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Regulation mechanism of ABCA1 expression by statins in hepatocytes

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ABSTRACT

ATP-binding cassette transporter A1 (ABCA1) is predicted to be involved in the control of apolipoprotein AI-mediated cholesterol efflux: biosynthesis of **high-density lipoprotein (HDL)**. However, the effects of **HMG-CoA reductase inhibitors (statins)** on ABCA1 in the liver and the precise mechanisms of their actions have been obscure. The aims of this study were to determine whether statins (**atorvastatin (Ato) and pitavastatin (Pit)**) affect hepatic ABCA1 expression and to clarify the mechanisms of their actions using HepG2 cells and the rat liver. We examined alterations in mRNA and protein levels of ABCA1 and peroxisome proliferator-activated receptors (PPARs) by quantitative real-time polymerase chain reaction (PCR) and Western blot analysis, respectively. In vitro and in vivo studies suggested that **Pit increases** ABCA1 mRNA level, but not **Ato**. **Pit** greatly increased Abca1 mRNA level and also increased the amount of plasma HDL and the mRNA level of PPARα. Clofibrate (PPARα agonist) increased ABCA1 expression in HepG2 cells and rat primary hepatocytes more than did PPAR β/δ and γ agonists. **Pit**-induced ABCA1 expression alteration was blocked by GW6471 (PPARα antagonist) and by PPARα knockdown. In this study, we demonstrated that **Pit** affect ABCA1 expression via PPARα in hepatocytes. The strategy to target a PPARα agonist in the liver can lead to increases in ABCA1 expression and HDL level.

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). Atherosclerotic diseases including cardiovascular disease are the main cause of death in individuals with metabolic syndrome. The reverse cholesterol transport system including ATP-binding cassette transporter A1 (ABCA1) and high-density lipoprotein (HDL) plays an important role in atherosclerosis (Chinetti et al., 2006). ABCA1 expression in liver was shown to be necessary for HDL formation. Inactivation of ABCA1 gene in mice leads to a severe HDL deficiency (Aiello et al., 2003), and targeted disruption of ABCA1 gene in mouse hepatocytes reduces plasma HDL level by 80% (Lee and Parks, 2005). On the other hand, overexpression of ABCA1 in mouse liver markedly increases plasma HDL level (Wellington et al., 2003). However, the effects of statins on ABCA1 in the liver and the precise mechanisms of their actions have been obscure.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily. Three subtypes of PPARs, PPARα, PPARβ/δ and PPARγ, have been identified. PPARα is predominantly expressed in the liver, kidney, heart and skeletal muscle, where it controls fatty acid metabolism. PPARβ/δ is ubiquitously expressed and controls brain lipid metabolism and **fatty acid**-induced adipogenesis and preadipocyte proliferation. PPARγ, which is highly expressed in brown and white adipose tissue and the intestine, triggers cellular differentiation, promotes lipid storage and modulates the action of insulin (Torra et al., 2001). **ABCA1 gene expression is known to be regulated by PPARα (**Ogata et al., 2009**) and PPARγ (**Chawla et al., 2001**). Although recent studies have demonstrated that PPARα mRNA level was affected by statins in an in vitro study (**Seo et al., 2008**), it has remained unclear that PPARα or the other isoforms, PPARβ/δ and PPARγ were affected by statins in vitro and in vivo.**

The aims of this study were to determine whether statins (atorvastatin (Ato) and pitavastatin (Pit)) affect hepatic ABCA1 expression and to clarify the mechanisms of their actions.

2. Materials and methods

2.1. Chemicals

Ato was kindly donated by Sankyo (Tokyo, Japan). **Pit** was kindly donated by Kowa (Tokyo, Japan). Clofibrate and GW6471 were

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obtained from Sigma-Aldrich (St. Louis, MO). All other reagents were of the highest grade available and used without further purification.

2.2. Cell culture

HepG2 cells were kept in Dulbecco's modified Eagle's medium (Sigma Aldrich Japan, Tokyo) with 10% fetal bovine serum (ICN Biomedicals, Inc., Aurora, OH) and 1% penicillin-streptomycin at 37 °C under 5% CO_2 as described previously (Kobayashi et al., 2008a). 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 5 mL of 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% CO_2). The density of isolated primary hepatocytes was 4×10^5 cells/cm^2. A minimum of two animal perfusions were used in the study.

2.3. Animals

Male Wistar rats, aged 7 to 9 weeks (260–320 g in weight), were obtained from Jla (Tokyo, Japan). The housing conditions were described previously (Kobayashi et al., 2008b). 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. Rats were starved for 24 h after administration and euthanized.

2.4. Quantitative real-time polymerase chain reaction (PCR)

Quantitative real-time PCR was performed using an Mx3000TM Real-time PCR System (STRATAGENE) with Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA) following the manufacturer's protocol as described previously (Kobayashi et al., 2005) and specific primers (sequences shown in Table 1) through 40 cycles of 94 °C for 15 s, 53–61 °C for 15–30 s, and 72 °C for 30 s. The PCR products were normalized to amplified **glyceraldehyde-3-phosphate dehydrogenase (GAPDH)**, which was the internal reference.

2.5. Western blot analysis

Western blot analysis was performed as described previously (Kobayashi et al., 2006). HepG2 cells and rat primary hepatocytes were seeded on 6-well plastic plates. Following cell attachment (24 h), various concentrations of clofibrate 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 **phosphate buffered saline (PBS)** and centrifuged at 1,300 g for 1 min at 4 °C. The resulting pellet was suspended in a 100 μL lysis buffer containing 1% Triton X-100, 0.1% **sodium dodecyl sulfate (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. (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% **bromo phenol blue (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 hABCA1 (ABM, Richmond, BC) (diluted 1:500), rAbca1 (NEUROMICS, Edina, MN) (diluted 1:2000) or mouse anti-actin monoclonal antibody (Chemicon, Temecula, CA) (diluted 1:500) 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 goat anti-mouse IgG_1-HRP secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:2000 or 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).

Table 1
Primer information.

Gene	Gene bank accession no.	Sequences		Product size (bp)
hABCA1	AF285167	Forward	5′-AAC AGT TTG TGG CCC TTT TG-3′	157
		Reverse	5′-AGT TCC AGG CTG GGG TAC TT-3′	
hPPARα	BC000052	Forward	5′-ACT TAT CCT GTG GTC CCC GG-3′	251
		Reverse	5′-CCG ACA GAA AGG CAC TTG TGA-3′	
hPPARβ/δ	NM_006238	Forward	5′-CAA CGC CCT GGA ACT TGA TG-3′	187
		Reverse	5′-CAG CAG CTT GGG GAA GAG GT-3′	
hPPARγ	BC006811	Forward	5′-CCT CCT TGA TGA ATA AAG ATG-3′	107
		Reverse	5′-GGG CTC CAT AAA GTC ACC AA-3′	
hGAPDH	NM_002046	Forward	5′-AAG GTC ATC CCT GAG CTG AA-3′	96
		Reverse	5′-TTC TAG ACG GCA GGT CAG GT-3′	
rAbca1	NM_178095	Forward	5′-CAG GCT GAT GTC AGT CTC CA -3′	194
		Reverse	5′-GGC TTC AGG ATG TCC ATG TT-3′	
rPparα	NM_013196	Forward	5′-TGA ACAAAG ACG GGA TG-3′	106
		Reverse	5′-TCA AAC TTG GGT TCC ATG AT-3′	
rPparβ/δ	NM_013141	Forward	5′-GAG GGG TGC AAG GGC TTC TT-3′	101
		Reverse	5′-CAC TTG TTG CGG TTC TTC TTC TG-3′	
rPparγ	NM_013124	Forward	5′-CAT GCT TGT GAA GGA TGC AAG-3′	131
		Reverse	5′-TTC TGA AAC CGA CAG TAC TGA CAT-3′	
rGapdh	AF106860	Forward	5′-ATG GGA AGC TGG TCA TCA AC-3′	221
		Reverse	5′-GTG GTT CAC ACC CAT CAC AA-3′	

2.6. Determination of serum HDL concentrations

Serum HDL was determined using DeterminerL HDL-C (Kyowa Medex, Tokyo, Japan). The assays were performed according to the manufacturer's instructions.

2.7. PPARα small interfering RNA (siRNA) and siRNA transfection

Silencer® Validated siRNA (ID: #5439) targeted to the PPARα gene and nontargeting siRNA as a Silencer® Negative control #1 siRNA were purchased from Ambion (Austin, TX). Delivery of siRNAs into HepG2 cells was performed by reverse transfection methods as per the manufacturer's protocol. **6 pmol nontargeting siRNA or PPARα siRNA with OPTI-MEM® I Reduced Serum Medium** (GIBCO, Grand

Island, NY) were mixed in a 12-well plate and incubated at room temperature for 10 min after addition of 2 μL Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA). Then 1 mL of suspended HepG2 cells (1.0×10^5 cells/mL) in growth medium without antibiotics was added. Following siRNA transfection (24 h), the medium was replaced with fresh normal growth medium and then the cells were used for analysis and experimentation at the times indicated.

2.8. Statistical analyses

Student's *t*-test was used to determine the significance of differences between two group means. Statistical significance among means of more than two groups was determined by one-way analysis of variance (ANOVA). Statistical significance was defined as $\mathbf{P}<0.05$.

3. Results

3.1. Effects of statins on ABCA1 expression in hepatocytes

First, we examined the alteration of ABCA1 mRNA level induced by **Ato** and **Pit** in HepG2 cells. **Pit** significantly up-regulated ABCA1 mRNA level. On the other hand, **Ato** had little effect (Fig. 1A). To investigate whether **Pit** regulated ABCA1 mRNA level in vivo, **Pit** were given to rats. **Pit** significantly increased Abca1 mRNA level in the rat liver, but not **Ato** (Fig. 1B). The results of in vitro and in vivo studies suggest that **Pit** increase ABCA1 expression in hepatocytes and that the effects of statins are different.

3.2. Effects of statins on ABCA1 function in hepatocytes

ABCA1 is predicted to play an important role in biosynthesis of HDL. To determine whether **Pit** which increased ABCA1 mRNA level in vitro and in vivo, affects ABCA1 function, we examined the amount of HDL in rats. **Pit** significantly increased plasma HDL concentration. On the other hand, **Ato** had no effect (Table 2).

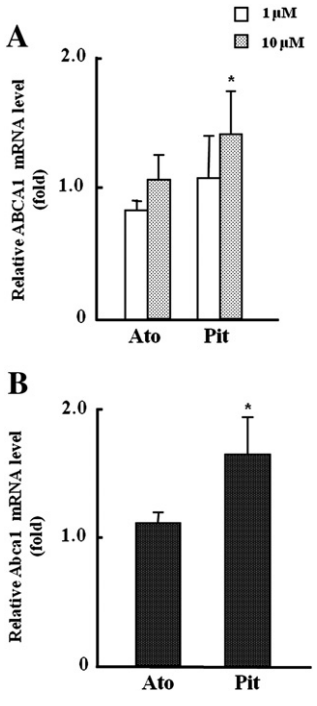

Fig. 1. Effects of **statins** on ABCA1 mRNA level in HepG2 cells (A) and the rat liver (B). (A) HepG2 cells were treated with statins (1, 10 μM) for 24 h. **The bar graphs (n = 4–7) are given as the means with S.D. of the more than two independent experiments. *P ≤ 0.05 was compared with vehicle control. (B)** Male Wistar rats were administered methylcellulose with or without statins (30 mg/kg) for 24 h. **The bar graphs (n = 3) are given as the means with S.D. of the three independent experiments. *P ≤ 0.05 was compared with vehicle control.**

3.3. Involvement of PPARs in statin-induced ABCA1 expression in hepatocytes

Recent studies have shown that statins affect the expression and activity of PPARs (Torra et al., 2001). Therefore, to clarify the mechanism by which statins induce ABCA1 expression, we focused on the expression of PPARs. We examined alterations of PPARα, β/δ, γ mRNA levels induced by **Pit**, which significantly increased ABCA1 mRNA level, and by **Ato**, which had little effect on ABCA1 mRNA level in HepG2 cells. As shown in Fig. 2A, Pit significantly increased PPARα mRNA level. **On the other hand, Ato decreased PPARα mRNA level in HepG2 cells and had no effect on any PPAR isoforms in the rat liver (Fig. 2B). The effect of Ato on PPARs expression was lower than those of Pit.** We examined whether PPARα agonists, clofibrate altered ABCA1 expression in HepG2 cells. Clofibrate significantly increased ABCA1 mRNA level in a concentration dependent manner (Fig. 3A). As shown in Fig. 3B, ABCA1 protein expression induced by clofibrate was associated with mRNA level. Moreover, the alteration of Abca1 protein expression induced by clofibrate in rat primary hepatocytes corresponded to those in HepG2 cells (Fig. 3C). These results suggest that **Pit** affect ABCA1 expression via a PPARα-dependent pathway.

3.4. Importance of PPARα in statin-induced ABCA1 expression in hepatocytes

We confirmed whether PPARα plays an important role in increment of ABCA1 mRNA level induced by pitavastain. We examined the effect of a PPARα antagonist (GW6471) on **Pit**-induced ABCA1 mRNA level in HepG2 cells and rat primary hepatocytes. As shown in Fig. 4, GW6471 completely abrogated increment of ABCA1 mRNA level by inhibition of PPARα. Next, we examined the effect of PPARα knockdown on **Pit** induced ABCA1 mRNA level in HepG2 cells. The siRNA (30 nM) for PPARα significantly decreased PPARα mRNA level in HepG2 cells at 72 h after transfection (Fig. 5A). And clofibrate and **Pit**-induced ABCA1 expression alteration was blocked by PPARα siRNA transfected into HepG2 cells (Figs. 5B, 6). These results indicate that **Pit** induce ABCA1 expression alteration by a PPARα-dependent pathway.

4. Discussion

In the present study, we found that **Pit** induced ABCA1 expression in both HepG2 cells and the rat liver. As tested in HepG2 cells, this inductive effect of **Pit** was mediated through a PPARα-dependent pathway. In an in vitro study, **Pit** increased mRNA level of ABCA1, but **Ato** had little effect on the mRNA level (Fig. 1). Moreover, **Pit** significantly increased plasma HDL concentration. On the other hand, **Ato** had no effect (Table 2). Yokote et al. (2008) reported that a significant increase in HDL-C was observed in the **Pit** and not in the **Ato** group in a clinical study. Our results were associated with this

Table 2
Effects of statins on plasma HDL concentration in rat.

	Relative HDL concentration (fold)
Control	1.01 ± 0.05
Ato	1.01 ± 0.05
Pit	1.22 ± 0.16[a]

Male Wistar rats were administered methylcellulose with or without 30 mg/kg statins for 24 h. **Each value (n = 4) are given as the means ± S.D. of the three independent experiments.**

[a] $P<0.05$ was compared with vehicle control.

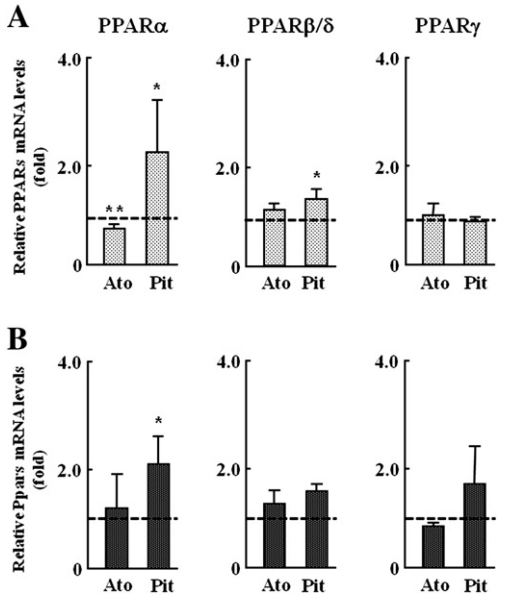

Fig. 2. Effects of **statins** on mRNA levels of PPARs in HepG2 cells (A) and the rat liver (B). (A) HepG2 cells were treated with statins (10 μM) for 24 h. **The bar graphs (n = 4–5) are given as the means with S.D. of the more than two independent experiments. *P ≤ 0.05 or **P ≤ 0.01 was compared with vehicle control.** (B) Male Wistar rats were administered methylcellulose with or without statins (30 mg/kg) for 24 h. **The bar graphs (n = 3) are given as the means with S.D. of the three independent experiments. *P ≤ 0.05 was compared with vehicle control.**

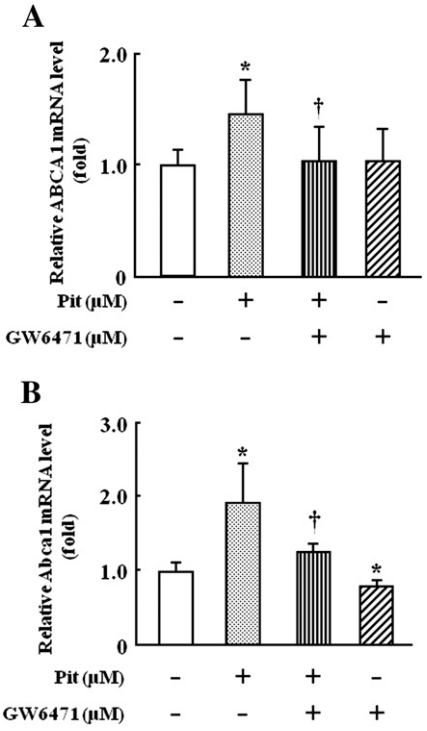

Fig. 4. Effects of GW6471 on **Pit**-induced ABCA1 mRNA level in HepG2 cells (A) and rat primary hepatocytes (B). (A) HepG2 cells were exposed to **Pit** (10 μM) in the absence or presence of GW6471 (10 μM) for 24 h. **The bar graphs (n = 5–11) are given as the means with S.D. of the more than two independent experiments. *P ≤ 0.05 was compared with vehicle control. †p ≤ 0.05 was compared with Pit (10 μM).** (B) Rat primary hepatocytes were exposed to **Pit** (10 μM) in the absence or presence of GW6471 (10 μM) for 24 h. **The bar graphs (n = 3–11) are given as the means with S.D. of the more than two independent experiments. *P ≤ 0.05 was compared with vehicle control. †p ≤ 0.05 was compared with Pit (10 μM).**

previous report. Recent studies have demonstrated that the effects of statins on ABCA1 regulated by Liver X Receptor (LXR) are biphasic: suppression through reduction of oxysterols (ligands for LXR) and enhancement through reduction of geranylgeranyl pyrophosphate (suppression factor of PPARs) in macrophage (Argmann et al., 2005). **So we examined the effects of Pit and Ato on LXRα mRNA level in HepG2 cells. Pit significantly increased LXRα mRNA level. On the other hand, Ato had little effect** (Supplementary Fig. 1). We suggest that the pathway via PPARs is superior to that via oxysterols, particularly in the case of increase in ABCA1 expression by **Pit** in the liver. Next, we tried to clarify the mechanism by which ABCA1 expression was induced by **Pit** in hepatocytes. Wong et al. (2004) reported that statins decreased the expression of ABCA1 and cholesterol efflux via an oxysterols-LXRα pathway in macrophages (Wong et al., 2004). Chawla et al. (2001) reported that PPARs had inductive effects on the expression of LXRα. Accordingly, we focused on the mechanism of **Pit**-induced ABCA1 expression involved in PPARs. PPARs are members of the nuclear hormone receptor superfamily represented by three subtypes, PPARα, PPARβ/δ and PPARγ (Dreyer et al., 1992). First, we confirmed statin-induced

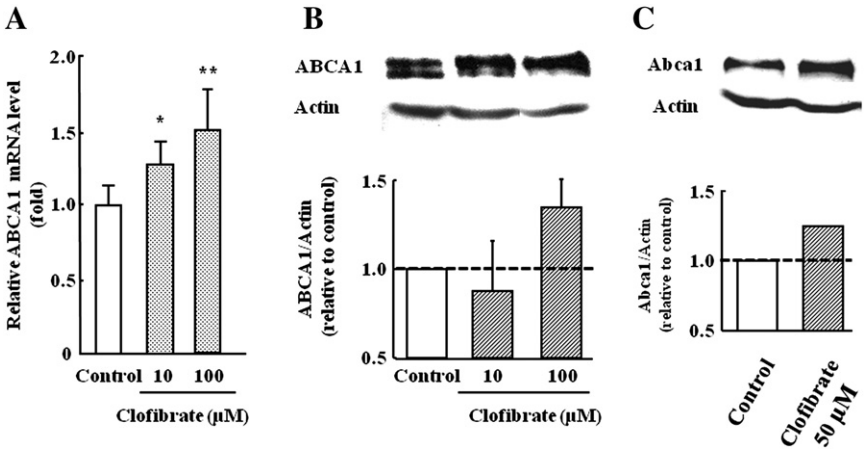

Fig. 3. Effects of clofibrate on ABCA1 mRNA (A) and protein level in HepG2 cells (B) and Abca1 protein level in rat primary hepatocytes (C). (A) HepG2 cells were treated with clofibrate (10, 100 μM) for 24 h. **The bar graphs (n = 5–6) are given as the means with S.D. of the more than two independent experiments. *P ≤ 0.05 or **P ≤ 0.01 was compared with vehicle control**. (B) HepG2 cells were treated with clofibrate (10, 100 μM) 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**. (C) Rat primary hepatocytes were treated with clofibrate (50 μM) for 24 h. Data shown are typical results of the more than two independent experiments. **The intensity of Western blot analysis was determined by densitometry using Scion image.**

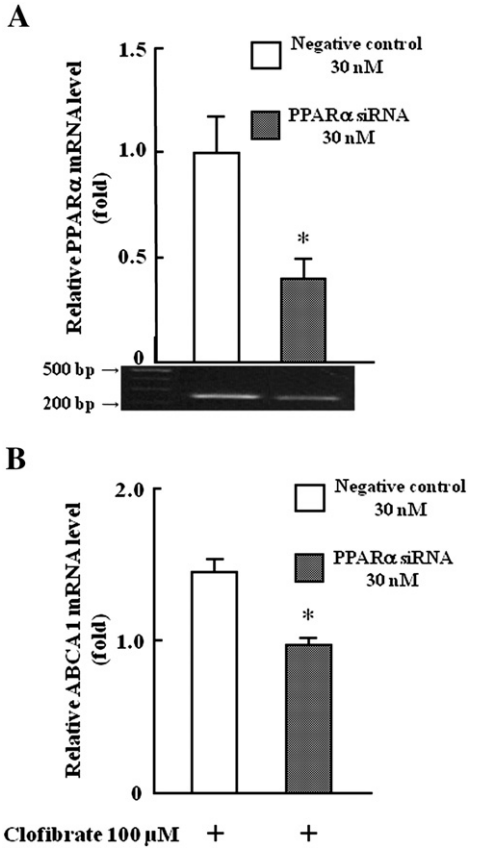

Fig. 5. Effects of PPARα siRNA on PPARα mRNA level (A) and clofibrate-induced ABCA1 mRNA level (B) in HepG2 cells. (A) HepG2 cells were transfected with PPARα siRNA (30 nM) for 72 h. **The bar graphs (n = 3) are given as the means with S.D. of the more than two independent experiments. *P ≤ 0.05 was compared with negative control.** (B) HepG2 cells were transfected with PPARα siRNA (30 nM) for 72 h and treated with clofibrate (100 μM) for 24 h. **The bar graphs (n = 3) are given as the means with S.D. of the more than two independent experiments. *P ≤ 0.05 was compared with negative control.**

alteration of the PPARs mRNA level in vitro and in vivo. As a result, **Pit** induced increment of PPARα mRNA level. **Ato**, however, had little effect on any PPAR isoforms (Fig. 2). Next, we examined the effects of

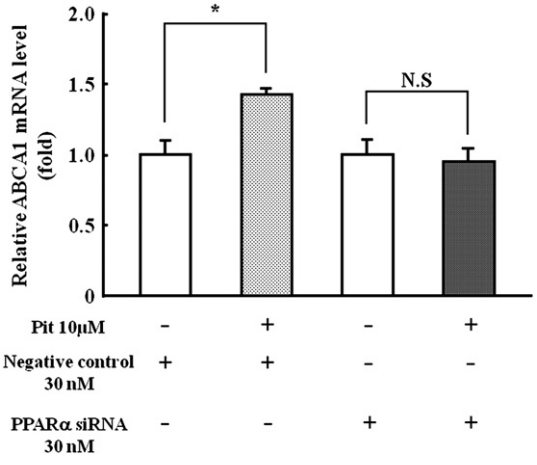

Fig. 6. Effects of PPARα siRNA on **Pit**-induced ABCA1 mRNA level in HepG2 cells. HepG2 cells were transfected with PPARα siRNA (30 nM) for 72 h and treated with **Pit** (10 μM) for 24 h. **The bar graphs (n = 3) are given as the means with S.D. of the more than two independent experiments. *P < 0.05 was compared with no Pit (10 μM). N.S; Not Significant.**

PPARα agonists on ABCA1 expression. Clofibrate (PPARα agonist) significantly increased ABCA1 expression in HepG2 cells and rat primary hepatocytes (Fig. 3). For these reasons, we hypothesized that PPARα plays an important role in statin-induced ABCA1 expression. Ogata et al. (2009) reported PPARα agonists enhanced ABCA1 gene in THP-1 macrophage, WI38 fibroblast and mouse fibroblast. To confirm whether our hypothesis is corrected, we examined the effects of a PPARα antagonist and siRNA on **Pit**-induced ABCA1 expression. **The 10 nM siRNA for PPARα significantly decreased PPARα mRNA level** (Supplementary Fig. 2)**. However, 30 nM PPARα siRNA had a strong knockdown effect and no effect on HepG2 cell viability (data not shown). Accordingly, we used 30 nM PPARα siRNA** (Fig. 5)**. Pit**-induced ABCA1 expression alteration was blocked by the PPARα antagonist and by knockdown (Figs. 4 and 6). These results indicated that **Pit** affected ABCA1 expression via a PPARα pathway in hepatocytes.

Though PPARγ and LXR regulate ABCA1 expression in macrophages (Argmann et al., 2005), we suggest that PPARα plays an important role in ABCA1 expression in hepatocytes. We consider that this difference is due to the presence of a liver-specific promoter of ABCA1 genes (Tamehiro et al., 2007). Further investigation of the differences in PPARs and ABCA1 between macrophages and hepatocytes is needed.

In this study, we demonstrated that statins induce not only increment of ABCA1 mRNA level but also facilitation of ABCA1 function and that PPARα plays an important role in the above-mentioned alteration in hepatocytes.

Supplementary materials related to this article can be found online at doi:10.1016/j.ejphar.2011.04.043.

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