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1 Regulation of multidrug resistance protein 2 (MRP2, ABCC2) expression by statins:

2 involvement of SREBP-mediated gene regulation

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ABSTRACT

Multidrug resistance protein 2 (MRP2, ABCC2) is localized to the apical membrane of hepatocytes and played an important role in the biliary excretion of a broad range of endogenous and xenobiotic compounds and drugs, such as pravastatin. However, the effects of statins on MRP2 in the liver and the precise mechanisms of their actions have been obscure. The goal of this study was to determine the regulatory molecular mechanism for statin-induced MRP2 expression in hepatocytes. *In vitro* and *in vivo* studies suggested that pitavastatin increased MRP2 expression. Pitavastatin promoted liver X receptor (LXR) α/β translocation from the cytosol to nuclei, resulting in LXR activation. Deletion and mutational analysis suggested that the potential a sterol regulatory element (SRE) played a major role in the observed modulation of MRP2 expression by pitavastatin. Furthermore pitavastatin increased the protein-DNA complex, and when SRE was mutated, stimulation of the protein-DNA complex by pitavastatin was decreased. It was demonstrated that pitavastatin upregulated MRP2 expression by an SREBP regulatory pathway in hepatocytes and that the actions of statins may lead to improve the biliary excretion of MRP2 substrates.

Keywords: Statin; hepatocytes; multidrug resistance protein 2; sterol regulatory element-binding protein

1. Introduction

HMG-CoA reductase inhibitors (statins) reduce low-density lipoprotein (LDL) cholesterol concentration through blockade of the mevalonate pathway and consequent increment of LDL receptor expression in the liver (Goldstein and Brown, 1990). Statins are the most widely used cholesterol-lowering agents for prevention of cardiovascular disease (Havel and Rapaport, 1995). Major functions of hepatocytes include sinusoidal extraction, intracellular metabolism and biliary excretion of endogenous and exogenous lipophilic compounds (Kim, 2002). Xenobiotics, unconjugated bilirubin and hormones are efficiently cleared from sinusoidal blood circulation into the liver by basolateral transporters, including OATP-C (SLCO1B1), and are also conjugated with phase II enzymes such as UDP-glucuronyltransferase (UGT1A1), sulfotransferase and glutathione transferase (Cui et al., 2001; Hagenbuch and Meier, 2004; Kullak-Ublick et al., 2004). After conjugation, the water-soluble metabolites are excreted primarily into bile by ATP-binding cassette (ABC) transporters such as multidrug resistance protein 2 (MRP2, ABCC2) (Paulusma and Oude Elferink, 1997). This transporter is localized to the apical membrane of hepatocytes and played an important role in the biliary excretion of a broad range of endogenous and xenobiotic compounds. Pravastatin, one of the statins, is a substrate of MRP2 using a double transporter (OATP1B1 and

MRP2)-expressing system (Sasaki et al., 2002). Cerivastatin is also a substrate of MRP2 (Matsushima et al., 2005). We previously reported that MRP2 expression is regulated via a cholesterol-sensing nuclear receptor liver X receptor α (LXR α)-dependent pathway (Chisaki et al., 2009). Several studies have shown that statins affect LXR activation in the liver. However, the effects of statins on MRP2 expression are obscure. It is important to investigate the effects of statins on MRP2 expression for considering the biliary excretion of MRP2 substrates.

The aim of this study was to determine the effects of statins on MRP2 expression in HepG2 cells and the rat liver.

2. Materials and methods

2.1. Chemicals

Pitavastatin Ca was kindly donated by Kowa (Tokyo, Japan). TO901317 was obtained from Cayman Chemical (Ann Arbor, MI). All other reagents were of the highest grade available and used without further purification.

2.2. Cell culture

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (Sigma Aldrich Japan, Tokyo) with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C under 5% CO₂ as described previously (Kobayashi et al., 2008). Rat primary hepatocytes were isolated by the collagenase perfusion technique as described previously with some modifications (Miyazaki et al., 1998). Collagen-coated plates were prepared by using 50 µg/mL collagen solution. The plates were allowed to dry in a laminar flow cabinet for 1 h. Isolated primary hepatocytes were plated onto the collagen-coated plates in William's E medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and left to attach for 6 h in an incubator (at 37°C under 5% CO₂). The density of isolated primary hepatocytes was 2.0×10^6 cells/well. A minimum of two animal perfusions were used in

the study.

2.3. *Animals*

Male Wistar rats, aged 7 to 8 weeks (265-400 g in weight), were obtained from Jla (Tokyo, Japan). The housing conditions were described previously (Kobayashi et al., 2008). The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the “Guide for the Care and Use of Laboratory Animals”. The dosage of statins suspended with methylcellulose was 30 mg/kg for rats as described previously (Kobayashi et al., 2011). Rats were starved for 24 hours after administration and euthanized.

2.4. *Quantitative real-time PCR*

Quantitative real-time PCR was performed using an Mx3000TM Real-time PCR System with Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. PCR was performed using human MRP2-specific primers through 40 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 30 s or using human GAPDH-specific primers after pre-incubation at 95°C for 15 min. The primers specific to hMRP2 and hGAPDH were designed on the basis of sequences

in the GenBankTM database (accession no.: NM_000392 and NM_002046). The sequences of the specific primers were as follows: the sense sequence was 5'-ACA GAG GCT GGT GGC AAC C-3' and the antisense sequence was 5'-ACC ATT ACC TTG TCA CTG TCC ATG A-3' for hMRP2, and the sense sequence was 5'-AAG GTC ATC CCT GAG CTG AA-3' and the antisense sequence was 5'-TTC TAG ACG GCA GGT CAG GT-3' for hGAPDH. PCR was performed using rat Mrp2-specific primers through 40 cycles of 94°C for 15 s, 52°C for 30 s and 72°C for 30 s or using rat Gapdh-specific primers after pre-incubation at 95°C for 15 min. The primers specific to rMrp2 and rGapdh were designed on the basis of sequences in the GenBankTM database (accession no.: NM_012833 and AF106860). The sequences of the specific primers were as follows: the sense sequence was 5'-TGA TCG GTT TCG TGA AGA GCT-3' and the antisense sequence was 5'-ACG CAC ATT CCC AAC ACA AA-3' for rMrp2, and the sense sequence was 5'-ATG GGA AGC TGG TCA TCA AC-3' and the antisense sequence was 5'-GTG GTT CAC ACC CAT CAC AA-3' for rGapdh. The PCR products were normalized to amplified GAPDH, which was the internal reference gene (housekeeping gene).

2.5. Western blot analysis

Western blot analysis was performed as described previously (Kobayashi et al., 2006). HepG2 cells were seeded on 6-well plastic plates. Following cell attachment (24 h), various concentrations of statins were added for 24 h. Total protein extracts were prepared from the cells. The cells were scraped and centrifuged at 1,300 g for 1 min at 4°C. The pellet was suspended in 1 mL of PBS and centrifuged at 1,300 g for 1 min at 4°C. The resulting pellet was suspended in 100 µL of lysis buffer containing 1% Triton X-100, 0.1% SDS and 4.5 M urea. The suspension was allowed to stand for 5 min and was sonicated for 15 min at 4°C. The suspension was then centrifuged at 12,000 g for 15 min at 4°C. The liver crude membrane was used for Western blot analysis (Johnson et al., 2006). The protein concentration in the clear supernatant was determined by the method of Lowry et al. (Lowry et al., 1951). The samples were denatured at 100°C for 3 min in a loading buffer containing 0.1 M Tris-HCl, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% BPB and 9 M urea and separated on 4.5% stacking and 10% SDS polyacrylamide gels. Proteins were transferred electrophoretically onto nitrocellulose membranes at 15 V for 90 min. The membranes were blocked with PBS containing 0.05% Tween 20 (PBS/T) and 10% non-fat dry milk for 1 h at room temperature. After being washed with PBS/T, the membranes were incubated with mouse monoclonal antibody to MRP2 (Abcam, Cambridge, UK) and mouse anti-actin monoclonal antibody

(Chemicon, Temecula, CA) for 24 h at room temperature and washed three times with PBS/T for 10 min each time. The membranes were subsequently incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:2000 or 1:4000 and washed three times with PBS/T for 10 min each time. The bands were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Biosciences Corp., Piscataway, NJ).

2.6. Immunohistochemistry

HepG2 cells were washed once with PBS and fixed with methanol for 5 min or 10% formaldehyde for 15 min. After the fixed cells had been washed three times with PBS, 10% FBS was added and the cells were incubated for 1 h at room temperature. The cells were then treated with LXR α/β (C-19), SREBP-1 (C-20) and SREBP-2 (H-164) (Santa Cruz Biotechnology, Santa Cruz, CA) for 24 h at room temperature. Then the cells were washed three times with PBS and treated with donkey anti-goat IgG-FITC or anti-rabbit IgG-FITC secondary antibody (Santa Cruz Biotechnology,

Santa Cruz, CA) at a dilution of 1:400. Nuclei were stained with DAPI. The samples were then visualized by using a confocal microscope (Zeiss LSM-510; Carl Zeiss Inc., Thornwood, NY).

2.7. Reporter plasmid construction

The human MRP2 promoter (-1200/+15) was amplified by PCR from human genomic DNA and then subcloned into a pGL3-basic vector (Promega, Madison, WI). Four 5'-deletion fragments (-701/+15, -427/+15 and -85/+15) were generated from -1200/+15 by the PCR amplification method and subcloned into a pGL3-basic vector. Four different forward primers each contained an internal site for Xho I restriction enzyme. The primer sequences were primer-1, 5'-CTA CTC GAG CTC CCA CAT TCT GGA TTT TGA-3'; primer-2, 5'- CAA CTC GAG ATG ATG GCA ACA CTG CAC TC -3'; primer-3, 5'- TAA CTC GAG GGC TCA CAC TGG ATA AGC TAT TTT -3'; and primer-4, 5'-GGT CTC GAG CCC TGT CCC TAG GGC TTT T-3'. In all cases, the sequence of the reverse primer, 5'-ACG AAG CTT ATG ACC TTT CAT CCC AAC CA-3', contained a site for HindIII enzyme. Vectors of the SRE mutant were generated from -1200/+15 by megaprimer PCR methods using forward and reverse primers for the MRP2 promoter and primers with a sense sequence of 5'-TAA CTC GAG GGC TCA

CAC TGG ATA AGC TAT TTT ATA CGC TGA CTT CTT CAA AGA A-3' for SRE

mutation. All PCR products and deletion constructs for reporter assays were sequenced using an ABI PRISM 3100 Genetic Analyzer with a BigDye Terminator v3.1 cycle sequencing kit according to the manufacturer's instructions.

2.8. Transient transfection and luciferase assay

HepG2 cells (1.0×10^5 cells/well) were seeded into 24-well plates and transfected while still in suspension with one of the MRP2 promoter-luciferase constructs using Lipofectin reagent (Invitrogen, Carlsbad, CA). Each well was transfected with 400 ng of pGL3-MRP2 promoter construct encoding a modified firefly luciferase gene (or empty pGL3 vector). After transfection, cells were transferred to a fresh medium supplemented with TO901317 or pitavastatin. Luciferase activity was measured by a luminometer according to the manufacturer's instructions using an assay kit from Promega. Relative firefly luciferase activities were normalized with renilla luciferase activities.

2.9. Electrophoretic mobility-shift assay (EMSA)

Probes for the gel shift assays were labeled with [γ - 32 P] ATP and T4 polynucleotide kinase. Labeled double-stranded oligonucleotides were prepared by

195 mixing the complementary single-stranded DNA that had been heated to 95 °C for 5
196 min. The labeled probe (30,000 cpm) was incubated with hepatic nuclear extract in a
197 mixture containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 1 mM EDTA,
198 5% glycerol, and 0.05 mg/mL poly (dI-dC) for 30 min on ice. The DNA-protein
199 complexes were resolved on a 5% PAGE gel at 100 V for 2 h. The gels were dried and
200 visualized by exposure to x-ray film.

202 2.10. Statistical analyses

203 Student's t-test was used to determine the significance of differences between two
204 group means. Statistical significance among means of more than two groups was
205 determined by one-way analysis of variance (ANOVA). Statistical significance was
206 defined as $p < 0.05$.

3. Results and Discussion

3.1. Effects of statins on expression of MRP2 in hepatocytes

First, we examined the alterations in MRP2 expression induced by atorvastatin and pitavastatin in HepG2 cells. Pitavastatin up-regulated MRP2 mRNA and protein level (Fig. 1A, C). To determine whether pitavastatin regulates MRP2 expression *in vivo*, statins were given to rats as described previously (Kobayashi et al., 2011). Pitavastatin significantly increased Mrp2 mRNA and protein level in the rat liver (Fig. 1B, D). Next, we examined the location of induced MRP2 molecule by statins in HepG2 cells using immunostaining. Pitavastatin increased MRP2 expression at the plasma membrane compared to that of non-treated or atorvastatin-treated cells (data not shown). **These results suggest that pitavastatin increases MRP2 expression in hepatocytes and the MRP2 expression alteration may improve the transport function.** Further investigations to measure this function using MRP2 over-expressed cells are in progress.

3.2. Involvement of LXR in MRP2 expression in hepatocytes

To clarify the mechanism by which pitavastatin induces alteration in MRP2 expression, we focused on liver X receptors (LXRs), members of the nuclear hormone

226 receptor superfamily. We previously reported that LXR α activation induced expression
227 of MRP2 in HepG2 cells and the rat liver (Chisaki et al., 2009). LXRs are members of
228 the nuclear hormone receptor superfamily represented by two subtypes, LXR α and
229 LXR β (Shinar et al., 1994; Willy et al., 1995; Miyata et al., 1996). Previous studies have
230 demonstrated that the effects of statins on ABCA1 regulated by LXR are biphasic:
231 suppression through reduction of oxysterols (ligands for LXR) and enhancement
232 through reduction of geranylgeranyl pyrophosphate (suppression factor of PPARs) in
233 macrophages (Argmann et al., 2005). Therefore, we investigated whether pitavastatin
234 activates LXR in HepG2 cells. We used TO901317, an LXR synthetic agonist, as a
235 positive control in this study. As shown in Fig. 2A, TO901317 promoted LXR α/β
236 translocation from the cytosol to nuclei, and pitavastatin also accelerated LXR α/β
237 transfer to nuclei. Next, an MRP2 promoter-luciferase construct was transiently
238 transfected into HepG2 cells and luciferase activity was measured after the addition of
239 TO901317. There was clearly an increase in luciferase activity from the MRP2
240 promoter construct by LXR activation. To confirm that LXR activation is associated
241 with MRP2 expression, we examined the effect of LXR α siRNA on alteration in MRP2
242 expression induced by pitavastatin. Pitavastatin-induced MRP2 expression alteration
243 was blocked by LXR α siRNA (10 nM) transfected into HepG2 cells (data not shown).

Moreover, to determine which region of the MRP2 promoter is responsible for TO901317-induced stimulation of MRP2 promoter activity, a series of MRP2 deletion constructs were transfected into HepG2 cells, and luciferase activity was measured. The full-length promoter construct (-1200/+15) exhibited approximately three-fold activation of promoter activity after TO901317 treatment compared with the control. Similarly, deletion constructs p-701/+15 and p-427/+15 showed an approximately two point five to three-fold increase after TO901317 treatment. On the other hand, deletion of nucleotides -85 to +15 significantly decreased both the basal and TO901317-induced promoter activities (Fig. 2B). These results suggest that the region between -427 and -85 is important for basal promoter activity and contains a TO901317-responsive element.

3.3. Importance of SREBP in statin-induced MRP2 expression

From deletion analysis, the region between -427 and -85 of the MRP2 promoter was found to be important for basal promoter activity and this region did not contain LXR response elements but contained a sterol regulatory element-binding proteins (SREBPs) binding site (Supplemental Fig. 1A). In the liver, SREBPs regulate the production of lipids for export into the plasma as lipoproteins and into the bile as micelles. An LXR-binding site in the SREBP-1c promoter activates SREBP-1c

transcription in the presence of LXR agonists (Repa et al., 2000). Therefore, to clarify the mechanism by which pitavastatin induces MRP2 expression, we focused on the involvement of SREBPs. As shown in Fig. 3A, the protein-DNA complex was increased in a TO901317 concentration-dependent manner and abolished by adding a 30-fold excess of non-radioactive SRE (Fig. 3B). SRE_m sequence was shown in (Supplemental Fig. 1B). Next, we examined the effect of SRE mutation, SRE_m, on the protein-DNA complex. When SRE was mutated, stimulation of the protein-DNA complex by TO901317 was decreased (Fig. 3B). These results suggest that SREBP binds to SRE in the MRP2 promoter and LXR activation promotes SRE binding ability. We examined the effects of statins on the protein-DNA complex in HepG2 cells. As shown in Fig. 4A, pitavastatin increased the protein-DNA complex. When SRE was mutated, stimulation of the protein-DNA complex by pitavastatin was decreased. Moreover, TO901317 and pitavastatin-induced stimulation of MRP2 promoter activity were also decreased (Supplemental Fig. 2). These results suggest that SREBPs play an important role in increment of MRP2 expression level induced by pitavastatin. The mammalian genome encodes three SREBP isoforms, designated SREBP-1a, SREBP-1c, and SREBP-2 (Brown and Goldstein, 1997). Therefore, to determine which SREBP isoform is involved in the increase of MRP2 expression induced by pitavastatin, we examined the

effects of TO901317 and statins on the expression of SREBPs. TO901317 and pitavastatin promoted SREBP-1 translocation from the cytosol to nuclei. On the other hand, SREBP-2 localized to the nuclei with or without TO901317 and statins (Fig. 4B). These results suggest that pitavastatin induces SREBP-1 activation and increases MRP2 expression mediated by the LXR-SREBP-1 pathway. MRP2 is a 190-kDa phosphoglycoprotein localized in the apical membrane of hepatocytes and is involved in the transport of a wide variety of organic anions such as bilirubin glucuronide and glutathione conjugates. MRP2/Mrp2 function plays a pivotal role in homeostasis of bilirubin, as shown in Dubin-Johnson Syndrome (DJS) and transport deficient (TR-) and Eisai hyperbilirubinemic rats (EHBR), all of which are caused by a congenital absence of MRP2/Mrp2 and show hyperbilirubinemia (Kartenbeck et al., 1996; Paulusma et al., 1996; Ito et al., 1997). Therefore, Hayashi et al suggest that that upregulation of MRP2 expression will improve hyperbilirubinemia in patients with DJS (Hayashi et al., 2012). Our results suggest that pitavastatin may lead to improve the biliary excretion of MRP2 substrates, such as bilirubin glucuronide.

296 **4. Conclusions**

297 In conclusion, our data demonstrated that pitavastatin upregulated MRP2
298 expression by an LXR-SREBP regulatory pathway in hepatocytes. Our findings suggest
299 that LXR and SREBP can be involved in the up-regulation of MRP2 expression in
300 hepatocytes.

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Figures Legends

Fig. 1. Effects of statins on MRP2 mRNA levels and protein expression in HepG2 cells (A, C) and the rat liver (B, D).

(A) HepG2 cells were treated with various concentrations of statins for 24 h.

The bar graphs (n=3-13) are given as means with S.D. of more than two independent experiments.

*P < 0.05 compared with vehicle control.

(B) Male Wistar rats were administered methylcellulose with or without 30 mg/kg statins for 24 h.

The bar graphs (n=3-10) are given as means with S.D. of more than two independent experiments.

*P < 0.05 compared with vehicle control.

(C) HepG2 cells were treated with 10 μ M statins for 24 h.

Data shown are typical results of the three independent experiments.

The intensity of Western blot analysis was determined by densitometry using Scion image.

(D) Male Wistar rats were administered methylcellulose with or without 30 mg/kg statins for 24 h.

Data shown are typical results of the three independent experiments.

The intensity of Western blot analysis was determined by densitometry using Scion image.

Fig. 2. Nuclear translocation of LXR α/β induced by LXR activation and pitavastatin (A) and functional analysis of MRP2 promoter in response to LXR activation (B) in HepG2 cells.

(A) HepG2 cells were treated with 10 μ M TO901317 and pitavastatin for 24 h.

Localization of LXR α/β was determined using an antibody against LXR α/β (green).

Nuclei were stained with DAPI (blue). Scale bar shows 10 μ m.

Data shown are typical results of three independent experiments.

(B) A series of MRP2 deletion promoter constructs were transfected into HepG2 cells for luciferase assay.

HepG2 cells were treated with 10 μ M TO901317.

The bar graphs (n=4-6) are given as means with S.D. of more than two independent experiments.

*P < 0.05 compared with vehicle control. N.S.: not significant.

Fig. 3. Electrophoretic mobility shift assay of nuclear extract of HepG2 cells binding to human SRE consensus sequence.

Double-stranded SRE consensus sequence (A) and SRE sequence of MRP2 promoter (B) were labeled with [γ - 32 P]-ATP.

These labeled SREs were incubated with nuclear extracts from HepG2 cells.

HepG2 cells were treated with various concentrations of TO901317 for 24 h.

Data shown are typical results of the more than two independent experiments.

Fig. 4. Nuclear extract of HepG2 cells binding to human SRE consensus sequence (A), SREBP-1 and 2 nuclear translocation (B) induced by TO901317 and statins in HepG2 cells.

(A) HepG2 cells were treated with 10 μ M TO901317 and statins for 24 h.

Data shown are typical results of the more than two independent experiments.

(B) HepG2 cells were treated with 10 μ M TO901317 and statins for 24 h. Localization of SREBP-1 and 2 was determined using an antibody against SREBP-1 and 2 (green). Nuclei were stained with DAPI (blue).

Data shown are typical results of the more than two independent experiments.

Supplemental Fig. 1. Sequence of MRP2 promoter (-427 to -85) (A) and SRE mutant

471 that was created to eliminate SREBP binding to the MRP2 promoter construct (B).

472

473 Supplemental Fig. 2. Effects of TO901317 (A) and pitavastatin (B) on SRE and SREm

474 promoter activity in HepG2 cells.

475 HepG2 cells were treated with 10 μ M TO901317 and pitavastatin for 3 and 6 hours.

476 The bar graphs (n=4-9) are given as means with S.D. of more than two independent

477 experiments.

478 *P < 0.05 compared with vehicle control. N.S.: not significant.

Fig. 1

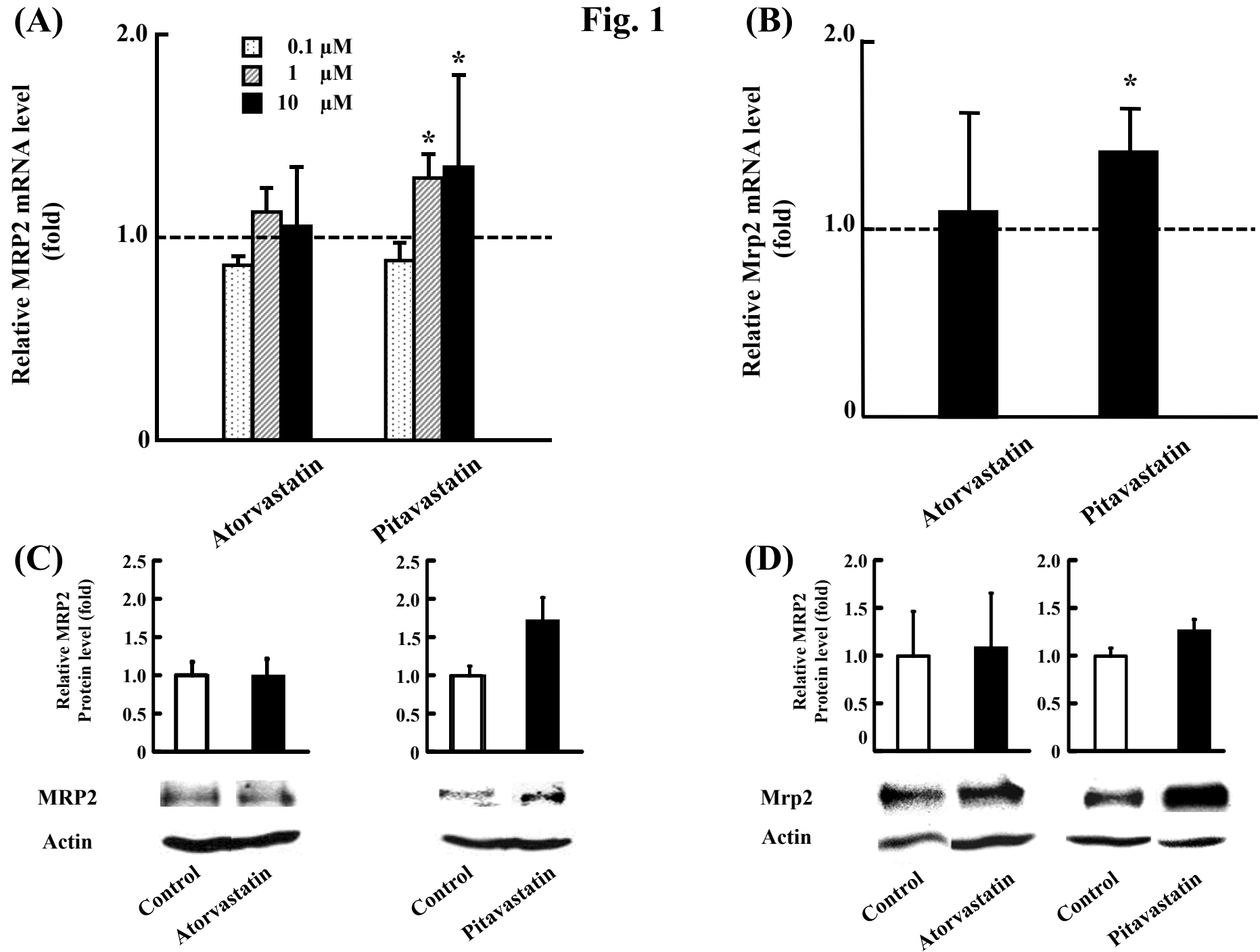
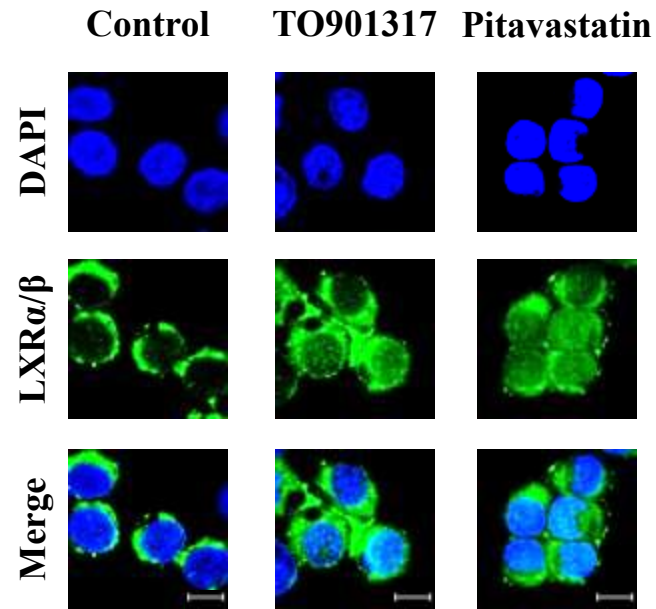


Fig. 2

(A)



(B)

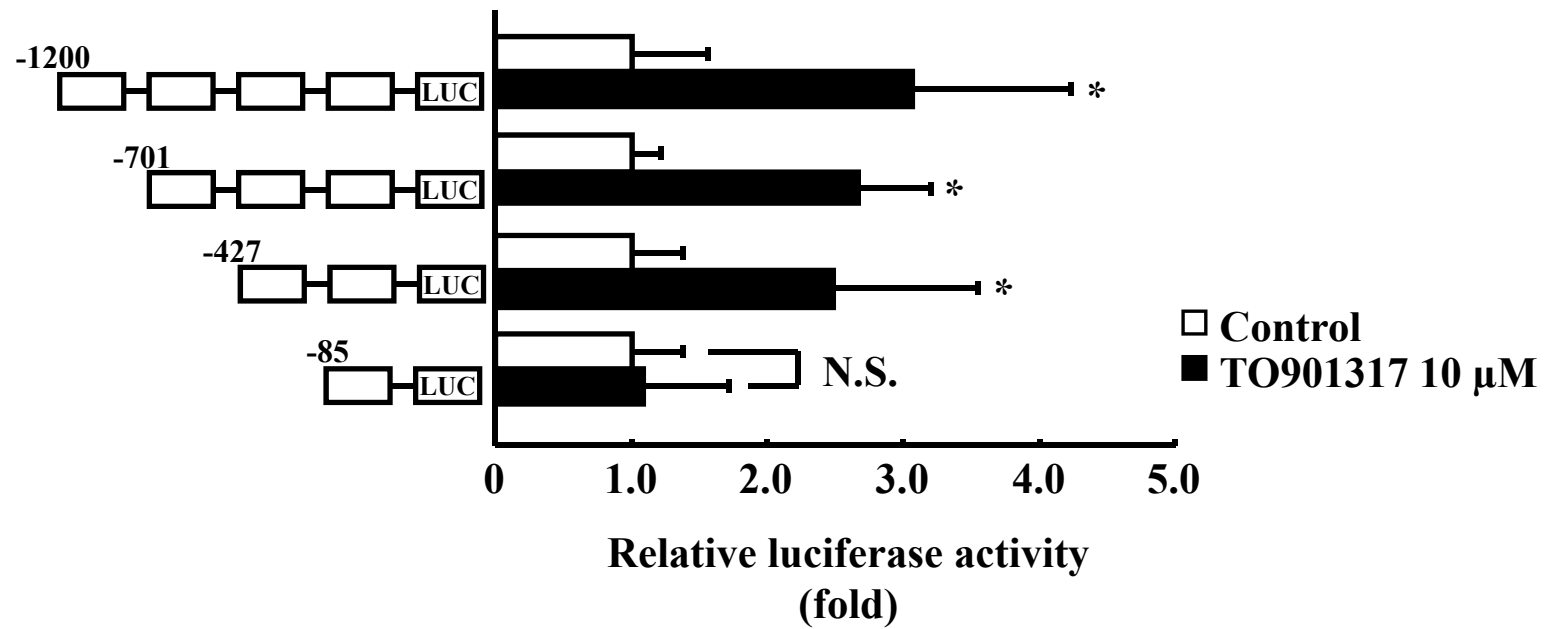
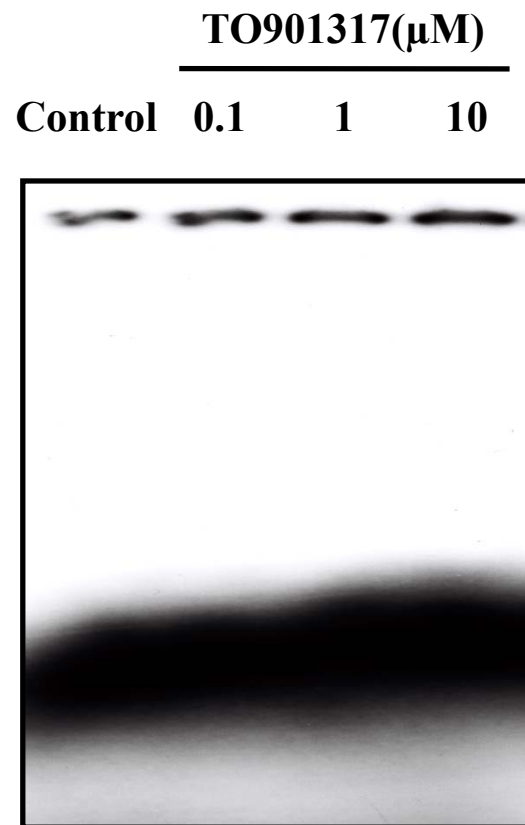


Fig. 3

(A)



(B)

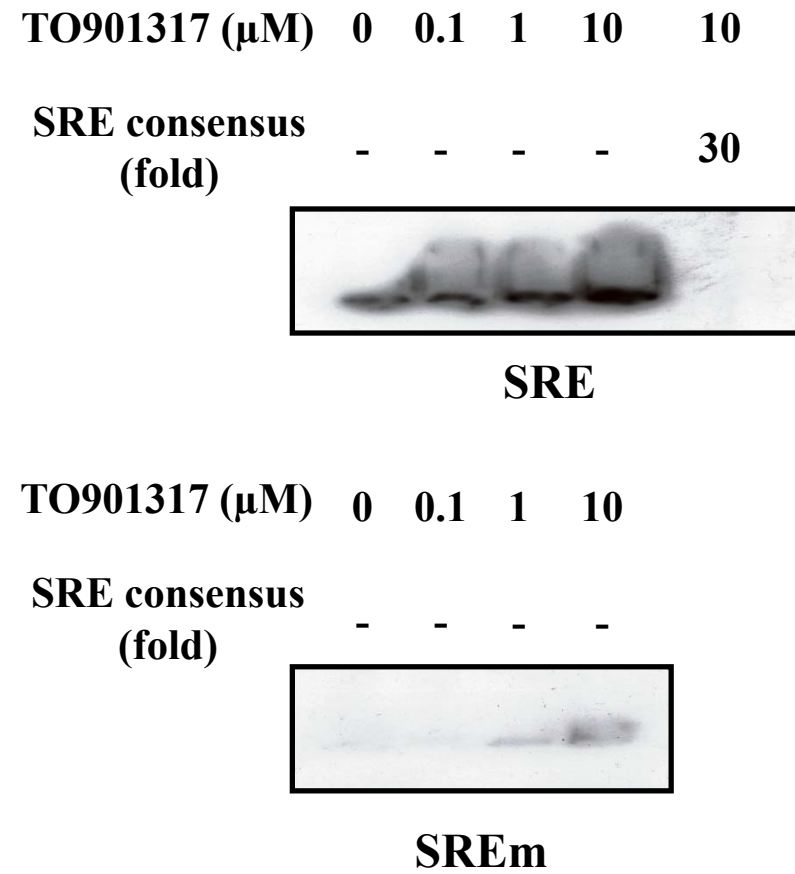
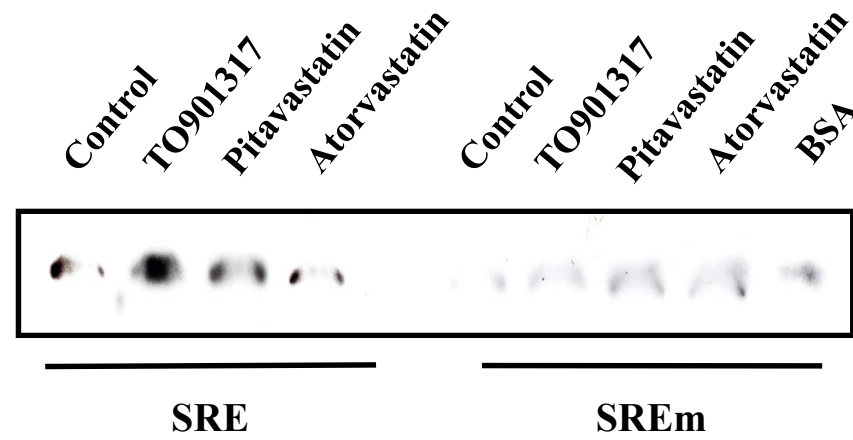
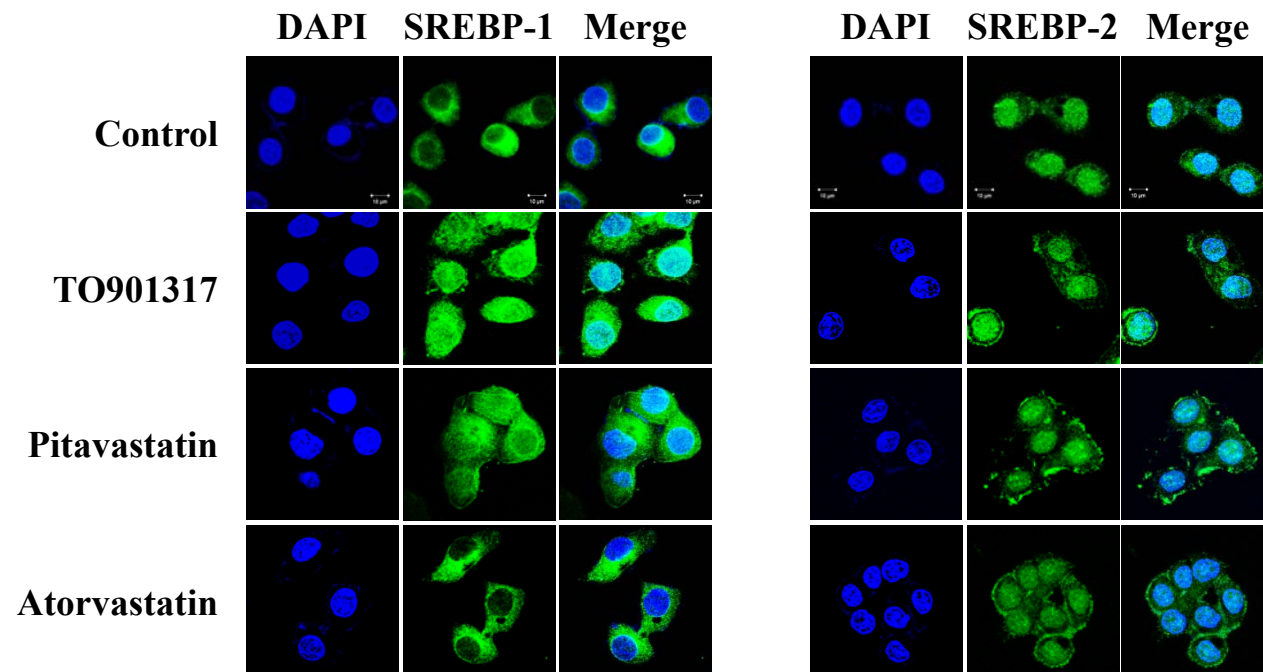


Fig. 4

(A)



(B)



Supplemental Fig. 1

(A)

-427 gctcacactggataagctat ttt **ataacctgact** tcttcaaagaaagttt
SREBP
acatcatgtttaaacatg ttttagattctatatttttaattaaaaatct
-327 aaggaagaaggatatttcacatttctataaaactctaagatcttgacagcag
aagcgaaactgcacatttaggggtgcctgcccccttactgatgctgccct
-227 ttgtgggtcatatgtccttaggaaaatgaaagactgtgcactcttgattt
gttggccagctctgttgacatctttcagtggttccttttatgtatggcca
-127 ctctacagaggcctcttgactttgggaactggtagtctc -85

(B)

SRE ata**ac**ctgact
SREm ata**c**gctgact

Supplemental Fig. 2

