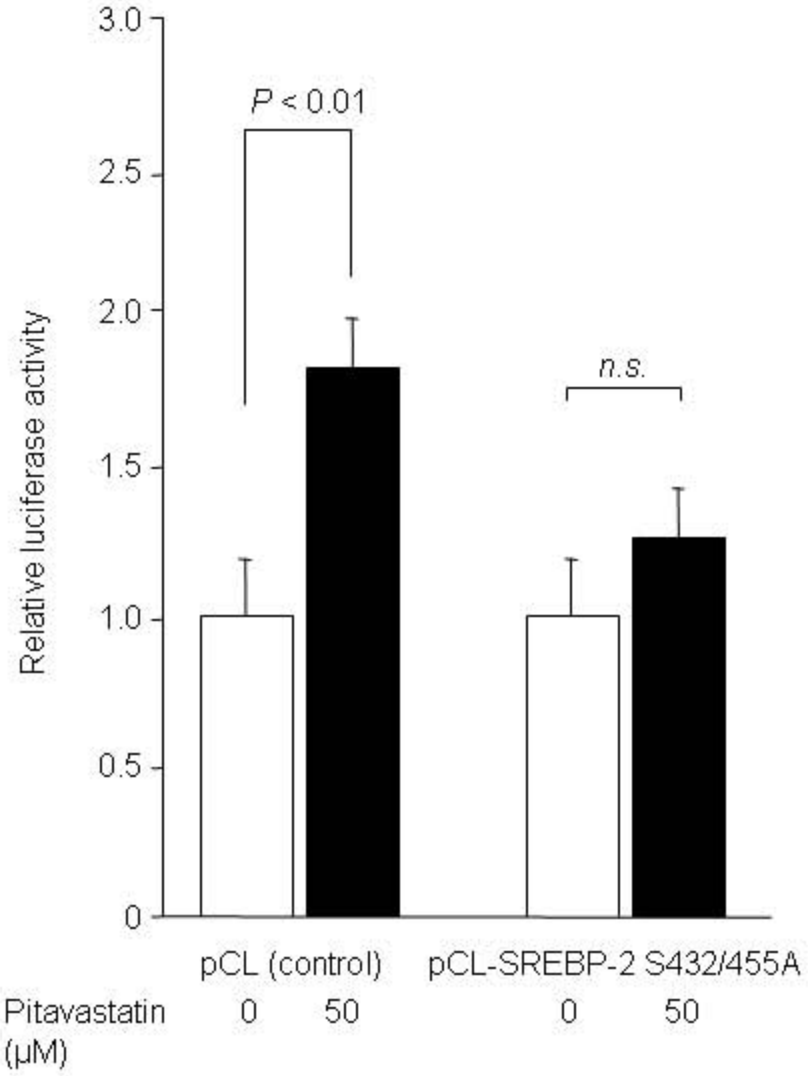
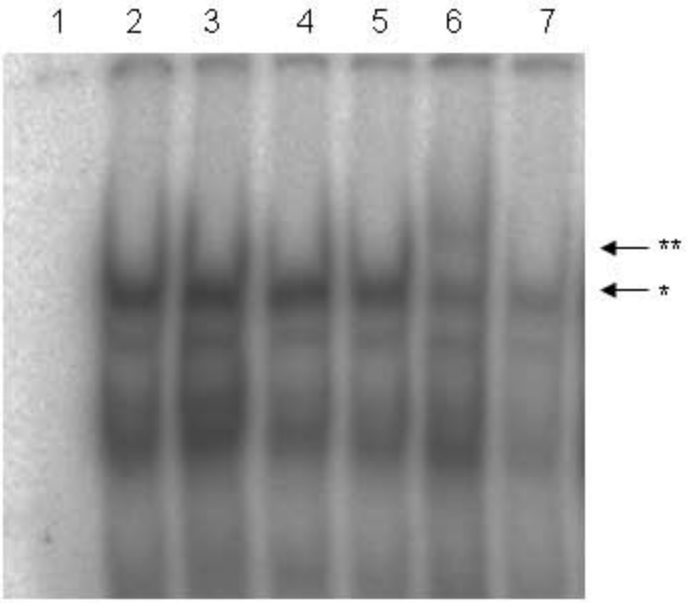

3.0
2.5
2.0
1.5
1.0
0.5
0
Relative luciferase activity
P < 0.01
n.s.
pCL (control)
pCL-SREBP-2 S432/455A
Pitavastatin (μM)
0
50
0
50

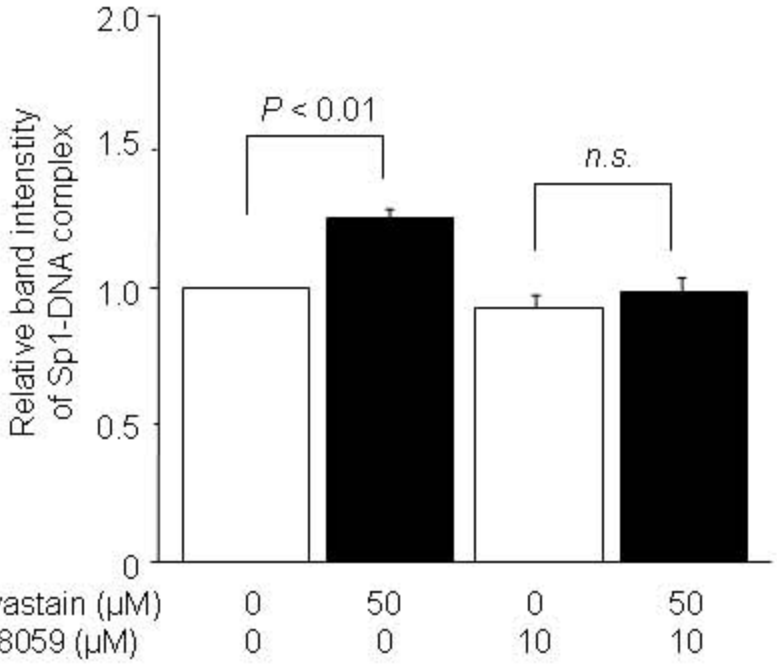
Figure. 5

A

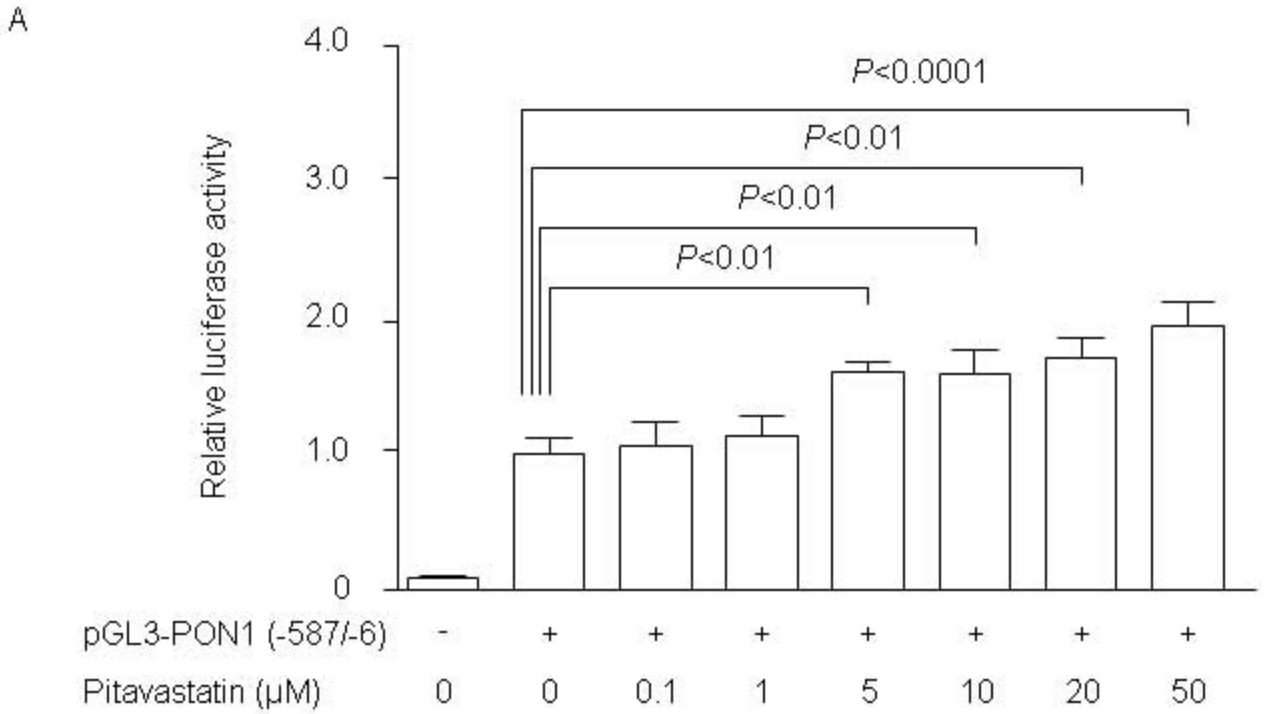


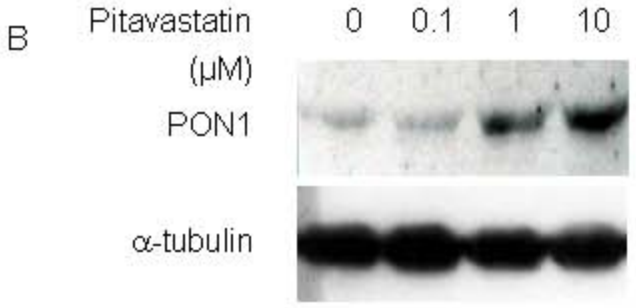
nuclear extracts	–	+	+	+	+	+	+
pitavastatin (50μM)	–	–	+	–	+	–	–
PD98059 (10μM)	–	–	–	+	+	–	–
anti-Sp1 antibody	–	–	–	–	–	+	–
Competitor	–	–	–	–	–	–	+

B



Supplemental file 1



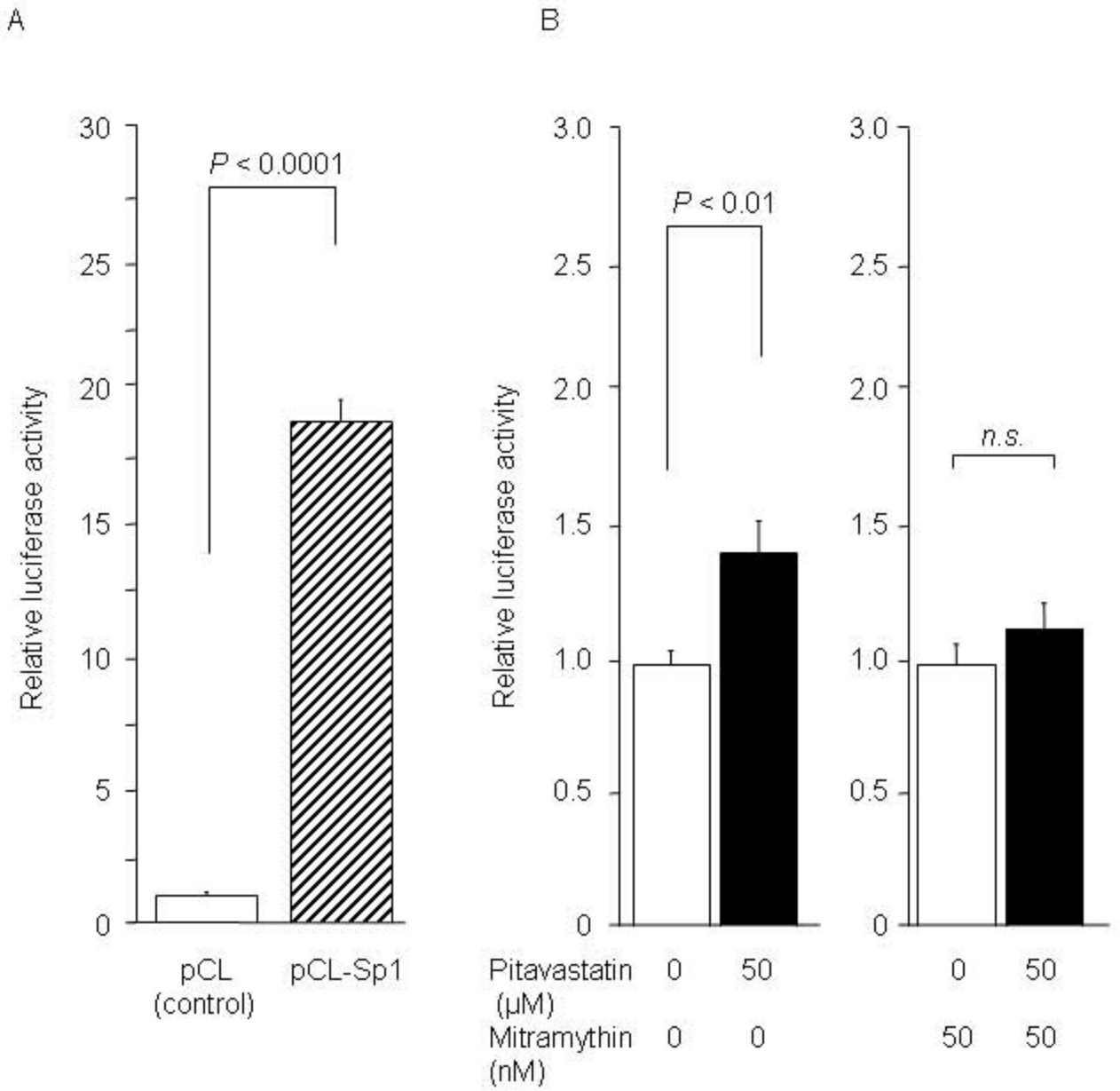


Dose-dependent effects of pitavastatin on PON1 promoter activities and PON1 protein expressions in Huh7 cells.

pGL3-PON1 (–587/–6) plasmid was transfected into Huh7 cells with various concentrations of pitavastatin, and the luciferase activity was measured at 24 h (A). Huh7 cells were stimulated with various concentrations of pitavastatin after 24 h. Aliquots of whole-cell lysate were obtained and immunoblotting was performed with the antibody to PON1 or α-tubulin (B). Internal control was evaluated by α-tubulin.

Pitavastatin 5 μM significantly increased the promoter activity ($P<0.01$), and it increased in a dose-dependent manner up to 50 μM ($P<0.0001$, one-way ANOVA). PON1 protein expression was increased dose-dependently in Huh7 cells by pitavastatin 0–10 μM after 24 h.

Supplemental file 2



Relationship of Sp1 to pitavastatin-enhanced PON1 promoter activation in Huh7 cells.

pGL3-PON1 (–587/–6) plasmid was transfected into Huh7 cells and the Sp1 expression plasmid (PCL-Sp1) or an empty vector was simultaneously cotransfected (A). pGL3-PON1 (–587/–6) plasmid was transfected and treated with 50 μM pitavastatin and/or 50 nM mithramycin, Sp1 inhibitor, at 60 min after transfection (B). Luciferase activity was measured at 24 h after transfection.

An overexpression of Sp1 significantly increased the PON1 promoter activity in Huh7 cells (p<0.0001). However, 50nM mithramycin, an Sp1 inhibitor, almost attenuated pitavastatin-enhanced PON1 promoter activity.