

Title: Role of protein kinase C in pitavastatin-induced human paraoxonase I expression in Huh7 cells

Authors: Kaoru Arii*, Tadashi Suehiro*, Yukio Ikeda*, Yoshitaka Kumon**, Mari Inoue*, Syojiro Inada*, Hiroshi Takata*, Ayako Ishibashi*, Kozo Hashimoto* and Yoshio Terada*

* Department of Endocrinology, Metabolism and Nephrology, ** Department of Laboratory Medicine, Kochi Medical School, Kochi University, Kochi, Japan.

Acknowledgments: We would like to thank Kowa Company, Ltd (Tokyo, Japan) for the provision of pitavastatin.

Conflict-of-Interest/Financial Disclosure: no conflict of Interest/financial disclosure

Corresponding author: Kaoru Arii, M.D., Ph.D.

Department of Endocrinology, Metabolism and Nephrology, Kochi Medical School, Kochi University, Kohasu, Oko-cho, Nankoku, Kochi, 783-8505, Japan

TEL: +81-88-880-2343, FAX: +81-88-880-2344

E-mail address: ariik@kochi-u.ac.jp

Abstract

Objective: We have demonstrated that pitavastatin, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, enhanced human serum paraoxonase (PON1) gene promoter activity and that protein kinase C (PKC) activated PON1 expression through Sp1, in cultured HepG2 cells. We investigated whether PKC was involved in pitavastatin-induced PON1 expression. *Methods:* PON1 gene promoter activity was assessed by a reporter gene assay using cultured Huh7 cells. PON1 protein expression and PKC activation were measured by Western blotting. The binding activity of Sp1 to the PON1 gene upstream was analyzed by electrophoretic mobility shift assay (EMSA). *Results:* Both PON1 gene promoter activity and PON1 protein expression were elevated by pitavastatin stimulation. The effects of pitavastatin on PON1 promoter activity and PON1 protein expression were attenuated by both bisindolylmaleimide IX (Ro-31-8220) and bisindolylmaleimide I. EMSA showed that pitavastatin increased the Sp1-PON1 DNA binding and this effect was attenuated by Ro-31-8220. Pitavastatin activated atypical PKC, but never conventional or

novel PKC. Myristoylated pseudosubstrate peptide inhibitor of PKC ζ abolished the pitavastatin-increased PON1 promoter activity, however calphostin C and Gö6976 (PKC inhibitors except for PKC ζ) did not influence on the promoter activity. Additionally an overexpression of dominant negative form of PKC ζ expression vector obviously decreased pitavastatin-induced PON1 promoter activation. *Conclusions:* These observations suggest that pitavastatin activates PKC, especially PKC ζ isoform, which increases the binding intensity of Sp1 to PON1 DNA promoter responsible for enhanced the transcription of PON1 gene and increased PON1 protein expression in Huh7 cells.

Introduction

Human serum paraoxonase (PON1) is an esterase, and associated with apolipoprotein A-I and J in high-density lipoprotein (HDL) [1, 2]. Previous data suggest that PON1 is a primary determinant of the antioxidant of HDL [3-5]. In PON1 knockout mice [6], atherosclerotic lesion formation was increased by feeding on a high fat and high cholesterol diet, meanwhile in PON1 transgenic mice [7], it was decreased. Mackness B et al reported that low paraoxonase activity is an independent risk factor for coronary events in male population [8]. In addition, we recent reported that PON1 concentration was related to cardiovascular mortality in patients on chronic hemodialysis [9]. These accumulating reports demonstrate that PON1 has effects against oxidative disorders and it plays an important role in the suppression of the development and progression of atherosclerosis.

The 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase inhibitors (statins) are widely prescribed to lower cholesterol levels in patients at risk of cardiovascular diseases. Recent studies show that statins have many

additional cardiovascular protective effects beyond the ability to lower serum cholesterol levels [10]. Anti-oxidant action is one of the pleiotropic effects of statins [11, 12]. We and other previously reported that statins enhanced the PON1 gene promoter activity in a human hepatocellular carcinoma cell line, HepG2 cells [13], or increased the serum PON1 concentrations and activities in patients with hypercholesterolemia [14].

We showed previously that PON1 gene promoter activity in HepG2 cells was modulated by an interaction between Sp1 and PKC [15]. Sp1 is an ubiquitous transcription factor and is well known to bind to GC-rich nucleotide sequences (GC boxes). Sp1 reportedly activates PON1 gene transcriptions [13, 16]. PKC family is a serine/threonine kinase, and divides into three classes (which consist of at least 12 isoforms); conventional PKC (PKC α , β I, β II and γ), novel PKC (PKC δ , ϵ , η , θ and μ) and atypical PKC (PKC ζ , and τ/λ). PKC play important roles in intracellular signal transduction mechanisms for hormones and growth factors, and individual isoforms have their distinct functional roles in the cells [17, 18]. Also, several investigators have reported interactions between

Sp1 and PKC, especially PKC ζ isoform, in the regulation of several gene expressions such as vascular endothelial growth factor gene, platelet-derived growth factor B-chain gene or insulin-like growth factor-II gene [19-21].

In the present study, we investigated whether the mechanism of PON1 gene promoter activation by pitavastatin was associated with PKC in cultured human hepatoma Huh7 cells *in vitro*. Here we demonstrate that pitavastatin increases PON1 gene promoter activity and PON1 protein expression and that these effects are regulated by PKC activation.

Materials and Methods

Cell culture

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 (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% CO₂. The cells were seeded into 90-mm plastic 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.

Reagents and Treatment

Pitavastatin was gifted from Kowa Company, Ltd (Tokyo, Japan). bisindolylmaleimide IX (Ro-31-8220), bisindolylmaleimide I (BIM), calphostin C and Gö6976 were all purchased from Calbiochem (La Jolla,

CA). Myristoylated pseudosubstrate peptide inhibitor of PKC ζ (MyrPKC ζ) was purchased from Biomol (Plymouth, PA). All of the above reagents were dissolved in dimethylsulfoxide (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. Before treatment with each reagent, the wells were washed twice with phosphate buffer saline, pH7.4 and then the medium was changed to fresh DMEM without fetal calf serum. Each reagent treatment was started at 120 min after transfection, and cultured cells were harvested at 24h for luciferase assay, Western blotting or electrophoretic mobility shift assay (EMSA). Pitavastatin was added at 120 min after pretreatment with each inhibitor.

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, Madison, WI) introduced DNA fragments of PON1 genes (-1230/-6)

[pGL3-PON1 (-1230/-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 constructed an expression vector of PKC ζ and mutated PKC ζ (PKC ζ DN), which had mutated form of ATP binding site in kinase domain for mammalian cells, as reported previously [15].

Transient transfection into Huh7 cells was performed using a cationic lipid method employing Tfx-20 (Promega), as reported previously [13, 15]. PON1 plasmid DNA was cotransfected with the pRL-TK vector (Promega), which expressed Renilla luciferase for an internal control. Cell extracts were prepared at 24h 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). 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, and subsequently harvested and lysed as described previously [15]. The protein concentration was adjusted (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA). Western blotting was performed as described previously [15]. First-antibodies for PON1 [23], α -tubulin (Sigma), PKC ζ (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-PKC $\alpha/\beta I/\beta II/\delta/\epsilon/\eta/\theta$ (Cell Signaling, Beverly, MA) and phospho-PKC ζ/λ (Cell Signaling) were used for blotting. Immunoreactive proteins were made visible using horseradish-peroxidase coupled secondary antibodies and ECL Plus Western Blotting Detection System (Amersham Pharmacia Biotech, Arlington Heights, IL). Each experiment was repeated at least three times, and representative results are shown.

Preparation of nuclear extracts and EMSA

Huh7 cells were grown to confluence and harvested, and the nuclear fraction was isolated and extracted as described previously [13, 15]. EMSA was

performed as described previously [13, 15]. The synthetic sense and antisense strands of oligonucleotides (-187/-159) were 5'-GGTGGGGGCTGACCGCAAGCCGCGC-3' and 5'-GGCGCGGCTTGCGGTCAGCCCCAC-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

Statistical differences among three groups or more were determined by analysis of variance (ANOVA). Comparisons for two groups were performed using the Fisher's test. *P* values less than 0.05 were considered statistically significant.

Results

Effects of PKC inhibitors on pitavastatin-enhanced PON1 promoter activity and PON1 protein expression

Pitavastatin 50 μ M significantly enhanced the promoter activity of PON1 gene in Huh7 cells (Fig 1). This result was consistent with our previous report in HepG2 cells [13]. Then we examined whether PKC pathway was involved in pitavastatin-induced PON1 promoter activation. Both 1 μ M Ro-31-8220 (a pan-PKC inhibitor) and 1 μ M BIM (a pan-PKC inhibitor) abolished pitavastatin-induced promoter activation (Fig 1A).

Next, we studied the effects of these PKC inhibitors on the PON1 protein expression in Huh7 cells. The PON1 protein expression in Huh7 cells was significantly increased by 10 μ M pitavastatin ($p < 0.01$), and both 1 μ M Ro-31-8220 and 1 μ M BIM attenuated this effect after 24h (Fig 1B).

Effect of PKC inhibitor on Sp1 binding to PON1 DNA

Since pitavastatin enhanced PON1 promoter activity through transcription

factor Sp1 [13, 15, 16], and PKC inhibitors declined pitavastatin-enhanced PON1 promoter activity and protein expression, we investigated the effect of PKC inhibitor on the binding of Sp1 to DNA fragments of the PON1 gene promoter (-187/-159). Treatment with 50 μ M pitavastatin increased the band intensity of Sp1-DNA complex ($p < 0.05$), however, pretreatment with 1 μ M Ro-31-8220 abolished the pitavastatin-increased band intensity (Fig. 2A, B). Sp1-DNA complex bands (indicated by an asterisk) were also attenuated by competitor (unlabeled DNA fragments) and supershifted by the anti-Sp1 antibodies (double asterisks) (Fig 2A).

Effect of pitavastatin on the activation of PKC isoforms

We carried out immunoblotting in order to identify which PKC isoforms were possibly participated in the regulation of PON1 gene transcription and PON1 protein expression in Huh7 cells. Pitavastatin 50 μ M phosphorylated PKC ζ/λ , meanwhile PKC α , β I, β II, δ , ϵ , η and θ were not phosphorylated by pitavastatin stimulation (Fig 3A and 3B). We calculated relative

phospho-PKC ζ/λ band intensities and demonstrated that pitavastatin 10 μ M significantly increased the band intensity after 60 min incubation ($p < 0.05$) (Figure 3C).

MyrPKC ζ , a specific inhibitor for PKC ζ but not PKC λ , 30 μ M abolished the pitavastatin-induced promoter activation (Fig 4A). However 200nM calphostin C and 1 μ M Gö6976 that were inhibitors of PKC except for atypical PKC ζ , did not influence on pitavastatin-induced PON1 promoter activation (Fig 4B and 4C).

Effect of dominant negative form of PKC ζ expression vector on pitavastatin-induced PON1 promoter activation

Finally, we studied the effect of co-transfection with dominant negative form of PKC ζ expression vector (PKC ζ DN) on pitavastatin-induced PON1 promoter activation. An overexpression of wild type PKC ζ did not influenced on pitavastatin-induced PON1 promoter activation in Huh7 cells (Fig 5A). However an overexpression of PKC ζ DN obviously decreased pitavastatin-induced PON1 promoter activation (Fig 5B).

Discussion

In the present study, pitavastatin enhanced PON1 gene promoter activity and PON1 protein expression through the activation of PKC in Huh7 cells. To the best of our knowledge, our result of PKC activation by pitavastatin is the first report. We also revealed that pitavastatin increased PON1 promoter activity through the activation of PKC ζ isoform but not other PKC isoforms.

Protein kinase is an enzyme family that phosphorylates various protein molecules and as intracellular signal transduction and metabolic modulating factor. Recently, statins were reported to have various effects through PKA, PKB, PKC or PKG [24-27]. We previously reported that PON1 gene promoter activity in HepG2 cells was regulated by PKC activation [15], and was enhanced by pitavastatin stimulation [13]. However it remained unclear whether pitavastatin-induced PON1 promoter activation was associated with PKC. To investigate the signal transduction pathway(s) involved in regulating PON1 gene transactivation in response to pitavastatin stimulation, we firstly examined the effects of chemical inhibitors of signaling intermediates on PON1 gene promoter

activity. We defined in this study that pitavastatin increased-PON1 gene promoter activity was associated with PKC activation (Fig 1). Moreover we demonstrated that PON1 protein expression in Huh7 cells was also increased by pitavastatin through PKC activation (Fig 1B).

Protein phosphorylation of transcription factor is one of the major mechanisms to regulate the binding activity of the factor to DNA either positively or negatively. It has been reported that Sp1 phosphorylation increases the capacity of Sp1 to bind DNA, and PKC, especially atypical PKC ζ isoform, plays a crucial role in Sp1 phosphorylation [19-21]. Sp1 was previous reported to activate PON1 gene transcriptions [13, 15, 16]. In the result, our EMSA showed that the binding intensity of Sp1 to DNA fragments of PON1 promoter was increased by treatment with pitavastatin and that Ro-31-8220 attenuated pitavastatin-increased band intensity of the Sp1-DNA complex (Fig 2). These results suggest that pitavastatin increases Sp1-DNA binding through activation of PKC.

We demonstrated that pitavastatin activated PKC ζ / λ isoforms, but not PKC α , β I, β II, δ , ϵ , η and θ isoforms. We previously reported that PON1 gene promoter activity in HepG2 cells was regulated by atypical PKC ζ as well as conventional PKC α [15], however we can hardly say with any finality that pitavastatin increased PON1 promoter activity through atypical PKC ζ .

Consequentially, we next set out to dissect the role of PKC ζ in pitavastatin-induced PON1 promoter activation. MyrPKC ζ , a specific PKC ζ inhibitor abolished the pitavastatin-induced PON1 promoter activation, but calphostin C and Gö6976, inhibitors of PKCs except for PKC ζ inhibition did not influence on the promoter activation (Fig 4). Additionally, PKC ζ DN abolished the pitavastatin-enhanced promoter activity (Fig 5). These observations suggest that pitavastatin-induced transcription is regulated not by classical or novel PKC but by atypical PKC ζ .

Some previous reports showed that statins influenced on the activity of various PKC isoforms. Atorvastatin inhibited PKC inhibitors-induced apoptosis of adult rat cardiac myocytes through PKC δ pathway [26]. On the other hand,

some studies in different cell systems showed that statins inhibited the activation of PKC. Ceolotto C et al. reported that pravastatin inhibited radical oxygen species production by inhibiting PKC δ in human fibroblast [28]. Maeda K et al. reported that pitavastatin suppressed the expression of PKC α , β 1 in polymorphonuclear leukocytes from hyperlipidemic guinea pig [29]. Yasunari K et al. also reported that statins have direct antimigratory effects via suppression of PKC α in human vascular smooth muscle cell [30]. The differences of those cell types and stimulation conditions may determine the outcome of PKC activation and may be responsible for incompatible function on the stimulating cells. Zhang Y et al. reported that PKC ζ was responsible for the marked Sp1 phosphorylation induced by trichostatin A in JAR cells [31]. Pal S et al. revealed that PKC ζ promoted the Sp1-mediated transcription of vascular permeability factor/vascular endothelial growth factor in human HT1080 and 786-0 cells [32]. Our observations were not inconsistent with these previous reports, consequently, we suppose that pitavastatin-activated PKC ζ may be increased Sp1-PON1 DNA binding.

Many pleiotropic effects of statins have been reported to depend on statins-induced depletion of isoprenoids in the mevalonic acid cascade. We reported that depletion of farnesyl pyrophosphate by pitavastatin was associated with pitavastatin-increased PON1 promoter activity in HepG2 cell [13]; moreover, it was recently reported that pitavastatin also induced PON1 expression through activation of the p44/42 mitogen-activated protein kinase signaling cascade in Huh7 cells [33]. To our best knowledge, it was only one report to refer the association with statins-influenced PKC activity and isoprenoids, which fluvastatin-decreased PKC activity was reversed by isoprenoids [30]. However, we determined that pitavastatin-activated PKC ζ was not reversed by supplement of isoprenoids, such mevalonic acid, farnesyl pyrophosphate and geranylgeranyl pyrophosphate in this study (data not shown). Furthermore, we could also not clarify the relationship between the p44/42 MAP kinase signaling cascade and PKC ζ in this study. That is to say, it may yet not be reasonable to presume that pitavastatin-activated PKC ζ is associated with depletion of isoprenoids in the mevalonic acid cascade and

more detailed studies are required to establish the relationship between pitavastatin-activated PKC ζ and the p44/42 MAP kinase signaling cascade. On the other hand, in contrast to our results, some statins, such as pravastatin, simvastatin and fluvastatin, have been reported previously, to decrease PON-1 expression [34]. We speculate that the differences in the type of statins and stimulation conditions may determine the outcome of the effect of statins on PON1 expression, and may explain their inconsistent actions on the Huh7 cells.

In conclusion, pitavastatin may activate atypical PKC ζ followed by increase in the binding of Sp1 to the PON1 gene promoter region, and pitavastatin enhances PON1 gene transactivation and PON1 protein expression.

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Figure Legends

Figure 1. Role of PKC in pitavastatin-enhanced PON1 promoter activity and PON1 protein expression in Huh7 cells.

(A) pGL3-PON1 (-1230/-6) plasmid was transfected into Huh7 cells and treated with PKC inhibitors; 1 μ M Ro-31-8220 or 1 μ M BIM, and with 50 μ M pitavastatin.

Each column represents mean \pm S.E.M. of data from 6 wells. (B) Cultured cells treated with 1 μ M Ro-31-8220 or 1 μ M BIM were stimulated with 10 μ M pitavastatin. Aliquots of whole-cell lysate were obtained, and immunoblotting was performed with antibody to PON1 or α -tubulin. Internal control was evaluated by α -tubulin. Relative PON1 protein expressions (PON1/ α -tubulin) were calculated. Each column represents mean \pm S.E.M. of data from 3 wells.

Figure 2. Role of PKC in the binding of Sp1 to the PON1 gene DNA fragment (-187/-159) in Huh7 cells.

(A) Lane 1, no nuclear extracts; lane 2, DNA fragments and nuclear extracts from Huh7 cells; lane 3, DNA fragments and nuclear extracts treated with 50 μ M

pitavastatin; lane 4, DNA fragments and nuclear extracts treated with 1 μ M Ro-31-8220; lane 5, DNA fragments and nuclear extracts treated with both pitavastatin and Ro-31-8220; lane 6, DNA fragments, nuclear extracts, and anti-Sp1 antibody; lane 7, DNA fragments, nuclear extracts, and competitor (unlabeled-DNA fragments). *, Sp1-DNA complex band; **, Sp1-DNA- Sp1 antibody complex band. (B) Calculated relative intensities of the Sp1-DNA complex band. Each column represents mean \pm S.E.M. of data from 3 wells.

Figure 3. Effect of pitavastatin on the activation of PKC isoforms in Huh7 cells.

Cultured cells were stimulated with 50 μ M pitavastatin. Aliquots of whole-cell lysate were obtained, and immunoblotting was performed with specific antibody for phospho-PKC ζ/λ (A) or phospho-PKC $\alpha/\beta I/\beta II/\delta/\epsilon/\eta/\theta$ (B). Antibodies for both α -tubulin and PKC ζ were used for internal control. Calculated relative intensities of the phospho-PKC ζ/λ band (phospho-PKC ζ/λ / α -Tubulin) after 1h incubation. Each column represents mean \pm S.E.M. of data from 3 wells (C).

Figure 4. Role of PKC ζ in pitavastatin-enhanced PON1 promoter activity in

Huh7 cells.

pGL3-PON1 (-1230/-6) plasmid was transfected into Huh7 cells and treated with

30 μ M MyrPKC ζ (A), 200nM calphostin C (B), 1 μ M Gö6976 (C), and with 50 μ M

pitavastatin. Each column represents mean \pm S.E.M. of data from 6 wells.

Figure 5. Effect of dominant negative form of PKC ζ on pitavastatin-induced

PON1 promoter activation in Huh7 cells.

pGL3-PON1 (-1230/-6) plasmid was transfected into Huh7 cells and the wild

type PKC ζ expression plasmid (PKC ζ), dominant negative type PKC ζ

expression plasmid (PKC ζ DN) or an empty vector (PCLneo) was

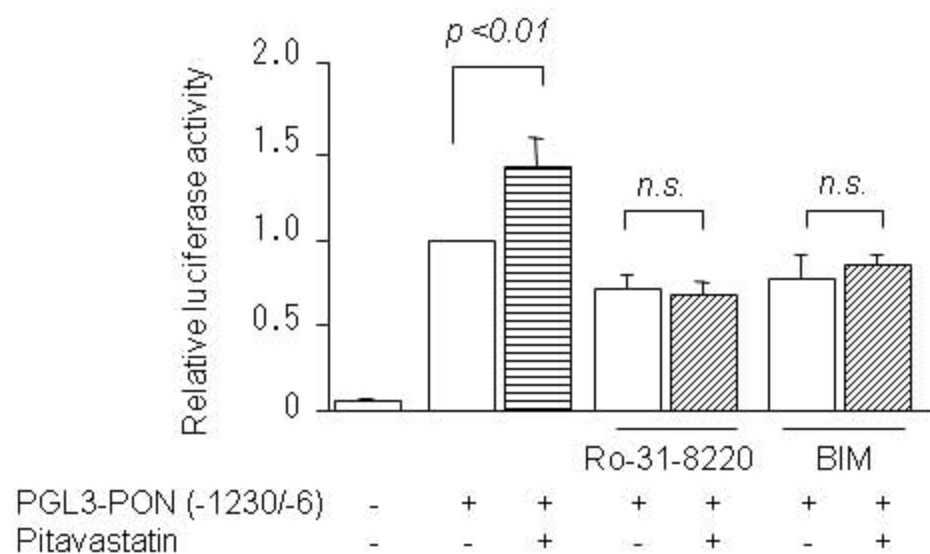
simultaneously cotransfected. The luciferase activity was measured 24hr after

50 μ M pitavastatin stimulation. Each column represents mean \pm S.E.M. of data

from 6 wells.

Figure 1

A



B

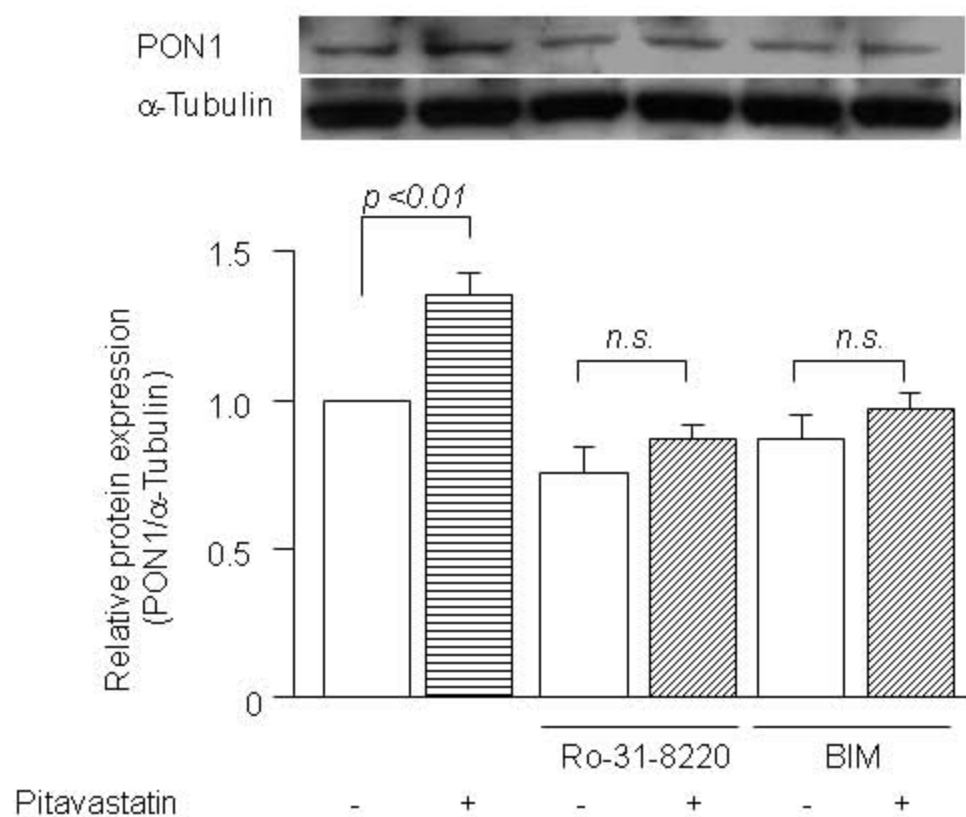
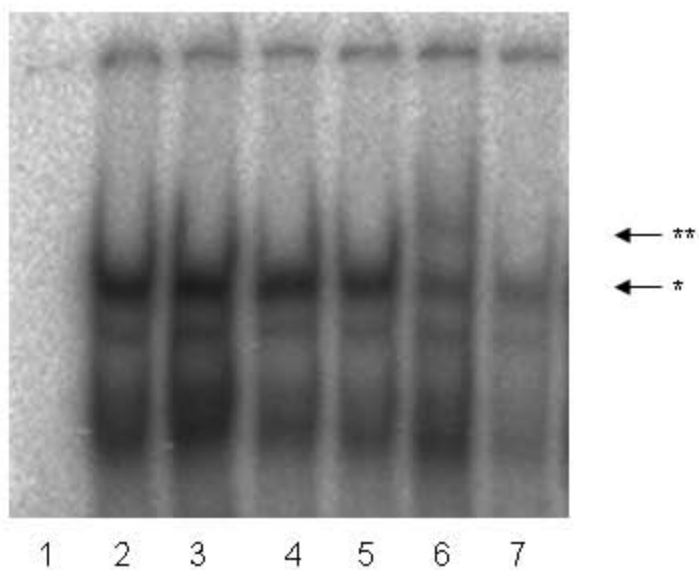


Figure 2

A

Nuclear extracts	-	+	+	+	+	+	+
Pitavastatin	-	-	+	-	+	-	-
Ro-31-8220	-	-	-	+	+	-	-
Anti-Sp1 antibody	-	-	-	-	-	+	-
Competitor	-	-	-	-	-	-	+



B

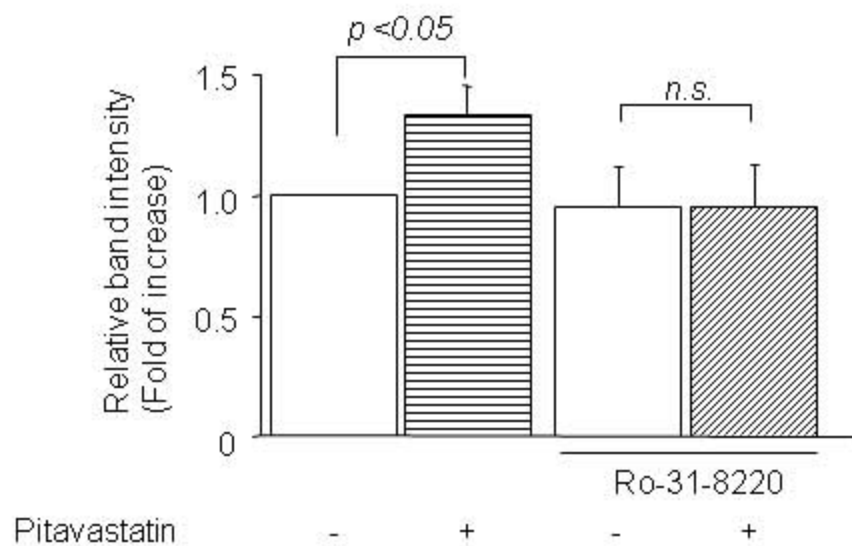


Figure 3

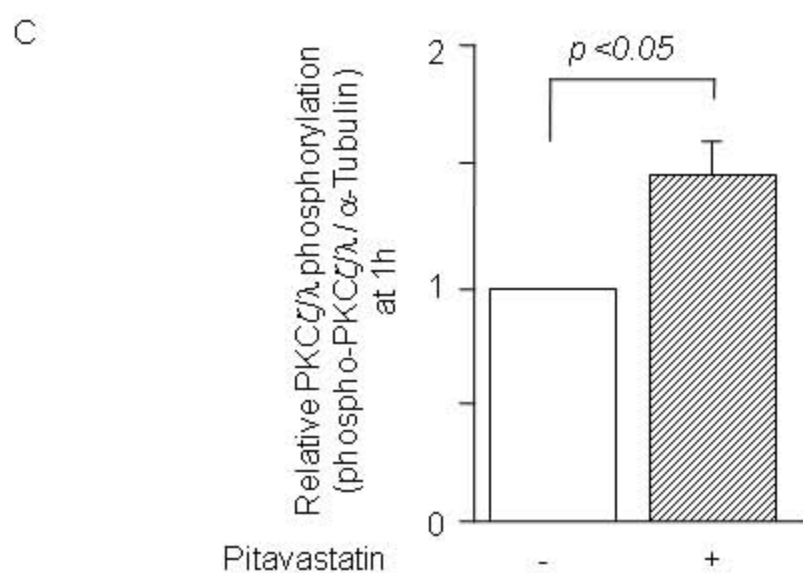
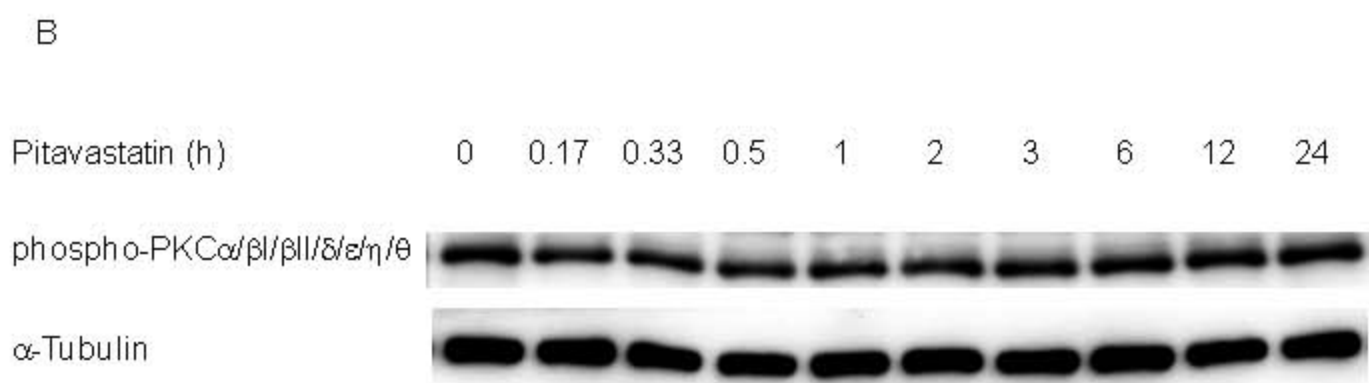
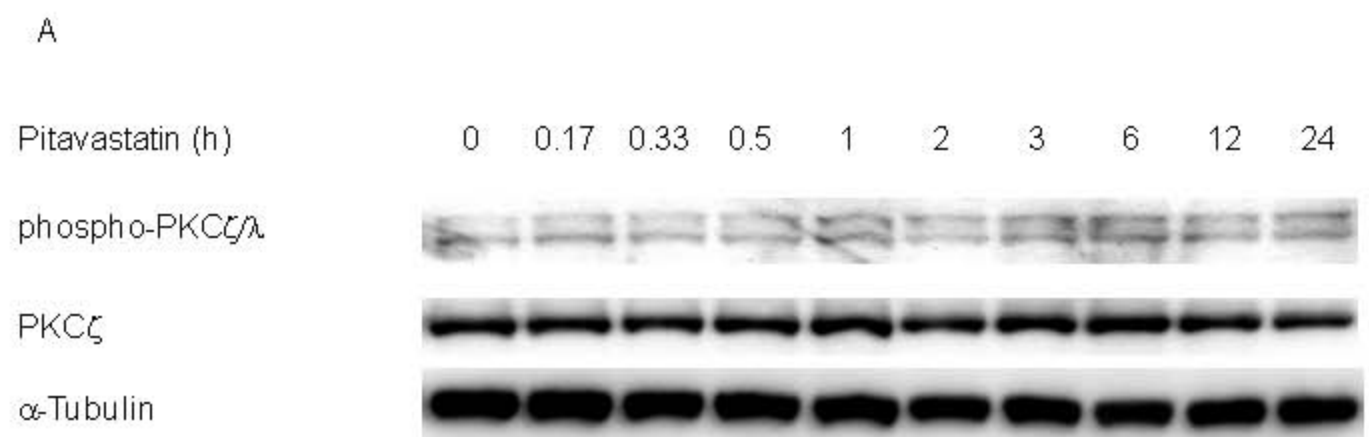


Figure 4

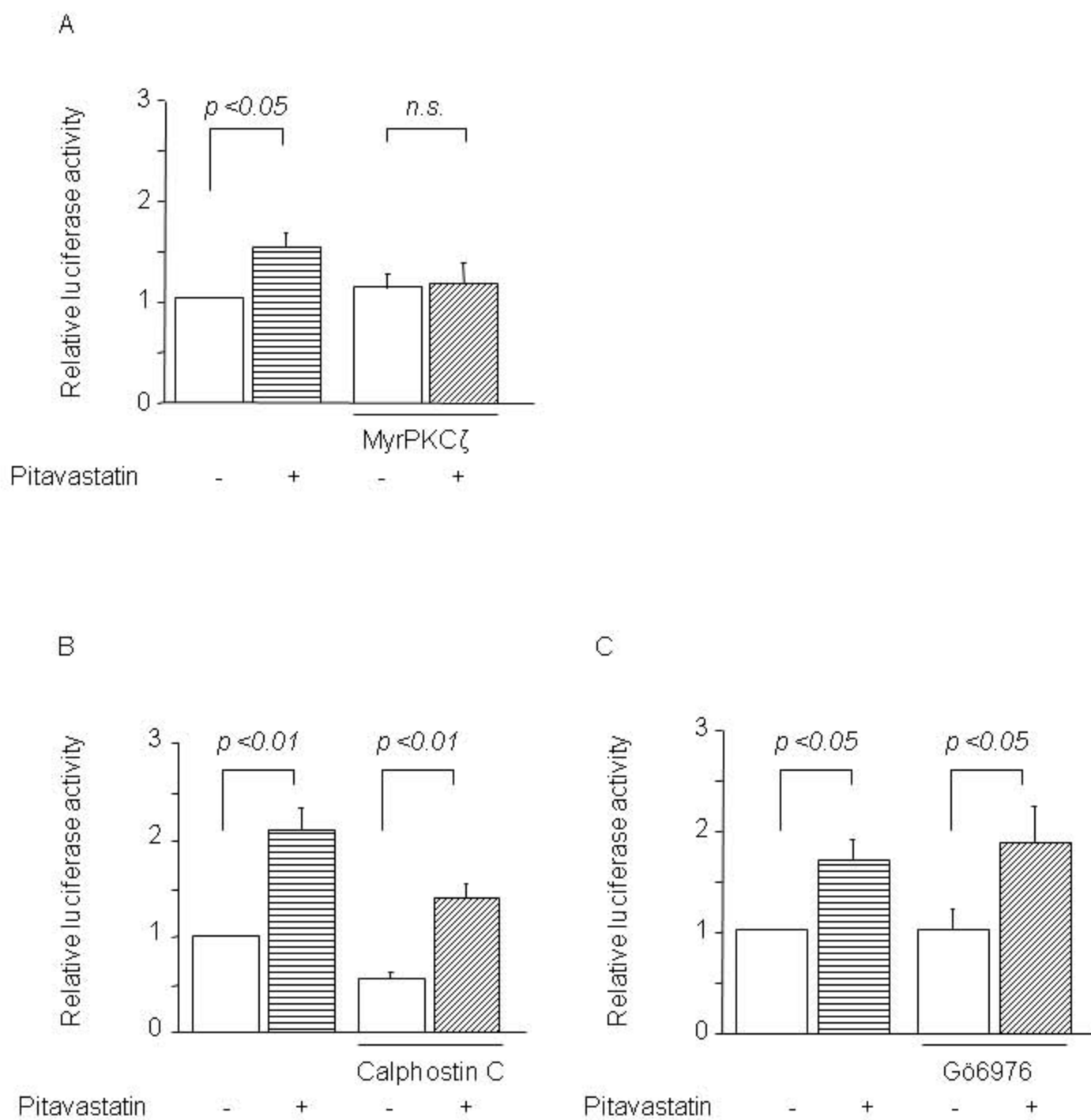
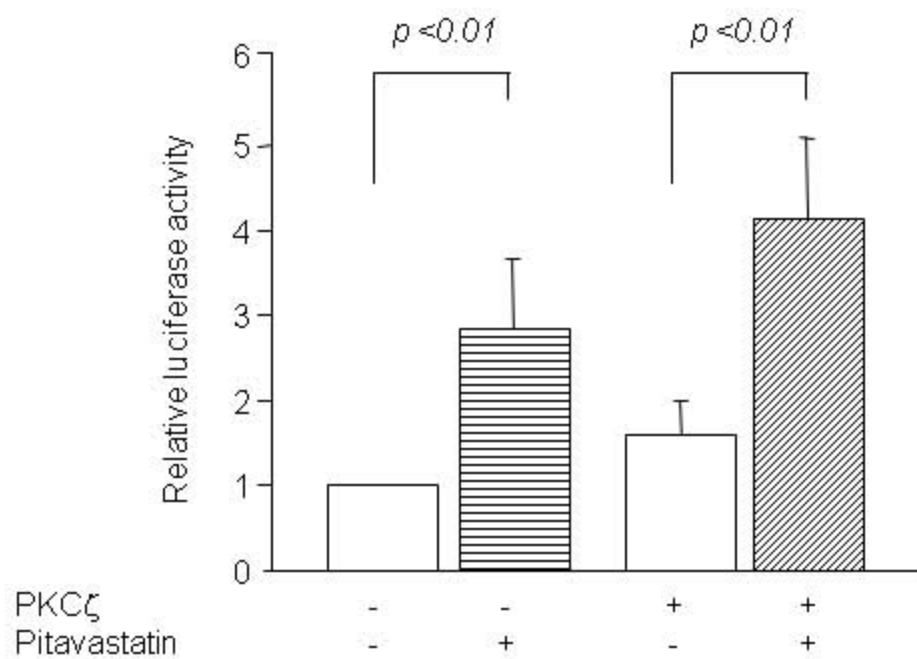


Figure 5

A



B

