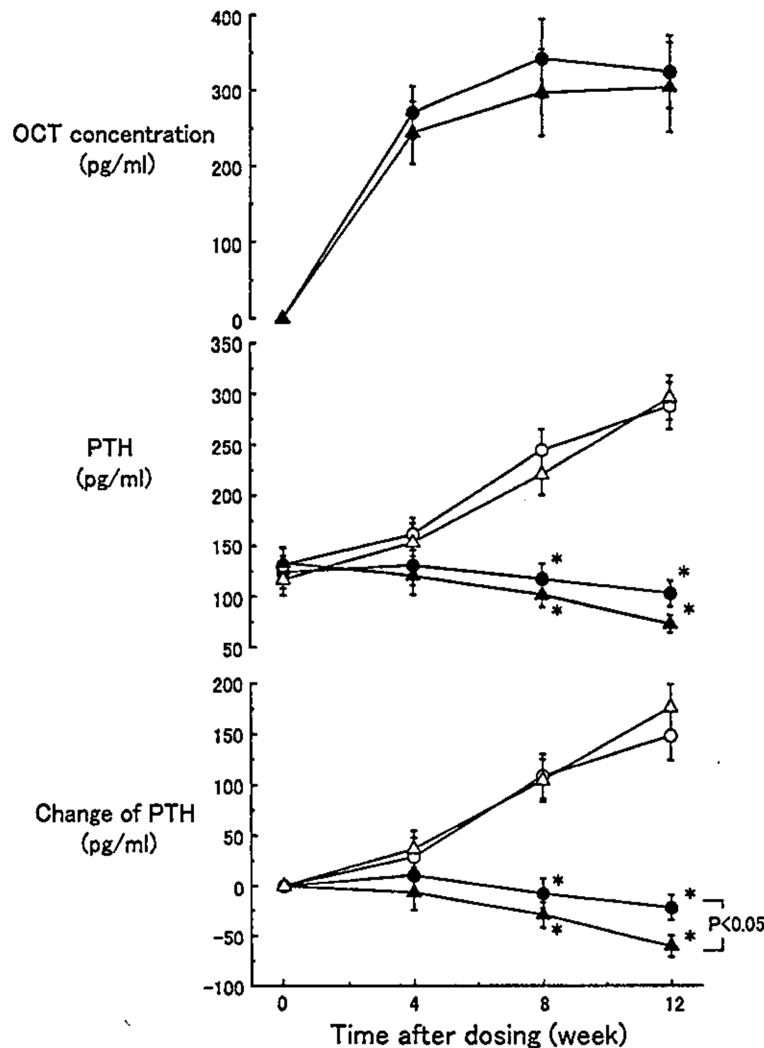


Fig. 5. Serum concentrations of OCT and PTH during a repeated dosing of OCT in 5/6 nephrectomized rats. Mean ± S.E., n = 10 in each. *P < 0.05 vs. each control, ● OCT (2HALO), ○ vehicle (2HALO). ▲ OCT (14HALO), △ vehicle (14HALO).

line (DPD) was also evaluated. Although this parameter was slightly higher in the 2HALO trial at baseline, the difference was not statistically significant. Urinary DPD gradually increased with the administration of vehicle alone. The reduction in the urinary DPD after OCT treatment was significantly greater in the 14HALO than in the 2HALO trials (Fig. 7).

*Bone density*

Bone density of the femur was determined by DEXA at the end of the drug treatment for three months (Fig. 8). Bone density increased during the repeated dosing with OCT in the 2HALO and 14 HALO trials. However, the increase in the 14HALO group was significantly greater than that in the 2HALO

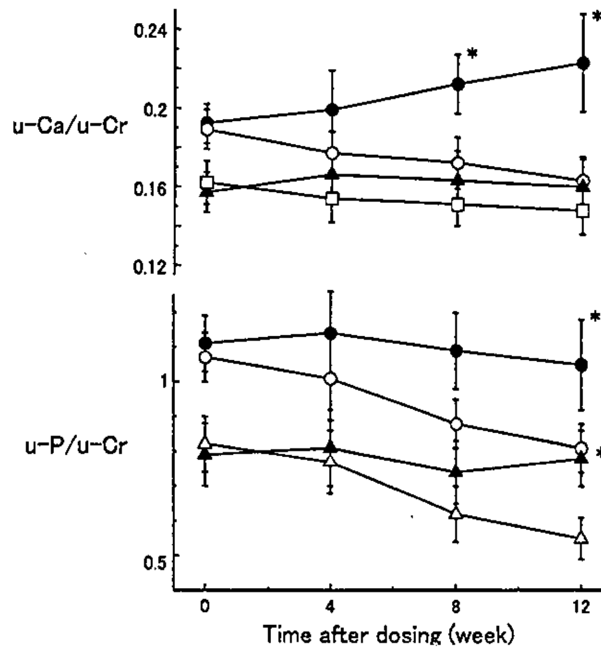


Fig. 6. The ratio of urinary Ca to creatinine (u-Ca/u-Cr) and urinary P to creatinine (u-P/u-Cr) during a repeated dosing of OCT in 5/6 nephrectomized rats. Mean ± S.E., n = 10 in each. *P < 0.05 vs. each control. ● OCT (2HALO), O vehicle (2HALO). ▲ OCT (14HALO), △ vehicle (14HALO).

trial. These results were similar to our previous findings with 1, 25(OH)2 vitamin D3 (Tsuruoka et al., 2003).

## Discussion

We have previously reported that dosing with 1,25(OH)2 vitaminD3 at 14HALO reduced the drug-related adverse reactions (hypercalcemia and hyperphosphatemia) and increased bone density both to a greater extent, than dosing at 2HALO in 5/6 nephrectomized rats (Tsuruoka et al., 2002). OCT was developed to avoid the drug-related hypercalcemia that is commonly observed for calcitriol (Brown et al., 1993; Kubrusly et al., 1993; Farach-Carson et al., 1993). Recently, in a clinical situation, the drug was used for the treatment of secondary hyperparathyroidism with renal osteodystrophy and osteoporosis. In this study, we found that the adverse reactions of OCT, such as hypercalcemic and hyperphosphatemic effects, also differed with its dosing time although OCT was reported to have less calcemic activity than other vitamin D preparations. We also found that the efficacy of the treatment was greater when the drug was given at 14HALO. Thus, OCT as well as other analogues of vitamin D have an optimal dosing time. However, this study showed that the chronopharmacological effects of OCT were different from those of other active vitamin D3 analogues in several aspects.

One of the most important findings in this study was that the hypercalcemic effect of OCT in the 14HALO trial was relatively small. The difference between OCT and vehicle in the area under the

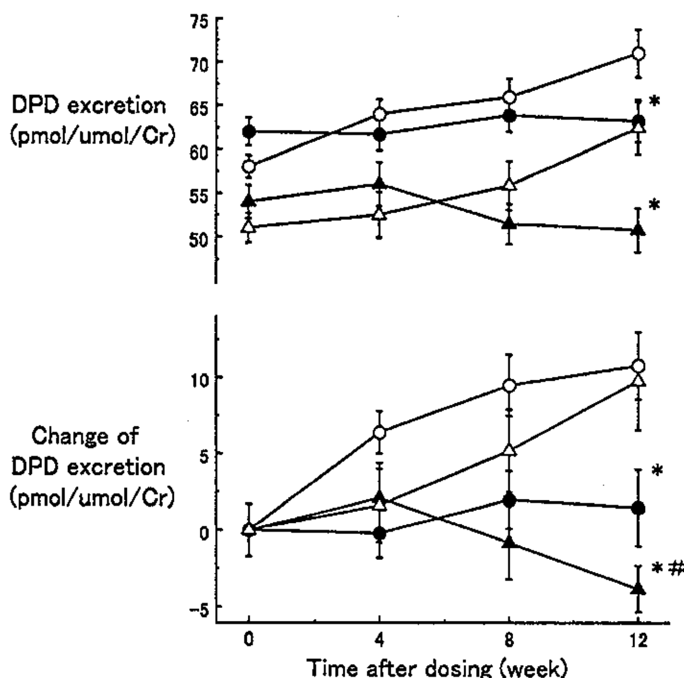


Fig. 7. Urinary deoxypyridinoline (DPD) excretion during a repeated dosing of OCT in 5/6 nephrectomized rats. Mean ± S.E., n = 10 in each. *P < 0.05 vs. each control. ● OCT (2HALO), ○ vehicle (2HALO). ▲ OCT (14HALO), △ vehicle (14HALO).

concentration-curve of serum Ca in the 14HALO trial was almost 1/4 of that in the 2HALO group in our single dosing study (14HALO; 1.8 ± 0.3 and 2HALO;6.3 ± 1.3 mg.hrs/dl). Our previous study with 1,25(OH)2 vitaminD3 showed that such differencee in the 14HALO group were about 1/2 of that in the 2HALO group (14HALO; 4.5 ± 0.9 and 2HALO; 8.0 ± 1.0 mg.hrs/dl) (Tsuruoka et al., 2000). Based on these findings, we believe that the chronopharmacological effect of hypercal-

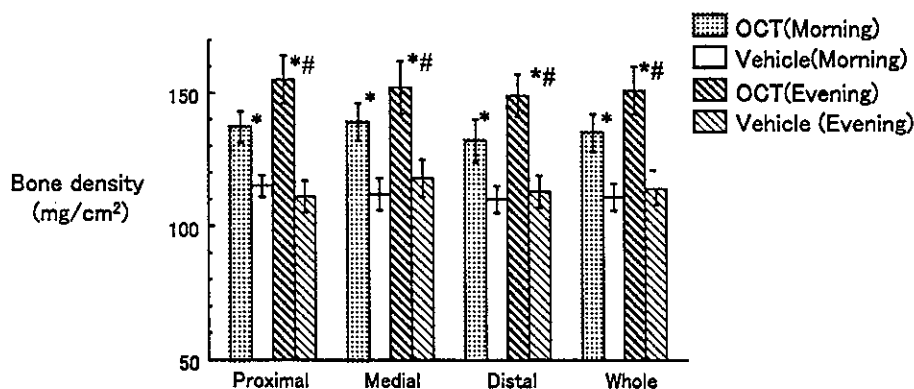


Fig. 8. Bone density of femur at the end of the study, Mean ± S.E., n = 10 in each. *P < 0.05 vs. vehicle. #P < 0.05 vs. Morning.

cemia was prominent for OCT compared to other analogues of vitaminD3. In the repeated dosing study, serum Ca concentration was not elevated at 14HALO but did at the 2HALO trial. In this study, the urinary ratio of Ca to creatinine was not elevated by OCT at 14HALO, but increased at 2HALO, which was similar to changes of serum Ca concentrations in both trials. Such chonopharmacological phenomena were not observed in a previous study using 1,25(OH)2 vitaminD3 in an identical animal model and 1-α(OH) vitaminD3 in an animal model of osteoporosis (Tsuruoka et al., 2001). Because u-Ca/u-Cr was also higher in 2HALO than 14HALO in this study, the higher serum Ca concentration in the 2HALO trial cannot be explained by the time-dependent difference in drug sensitivity to renal Ca handling. Moreover, the reduction of DPD excretion and increase of bone density were rather prominent at the 14HALO dosing. Therefore, although the precise mechanism was not certain, a dosing time-dependent prevention of Ca release from bone may be the reason of this phenomenon. We performed the study under a free-fed condition and therefore cannot evaluate a contribution of intestinal Ca transport. On the other hand, the dosing-time dependent change in the hyperphosphatemic effect of OCT was not as prominent as that of serum Ca. Urinary phosphate excretion showed a similar tendency to the change of serum phosphate concentration. This finding supports previous observations that OCT exerts a similar hyperphosphatemic effect as do other vitaminD3 analogues but less hypercalcemic effect (Brown et al., 1993; Kubrusly et al., 1993; Farach-Carson et al., 1993).

Another important finding is that the efficacies of OCT (prevention of the increase of and loss of bone density) were greater in the 14HALO group. In our previous study using 1,25(OH)2vitaminD3 in the same animal model, PTH reduction did not differ between the two dosing schedules(Tsuruoka et al., 2002). The mechanism of this difference between OCT and other vitamin D preparations is not obvious at the present time. Serum concentrations of trough OCT and albumin did not significantly differ between the 2 and 14HALO groups. Therefore, although unbound OCT, the active form(Kobayashi et al., 1994), was not determined in this study, we believe that serum concentrations of active OCT of the two trials did not differ significantly. Thus, a pharmacokinetic-related mechanism might not be involved in this phenomenon, which is similar to our previous study using 1-α(OH) vitaminD3 (Tsuruoka et al., 2001). As with the dosing-time dependent difference of serum Ca concentration, a dosing-time dependent sensitivity to OCT of bone may be the explanation for this phenomenon. It was recently reported that clock genes exist (Balsalobre, 2002); thus, we hypothesize that clock genes also exist in the osteoclast and their expressions are changed with the time of day, which in turn, might affect the sensitivity to OCT. Further studies are needed to resolve this hypothesis.

It is generally accepted that rats tend to be active at night (dark phase), while humans are active during the day. However, it is also accepted that serum concentrations of Ca and phosphate show similar fluctuations in the two species (i.e. higher in light phase and lower in dark phase). When we apply the present findings to the treatment of patients with osteoporosis, we need to consider these differences.

In conclusion, OCT exerts chronopharmacological effects in 5/6 nephrectomized rats. Adverse reactions (hypercalcemia and hyperphosphatemia) were mild, and efficacies (increase of bone density and reduction of PTH) were prominent when OCT was given at 14HALO. In addition, the degree of hypercalcemia at 14HALO was slight compared to other vitaminD3 analogues. The mechanism of this difference seems to be caused by the change of drug sensitivity in bone cells. These findings may be important in reducing adverse reactions in the treatment of osteoporosis.

## Acknowledgements

We thank Ms. Mariko Ando for her technical assistance.

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# $\beta_2$-Microglobulin Adsorption Column Reduces Digoxin Trough Level During Hemodialysis

## *Three Case Reports*

*Shuichi Tsuruoka,* Michi Wakaumi,* Hisashi Yamamoto,* Hitoshi Ando,* Tetsuo Saito,† and Akio Fujimura**

**Abstract:** We have previously reported that a $\beta_2$-microglobulin adsorption column for the treatment of dialysis-related amyloidosis decreased serum digoxin concentration in renal failure patients. Because the distribution volume of digoxin is high, it is uncertain whether the repetitive use of this column influences the pharmacokinetics of digoxin in renal failure patients. We have observed 3 renal failure patients whose trough serum digoxin concentrations were significantly reduced by the repetitive use of tandem $\beta_2$-microglobulin adsorption columns for treatment of dialysis-related amyloidosis. These patients experienced symptomatic elevation of their heart rates in parallel with a significant reduction in serum digoxin concentrations. Termination of the use of the adsorption column improved the symptoms in 1 patient; however, severe arthritic pain caused by amyloidosis relapsed. Dosage of digoxin was increased in 2 other patients with continuous treatment by the column. Their digoxin concentrations increased, and their heart rates decreased without any deterioration of joint pain. We have demonstrated that the repetitive use of the $\beta_2$-microglobulin adsorption column in tandem with standard hemodialysis actually decreases trough digoxin concentration in renal failure patients. Careful monitoring and alteration of digoxin dosage regimens are needed under these circumstances.

**Key Words:** dialysis-related amyloidosis, digoxin, adsorption

(*Ther Drug Monit* 2004;26:450–452)

Digitalis is used for the treatment of atrial fibrillation and heart failure. Because of its narrow therapeutic range and high renal elimination,[1] drug monitoring is recommended, especially for the treatment of renal failure patients.[2] Despite this precaution, overdosing of the drug is still observed.

Dialysis-related amyloidosis, caused by the accumulation of $\beta_2$-microglobulin,[3] is one of major complications during long-term hemodialysis.[4] A specific adsorption column (Lixelle™) was developed to remove this substance from circulating blood and thereby reduces its accumulation in the organs.[5] Use of this column in tandem with usual maintenance hemodialysis reduces serum $\beta_2$-microglobulin concentrations and relieves the pain induced by the dialysis-related amyloidosis.[6]

We have previously reported a case in which the $\beta_2$-microglobulin adsorption column was used for the successful treatment of digoxin intoxication in a renal failure patient.[7] We have also reported that serum digoxin concentrations decreased after a single use of this column during hemodialysis.[8] We observed a higher capacity for digoxin removal (80% of plasma flow rate).[8] On the other hand, digoxin is believed to have low dialyzability because of its large distribution volume.[9] Therefore, it is uncertain whether the repeated treatment of dialysis-related amyloidosis with this column would significantly alter the pharmacokinetics of digoxin in patients with renal failure.

We studied 3 patients who developed significant reduction in trough serum digoxin concentrations during the repeated use of a $\beta_2$-microglobulin adsorption column with hemodialysis in chronic renal failure. Each patient became symptomatic and complained of tachycardia in parallel with the reduction in trough serum digoxin levels. After modification of regimens, trough digoxin concentration increased, and their heart rate decreased.

## CASE PRESENTATION

Three patients suffered from atrial fibrillation as well as chronic renal failure. Their clinical characteristics are shown in (Table 1). They were anuric and underwent a maintenance hemodialysis program (3 times a week, 4 hours a session) for more than 8 years. They took digoxin (3 times a week, after each hemodialysis session) for at least 5 years. Their serum digoxin concentration was monitored every month before the initiation of the hemodialysis session. Two patients received a

Received for publication July 2, 2003; accepted November 17, 2003.

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**TABLE 1.** Characteristics of 3 Patients

| | Age | Gender | Etiology of Renal Failure* | s-Creatinine (mg/dL) | $\beta_2$-Microglobulin (mg/L) |
|---|---|---|---|---|---|
| Patient 1 | 56 | Male | CGN | 9.6 | 25.5 |
| Patient 2 | 68 | Female | CGN | 11.2 | 31.7 |
| Patient 3 | 57 | Male | DM | 7.5 | 29.3 |

*CGN, chronic glomerulonephritis; DM, diabetic nephropathy.

Ca-channel blocker (amlodipine 5mg a day), and the other patient received a β-blocker (atenolol 12.5 mg a day). Their drug regimen was not altered during the observation period except for the amount of $CaCO_3$ used as a phosphate binder. All the patients had a history of surgical release for carpal tunnel syndrome, which was confirmed as dialysis-related amyloidosis by Congo red staining of the specimen. Each patient complained of symptoms, such as joint pain and numbness of hands, induced by dialysis-related amyloidosis. Treatment with an adsorption column for $\beta_2$-microglobulin was therefore started in August 2001 through September 2002 (Fig.1). Although their pain was remarkably relieved within a few days, trough levels of digoxin concentration (TDx, autoanalyzer, Dinabot Co, Ltd, Japan[10]) gradually decreased, and their heart rate gradually increased and became symptomatic. Because of these symptoms, the use of the adsorption column was terminated in Patient 1. Thereafter, serum digoxin concentration increased, and the heart rate gradually decreased, within a week. However, severe joint pain also relapsed. We measured serum digoxin concentrations at the beginning and at the end of 2 hemodialysis sessions in this patient. These 2 sessions were the last hemodialysis session with the $\beta_2$-microglobulin adsorption column and the first hemodialysis session without the adsorption column. The reduction in serum digoxin concentration during the dialysis session was 0.3 ng/mL/4 h (0.075 ng/mL/h; from 0.5 ng/mL to 0.2 ng/mL) with the adsorption column and 0.1 ng/mL/4 h (0.025 ng/mL/h; from 0.6 ng/mL to 0.5 ng/mL) without the adsorption column.

Because we confirmed the enhanced decrease of serum digoxin by the repetitive use of the adsorption column, larger dosages of digoxin were given to the other 2 patients. As shown in Fig.1, the trough level of digoxin concentration with concomitant administration of increased digoxin wand use of the adsorption column was increased within a month. Their heart rate decreased, and severe pain by amyloidosis was not recorded during this period.

## DISCUSSION

We have previously reported that a single use of a $\beta_2$-microglobulin adsorption column increases digoxin clearance in a crossover study.[8] However, it was still uncertain whether repetitive use of the column would influence the pharmacokinetics of digoxin in renal failure patients. This question was unclear because digoxin has a large volume of distribution (6 L/kg).[9] In this report, we have confirmed that repeated exposure to a $\beta_2$-microglobulin adsorption column actually increases digoxin removal and reduces trough digoxin concen-

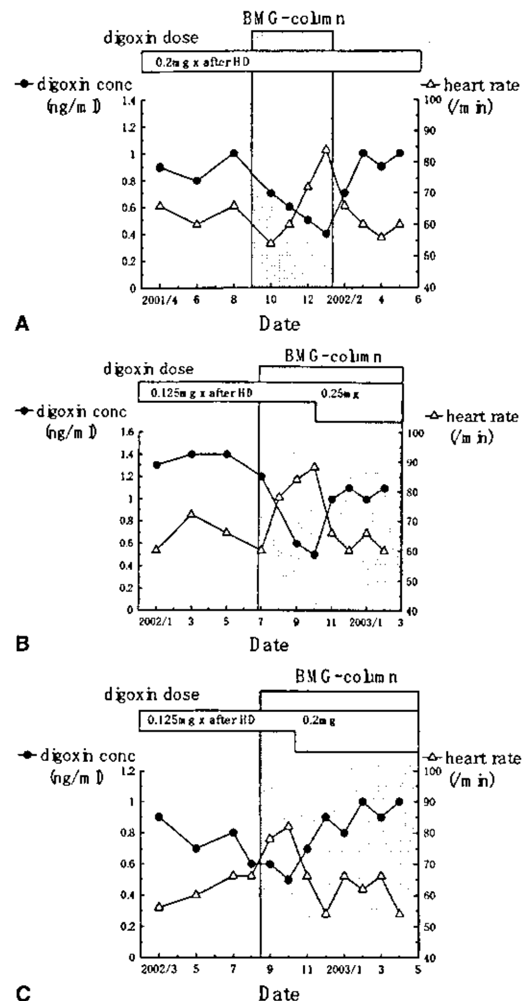


**FIGURE 1.** Clinical course of 3 patients: (A) patient 1; (B) patient 2; (C) patient 3.

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tration in renal failure patients. The decrease in the serum digoxin concentration during a single hemodialysis session with this adsorption column in Patient 1 was 0.075 ng/mL/h. This decrease was 3 times the magnitude that was observed with hemodialysis alone. This is almost comparable with that observed in our previous single-use study.[8]

The mechanism of this digoxin adsorption is still uncertain. The column consists of cellulose beads attached to hexadecyl residues and has a high affinity for the highly lipophilic residue of $\beta_2$-microglobulin.[6] Because digoxin is also highly lipophilic, we speculate that the beads might attach the similar lipohilic domain of the drug. However, recent reports provide an alternative mechanism. Takenaka and Suzuki reported that the use of the column improved platelet counts in patients with idiopathic thrombopenic purpura, suggesting adsorption of the platelet autoantibodies.[11] Tsuchida et al have reported that the column also adsorbs LPS-induced endotoxin in vitro.[12] Both reports indicate that the column might adsorb some protein. Although protein binding of digoxin is reported to approach 20%,[9] adsorption of an as-yet unidentified digoxin binding protein might also occur. Future studies will resolve this problem.

We demonstrated that the decrease in serum digoxin concentration could be avoided by simply increasing its dosage without stopping treatment of the dialysis-related amyloidosis. The incidence of dialysis-related amyloidosis in end-stage renal failure increases, especially at 5–7 years after the initiation of hemodialysis, and pain induced by this disease remarkably decreases the quality of life.[4] At present, there is no medication for prevention of this condition even though the accumulation of $\beta_2$-microglobulin is the cause of this disease. Although surgical removal of affected tissue may help,[4] it is not generally attempted because of the nature of high-risk patients. Use of an adsorption column for $\beta_2$-microglobulin is less invasive therapy and widely used in some countries including Japan. The survival period after the initiation of maintenance hemodialysis has dramatically increased, and the number of renal failure patients who have cardiac complication is also increased. Therefore, concomitant use of the column in patients who are prescribed digoxin will probably continue to increase in the future. Although a large trial is needed to confirm our finding, this case report may be useful for such occasions. In conclusion, this report demonstrates that trough digoxin concentrations are reduced by the repeated use of $\beta_2$-microglobulin adsorption columns in hemodialysis patients. Careful monitoring of digoxin concentration is required to avoid an adverse arrhythmia-related outcome.

## ACKNOWLEDGMENT

The authors thank Dr. David C. Kem (Department of Medicine, University of Oklahoma) for critical reading of our manuscript.

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DOI:10.1111/j.1365-2125.2004.02171.x



# Drug interaction between St John's wort and quazepam

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**Keywords**
CYP3A4, quazepam, St. John's Wort

**Received**
13 August 2003
**Accepted**
23 February 2004

**Aim**
St John's Wort (SJW) enhances CYP3A4 activity and decreases blood concentrations of CYP3A4 substrates. In this study, the effects of SJW on a benzodiazepine hypnotic, quazepam, which is metabolized by CYP3A4, were examined.

**Methods**
Thirteen healthy subjects took a single dose of quazepam 15 mg after treatment with SJW (900 mg $day^{-1}$) or placebo for 14 days. The study was performed in a randomized, placebo-controlled, cross-over design with an interval of 4 weeks between the two treatments. Blood samples were obtained during a 48 h period and urine was collected for 24 h after each dose of quazepam. Pharmacodynamic effects were determined using visual analogue scales (VAS) and the digit symbol substitution test (DSST) on days 13 and 14.

**Results**
SJW decreased the plasma quazepam concentration. The $C_{max}$ and $AUC_{0-48}$ of quazepam after SJW were significantly lower than those after placebo [$C_{max}$; −8.7 ng $ml^{-1}$ (95% confidence interval (CI) −17.1 to −0.2), $AUC_{0-48}$; −55 ng h $ml^{-1}$ (95% CI −96 to −15)]. The urinary ratio of 6β-hydroxycortisol to cortisol, which reflects CYP3A4 activity, also increased after dosing with SJW (ratio; 2.1 (95%CI 0.85–3.4)). Quazepam, but not SJW, produced sedative-like effects in the VAS test (drowsiness; $P < 0.01$, mental slowness; $P < 0.01$, calmness; $P < 0.05$, discontentment; $P < 0.01$). On the other hand, SJW, but not quazepam impaired psychomotor performance in the DSST test. SJW did not influence the pharmacodynamic profile of quazepam.

**Conclusions**
These results suggest that SJW decreases plasma quazepam concentrations, probably by enhancing CYP3A4 activity, but does not influence the pharmacodynamic effects of the drug.

## Introduction

St. John's Wort (SJW) is one of the most commonly used herbal medicines for the treatment of mild to moderate depression in the countries of the European Union and in the United States [1, 2]. The internet marketing sales of SJW were reported to be 235 million dollars in 2000 [3]. More than 2000 products that contain SJW are also consumed as dietary supplements or food products in Japan. SJW is a potent inducer of cytochrome P450 (CYP) 3A4 in the intestinal wall and liver [4], and it reduces the plasma concentrations of some CYP3A4 substrates, which, in turn, may influence the outcome of drug therapy [5, 6].

Hypnotic drugs are used for treating insomnia, which is one of the common symptoms of depressive patients and is involved in the diagnostic criteria for depression

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[7]. Therefore, it is likely that subjects with depressive states may simultaneously take SJW and a hypnotic drug. A recent epidemiological study demonstrated that more than 7% patients taking benzodiazepine hypnotics also used herbal preparations/supplements concomitantly [8]. The substantial overlap between use of benzodiazepines and herbal preparations/supplements such as SJW raises concern about unintended interactions. Drug interactions between SJW and midazolam [9] and alprazolam [10] have already been examined. Quazepam is a trifluoethylbenzodiazepine, and has significant effects on the induction and maintenance of sleep without major effects on sleep architecture [11, 12]. Quazepam is metabolized to 2-oxoquazepam, an active metabolite, which is further converted to other less active metabolites [13]. Because the metabolism of quazepam and 2-oxoquazepam is mediated by CYP3A4 and CYP2C9 [13], it is likely that SJW decreases plasma concentrations of quazepam and 2-oxoquazepam and consequently diminishes the pharmacodynamic effects of the drug. This study was undertaken to examine this hypothesis. The effect of SJW on the pharmacokinetics and pharmacodynamics of quazepam was evaluated in a double-blind, placebo-controlled, cross-over study in healthy subjects.

## Methods

### *Subjects*

To detect a difference of 30% in the area under the plasma concentration-time curve (AUC) of quazepam between with and without SJW with a power = 80% and $\alpha = 0.05$, 12 subjects were required. Therefore, 13 healthy men [age mean ± s.d., 34 ± 6 years (range, 25–45); weight ± s.d., 66 ± 7 kg (range, 56–75)] were enrolled in this study. As the SJW-mediated induction of CYP3A4 activity is reported to differ between males and females [14], only male subjects were studied. Their biochemical and haematological functions were normal at screening. They were non-smokers and did not receive any continuous medications. Subjects were requested to abstain from grapefruit, grapefruit juice, herbal dietary supplements, and herbal tea during the study period. Caffeine-containing beverages, including coffee and green tea, were withheld from the night before the study day until the final blood sample. The study protocol was approved by the Ethics Committee of Jichi Medical School (Tochigi, Japan). All volunteers gave written informed consent.

### *Study design*

A randomized, double-blind, cross-over design, with an interval of 4 weeks between treatments, was used in this study. In each phase, the subject took a 300 mg caplet of SJW (lot NO.265112) (TruNature, Carson, California, USA) or matching placebo orally three times a day for 14 days, according to a randomization schedule. The SJW caplet used in this study was labelled to be standardized to 0.3% hypericin. The dose of SJW was chosen on the basis of previous reports [4, 15]. Our previous study showed that 900 mg of SJW, which was the same caplet used in this study, for 14 days decreased the blood concentration of the CYP3A4 substrate, simvastatin [16]. In Japan the recommended dose of quazepam is 15–30 mg for the treatment of sleep disorders. Because 15 mg quazepam is used more often than 20 or 30 mg, we chose this dose for our study. Adverse symptoms were checked during repeated dosing with SJW or placebo. On day 14, a single oral dose of 15 mg quazepam (Mitsubishi Pharma Co. Ltd, Tokyo, Japan) was given to the subjects, with 150 ml water at 08:00. The subjects fasted overnight before the dose of quazepam and were allowed a meal 4 h afterwards. On days 7 and 14, we checked the number of remaining SJW caplets. CYP2C9 plays a role in the disposition of quazepam [13]. However, because polymorphisms in CYP2C9 are not common in the Asian population [17], genotyping for CYP2C9 was not performed in this study.

### *Blood and urine sampling*

On day 14, blood samples (2 ml in each) were collected in heparinized tubes just before and at 0.5, 1, 2, 3, 4, 6, 8, 12, 24 and 48 h after the dose of quazepam. Urine was collected for 24 h. Plasma and urine samples were stored at −80 °C until analysis.

### *Pharmacodynamic measurements*

The effect of SJW and/or quazepam on sedative-like self-rated moods was determined by visual analogue scale (VAS) and on psychomotor performance by the digit symbol substitution test (DSST) immediately before blood sampling at 0, 2, 4, 8 and 12 h after dosing on day 14. These tests were also performed at identical clock-times on day 13. The subjects had been fully trained to perform the tests before the start of the study. A 100-mm long horizontal VAS was used to measure sedative-like self-rated moods, which were the pairs of adjectives such as drowsy/alert, calm/nervous, mentally slow/quick-witted, and discontented/contented. In the DSST, the number of digits correctly substituted in 2 min was recorded.

### *Determination of quazepam and 2-oxoquazepam*

Plasma concentrations of quazepam and 2-oxoquazepam were measured by a column-switching

high performance liquid chromatography (HPLC) analysis, as described by Hikida *et al.* [18]. Aliquots of each plasma sample (1.5 ml), to which 0.1 ml of cisapride (800 ng $ml^{-1}$) was added as an internal standard, were alkalinized with 500 kl of 0.5 M NaOH and then, 0.4 ml of water and 5 ml of toluene/chloroform (85 : 15, v/v) were added. The mixture was shaken vigorously for 15 min and then centrifuged at 2000 g for 10 min. A 4.5 ml portion of the organic layer was evaporated to dryness in vacuo at 45 °C. The residue was reconstituted with 0.8 ml of eluent A (see below) and used as an extract. A 0.5-ml portion of the extract was injected onto the column-switching HPLC system. The HPLC system consisted of a chromatography pump (LC-10 A; Shimadzu, Tokyo, Japan), an autoinjector (AS-8020, Toshoh Co., Tokyo, Japan), and an ultraviolet detector (SPD-10 A, Shimadzu, Tokyo, Japan). Column I (TSK-BSA-C8, 5 µm, Tosoh, 10 mm × 4.6 mm) was used for pretreatment and column II (STR-ODS II, 5 µm, Shimadzu, 150 mm × 4.6 mm) for the column oven module. Between 0 and 13 min after a sample injection, cisapride was separated from the interfering substances existing in the extract on column I with a mobile phase solvent (eluent A) consisting of acetnitrile/0.02 mol $l^{-1}$ $KH_2P_{04}$ (13 : 87, v/v). Between 13 and 20 min after the injection, quazepam and 2-oxoquazepam retained on column I were eluted with a mobile phase (eluent B) consisting of acetnitrile/perchloric acid/0.02 mol $l^{-1}$ $KH_2P_{04}$ (41 : 0.05: 58.95, v/v/v), and the effluent from column I was switched to column II. Quazepam and 2-oxoquazepam were separated on column II by eluting with a mobile phase solvent (eluent C) consisting of acetnitrile/0.02 mol $l^{-1}$ $KH_2P_{04}$ (62.5 : 37.5, v/v) between 32.0 and 46.5 min. The mobile phase was pumped at a flow rate of 0.6 ml $min^{-1}$. The absorbance of the effluent from column II was monitored at 254 nm for 2-oxoquazepam and 286 nm for quazepam. The limits of quantification for quazepam and 2-oxoquazepam were 0.5 ng $ml^{-1}$. The coefficient of variation for intra- and inter reproducibility was better than 3.7% at 2, 20 and 40 ng $ml^{-1}$.

*Determination of cortisol and 6β-hydroxycortisol*

Cortisol, 6β-hydroxycortisol and 6α-methylprednisolone were purchased from Sigma (St. Louis, MO, USA). O-methylhydroxylamine hydrochloride was purchased from Tokyo Chemical Industry (Tokyo, Japan) and N-(trimethylsilyl) imidazole (TMSI) from Nacalai Tesque (Kyoto, Japan). All other chemicals and solvents were of analytical grade. A 1.0-ml of urine sample with 6α-methylprednisolone (as an internal standard, 500 ng $ml^{-1}$) was loaded onto a preconditioned Sep–Pak C18 cartridge (Waters, Milford, MA, USA). The cartridge was washed with 5 ml of distilled water and then eluted with 2 ml of ethyl acetate into a glass tube. The eluate was evaporated to dryness at 60 °C under reduced pressure. Derivatization was performed according to the general procedures as described previously [19]. The dried residue was dissolved into 100 µl of a 2% solution of O-methylhydroxylamine hydrochloride in pyridine. After 2 h at 60 °C, the pyridine was evaporated and 50 kl of TMSI was added. This was kept at 100 °C for 15 h to yield methyloxime-trimethylsilyl esters. Gas chromatography-mass spectrometry (GC-MS) analysis was carried out on a GCMS-QP5050A gas chromatography-mass spectrometer (Shimadzu, Kyoto, Japan). Gas chromatography was performed on an Ultra Ally 5 fused silica capillary column (30 m × 0.25 mm I.D., film thickness 0.25 µm, Frontier Laboratories Ltd, Fukushima, Japan) with splitless injection mode. Two µl of derivative were injected (injector:280 °C). The temperature program was as follows: the initial temperature 230 °C was held for 2 min, and then increased to 260 °C at 15 °C $min^{-1}$. After a steady period for 1 min, the temperature was increased to 320 °C at 2 °C $min^{-1}$, and held for 5 min. The selected ion monitoring was used for the detection of cortisol (m/z = 605), 6β-hydroxycortisol (m/z = 694) and 6α-methylprednisolone (m/z = 617). The quantifications of cortisol and 6β-hydroxycortisol were performed by measuring the peak-area ratios of these compounds and of the internal standard. The limit of quantification was 0.2 ng $ml^{-1}$ for cortisol and 0.5 ng $ml^{-1}$ for 6β-hydroxycortisol. The coefficient of variation for intra- and inter reproducibility was better than 5.9%.

*Pharmacokinetic calculations*

The pharmacokinetics were characterized by maximum plasma concentration ($C_{max}$), time to maximum concentration ($t_{max}$), elimination half-life ($t_{1/2}$), and area under the plasma concentration-time curve from 0 to 48 h after dosing ($AUC_{0-48}$). Elimination rate constant (ke) was determined by a linear regression analysis of a log-linear phase of plasma drug concentration-time curve. The elimination half-life ($t_{1/2}$) was calculated as follows: $t_{1/2} = \ln 2/ke$. The $AUC_{0-48}$ was calculated by the trapezoidal rule.

*Statistical analysis*

Data are expressed as the mean ± SE. Pharmacokinetic parameters were analysed by one-way ANOVA. Pharmacodynamic effects [without (on day 13) *vs.* with (on day 14) quazepam] were analysed by repeated measures ANOVA and pharmacodynamic effects (with *vs.* without

SJW) were analysed by repeated measures ANOVA of a cross-over design with the adjustment of Huynh-Feldt. Differences were considered to be statistically significant for $P$-values < 0.05. Calculations were performed by SAS software (SAS Institute Inc, Cary, NC, USA).

## Results

All enrolled subjects took the full course of SJW and no adverse symptoms were observed during the study.

*Plasma concentrations of quazepam and 2-oxoquazepam*

Plasma concentrations of quazepam and 2-oxoquazepam decreased after pretreatment with SJW (Figure 1a,b). The values of $C_{max}$ and $AUC_{0-48}$ for quazepam with SJW were significantly lower than those after placebo (Table 1). The values of 2-oxoquazepam were also lower after SJW, but these differences did not reach statistical significance (Table 1). The ratio of 2-oxoquazepam to quazepam in the $C_{max}$ was significantly greater in the SJW period (placebo; 0.40 ± 0.04, SJW; 0.47 ± 0.04, $P < 0.01$). No significant differences were observed in the $t_{1/2}$ or $t_{max}$ (Table 1).

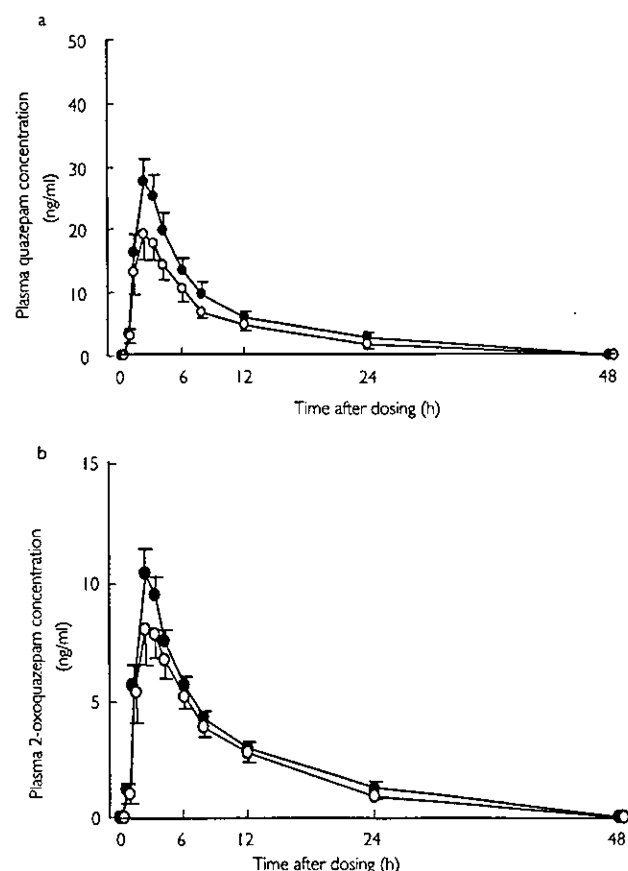


**Figure 1**

Mean ± SE plasma concentrations of quazepam (1a) and 2-oxoquazepam (1b) after a single oral dose of 15 mg quazepam following pretreatment with placebo (●) or SJW (900 mg $day^{-1}$ for 14 days) (○) in 13 healthy males

*Urinary ratio of 6β-hydroxycortisol/cortisol (6β-OHC/C)*

The ratio of 6β-OHC/C significantly increased after repeated dosing with SJW for 14 days (placebo; 9.4 ± 4.8, SJW; 18.4 ± 11.7: ratio; 2.1, 95% confidence interval, 0.85–3.4, $P < 0.05$).

*Pharmacodynamics*

No significant change was detected in the VAS test with SJW alone (Figure 2). On the other hand, quazepam produced significant sedative-like effects (drowsiness; $F$-value 23.2, $P < 0.01$, mental slowness; $F$-value 8.1, $P < 0.01$, calmness; $F$-value 6.9, $P < 0.05$, discontentment; $F$-value 14.4, $P < 0.01$). SJW did not influence the subjective effects of quazepam.

SJW significantly ($F$-value 4.9, $P < 0.05$) impaired psychomotor performance in the DSST while quazepam did not (Figure 3). A carry-over effect was detected in the DSST ($F$-value 16.5, $P < 0.01$). SJW did not influence performance after quazepam.

## Discussion

This study showed that the concomitant use of SJW decreased plasma concentrations of quazepam, although the effect was smaller than that for alprazolam [10]. Although the label stated that the SJW caplet contained 0.3% hypericin, this was not confirmed. However the same caplet of SJW was used by us in a previous study which showed that SJW substantially altered the pharmacokinetics of simvastatin [16]. In a previous report, SJW lowered the trough cyclosporin concentration in whole blood, but this returned to normal 7 days after discontinuation of SJW [20]. It therefore seems unlikely that the carry-over effect of SJW lasts for 4 weeks.

The baseline pharmacokinetic profiles of quazepam in Japanese male subjects were similar in this and other studies, except for $t_{1/2}$ [21]. The difference in the mean $t_{1/2}$ (21.2 *vs* 8.8 h) may be due to the difference in the duration of the blood-sampling period.

Quazepam is a relatively weak benzodiazepine-receptor ligand and binds selectively to the type-1 benzodiazepine-receptor [22, 23]. An *in vitro* study showed that quazepam and 2-oxoquazepam are metabolized by CYP3A4 and CYP2C9 [13]. Because SJW induces the activity of CYP3A4, but not that of CYP2C9 [4, 15, 24], it is likely that the enhancement of the CYP3A4-mediated metabolism of quazepam and 2-oxoquazepam

**Table 1**
Effect of St. John's Wort on the pharmacokinetics of quazepam and its metabolite, 2-oxoquazepam (Mean +/− SE, $n$ = 13)

| | Treatment | | ANOVA Mean value of difference | |
|---|---|---|---|---|
| | Quazepam + placebo | Quazepam + SJW | (95% CI) | $P$-value |
| parameter | | | | |
| quazepam | | | | |
| $C_{max}$ (ng/ml) | 30.5 ± 3.9 | 21.8 ± 3.9 | −8.7 (−17.1 ~ −0.2) | $P < 0.05$ |
| $t_{max}$ (h) | 2.2 ± 0.2 | 2.5 ± 0.2 | 0.3 (−0.3 ~ 0.9) | NS |
| $t_{1/2}$ (h) | 8.8 ± 0.5 | 8.4 ± 0.4 | −0.3 (−1.7 ~ 1.0) | NS |
| $AUC_{0-48}$ (ng h ml$^{-1}$) | 217 ± 28.7 | 161 ± 25.2 | −55 (−96.0 ~ −15.0) | $P < 0.05$ |
| 2-oxoquazepam | | | | |
| $C_{max}$ (ng ml$^{-1}$) | 10.9 ± 1.0 | 9.1 ± 1.4 | −1.8 (−4.7 ~ 1.2) | NS |
| $t_{max}$ (h) | 2.2 ± 0.2 | 2.8 ± 0.3 | 0.6 (−0.1 ~ 1.4) | NS |
| $t_{1/2}$ (h) | 9.5 ± 0.5 | 9.1 ± 0.5 | −1.0 (−3.0 ~ 1.0) | NS |
| $AUC_{0-48}$ (ng h ml$^{-1}$) | 92 ± 5.7 | 80 ± 9.5 | −12 (−32.6 ~ 9.6) | NS |

*Cmax, maximum plasma concentration; tmax, time to maximum concentration; $t_{1/2}$, elimination half-life; $AUC_{0-48}$, area under the plasma concentration-time curve from 0 to 48 h after dosing; CI, confidence interval; NS, not significant.*

is involved in the SJW-related reductions in plasma concentrations of these two drugs.

CYP3A4 is the most abundantly expressed CYP (approximately 30% to 40% of the total CYP content in the human adult liver and small intestine) and plays a major role in the metabolic pathways of various drugs [25, 26]. SJW has been shown to induce hepatic and intestinal CYP3A4 activity [4, 15], probably through the activation of the pregnane X receptor, a human orphan nuclear receptor [27]. The activated CYP3A4, in turn, has enhanced the metabolism of several drugs such as indinavir and cyclosporin and diminished their efficacy during repeated dosing with SJW [5, 6]. In this study, the urinary ratio of 6β-hydroxycortisol to cortisol increased after dosing with SJW for 14 days, which indicates that the hepatic CYP3A4 activity was activated under the present study conditions [28]. Similar data have already been reported [15, 29]. However, because the elimination $t_{1/2}$ of quazepam and 2-oxoquazepam were not significantly changed in this study, the degree of the SJW-mediated induction might be relatively small for hepatic CYP3A4. On the other hand, the ratio of $C_{max}$ of 2-oxoquazepam to quazepam, which might reflect CYP3A4 activity in the liver and intestine, significantly increased after the repeated dosing of SJW in this study. Therefore, although there is no evidence indicating intestinal metabolism of quazepam, we believe that SJW activated intestinal and hepatic CYP3A4 activity and consequently, enhanced the conversion of quazepam to 2-oxoquazepam.

SJW is also reported to induce P-glycoprotein expression in human subjects [4, 30]. Induction of P-glycoprotein would decrease the extent of absorption of the substrates for this transporter. Although benzodiazepines, such as flunitrazepam and midazolam, are not substrates for P-glycoprotein [31, 32], it is not known whether quazepam is a substrate for this transporter. The possibility that the SJW-related reduction in plasma quazepam concentration was caused by an induction of P-glycoprotein remains to be determined.

Meta-analyses of randomized clinical trials have demonstrated that SJW is as effective as standard antidepressants in the treatment of mild to moderate depression [1, 2]. Although SJW is reported to be as safe as, or possibly safer, than standard antidepressants, it also causes adverse effects including central nervous system (CNS)-related symptoms [33]. To our knowledge, well-controlled clinical trials, which were undertaken to examine the CNS-related symptoms caused by SJW, are limited [34]. In this study, SJW had no effect on the sedative-like self-rated moods, which agrees with previous data [34]. SJW impaired psychomotor performance measured by the DSST test although it has been reported that it did not impair cognitive function [34] as determined using the Block Board tapping test [35]. The diverse effects of SJW could be explained by the different methodologies used. In this study, a carry-over effect was detected in the DSST test. However, as the trial was performed with a cross-over design, the influence of such an effect on the data should be small. Further

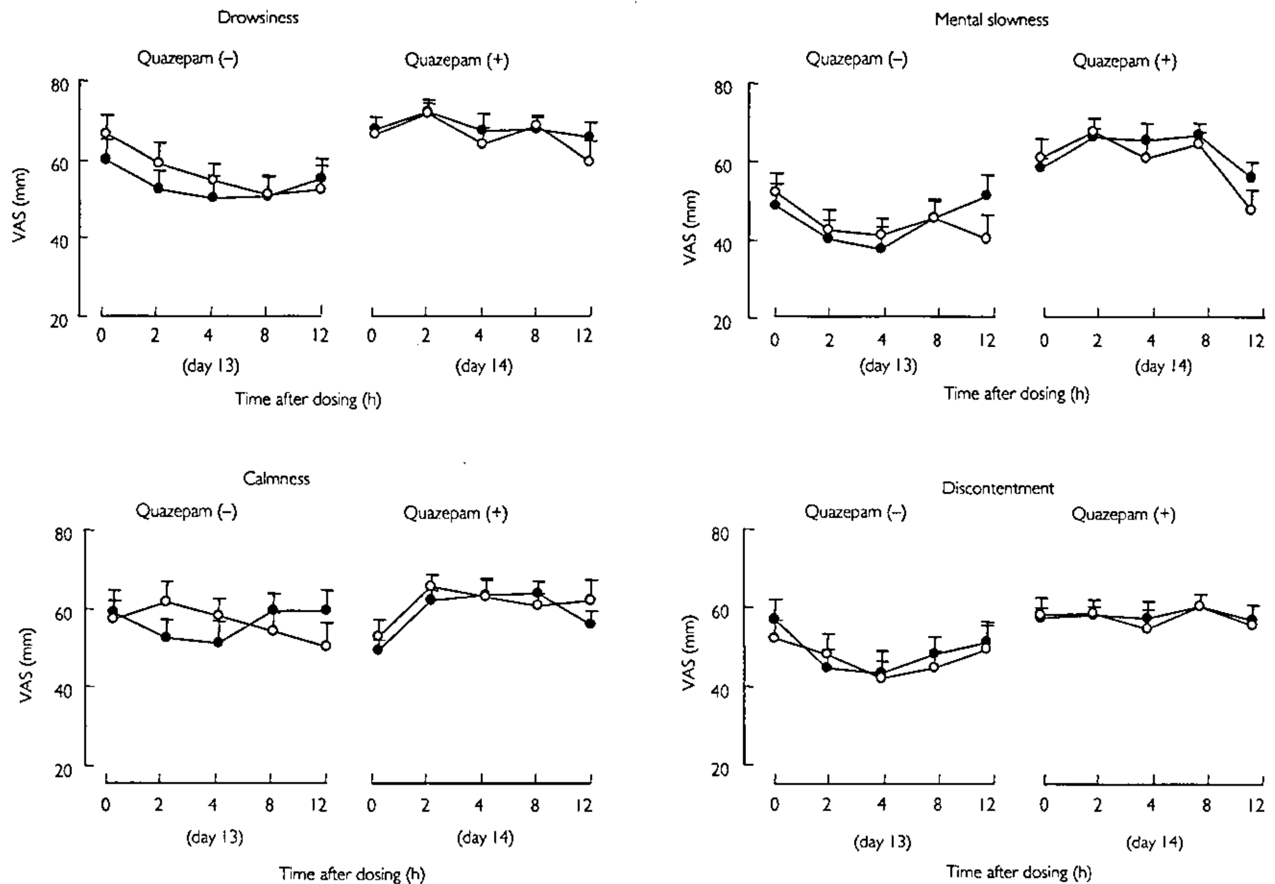


**Figure 2**
Mean ± SE subjective drug effects [based on scores from a 100-mm visual analogue scale (VAS)] before (on day 13) and after (on day 14) a single oral dose of 15 mg quazepam in 13 healthy males. Pretreatment with placebo (●); pretreatment with SJW (O) (900 mg day–1 for 14 days)

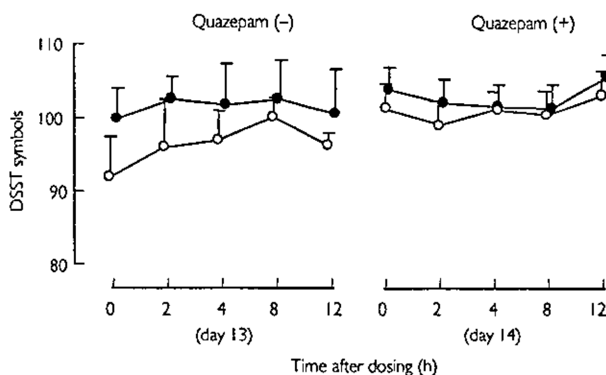


**Figure 3**
Mean ± SE digit symbol substitution test (DSST) scores before (on day 13) and after (on day 14) a single oral dose of 15 mg quazepam in 13 healthy males. Pretreatment with placebo (●); pretreatment with SJW (O) (900 mg day–1 for 14 days)

studies are needed to conclude that SJW impairs psychomotor function after repeated use.

The mechanism of the antidepressant activity of SJW is not fully understood, but inhibition of the GABA receptor-mediated response is involved [33, 36]. On the other hand, benzodiazepines including quazepam stimulate the GABA receptor-mediated response [37]. Therefore, the effect of SJW on the CNS seems to differ from that of benzodiazepines. In fact, the quantitative electroencephalogram showed that SJW increases slow wave activity while benzodiazepines enhance fast wave activity [34, 37]. These data led us to speculate that the pharmacodynamic effects of quazepam might be altered by the repeated dosing with SJW. In this study, quazepam 15 mg produced significant sedative-like effects. Although plasma concentrations of quazepam and 2-oxoquazepam decreased after repeated dosing with SJW, SJW did not reduce the sedative-like effects of quazepam.

In summary, this study showed that SJW decreased plasma concentrations of quazepam, probably by activating CYP3A4, but it did not diminish the pharmacodynamic effects of the drug.

*The authors gratefully thank Dr T. Kotegawa of the Oita Medical University for his helpful suggestions and Ms. C. Fukushima, Ms. T. Kawaguchi, and Ms. M. Hojo for their expert assistance.*

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0022-3565/04/3111-420–425$20.00
The Journal of Pharmacology and Experimental Therapeutics
Vol. 311, No. 1
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68213/1172681
JPET 311:420–425, 2004
Printed in U.S.A.

# Effects of Pravastatin on the Expression of ATP-Binding Cassette Transporter A1

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Received March 11, 2004; accepted June 17, 2004

**ABSTRACT**

In vitro inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase causes the suppression of liver X receptor (LXR) activity. Because LXR regulates the expression of ATP-binding cassette transporter (ABC) A1, which is involved in the high-density lipoprotein-related reverse cholesterol transport pathway, we examined the effects of an HMG-CoA reductase inhibitor pravastatin on ABCA1 expression in vitro and in vivo. Pravastatin (10 μM) significantly reduced the transcript levels of murine ABCA1 gene by 35% in RAW264.7 macrophages under a lipoprotein-deficient condition. The inhibition was due to the decreased mevalonic acid production because addition of exogenous mevalonic acid restored ABCA1 mRNA levels. In addition, cholesterol and 22(*R*)-hydroxycholesterol thoroughly blunted the inhibition. Furthermore, pravastatin did not decrease ABCA1 mRNA and protein levels in HepG2 hepatocytes even in the absence of exogenous LXR agonists. Oral dosing of pravastatin (0.1% concentration in drinking water) for 24 h or 2 weeks to mice did not decrease ABCA1 mRNA and protein levels in the liver and leukocytes. Interestingly, pravastatin significantly increased both hepatic and leukocyte LXRα mRNA levels. Thus, although HMG-CoA reductase inhibitors suppress ABCA1 mRNA expression in the absence of LXR agonists, in vivo inhibition of HMG-CoA reductase is unlikely to cause such suppression.

For over three decades, it is believed that high-density lipoprotein (HDL) protects against atherosclerosis through the action of the reverse cholesterol transport pathway (Glomset, 1968). In this pathway, HDL or its apolipoproteins mediate the removal of excess cholesterol from peripheral cells back to the liver for subsequent excretion into the bile. The recent discovery of the role of ATP-binding cassette transporter (ABC) A1 has given further support for the reverse cholesterol transport hypothesis. Loss of ABCA1 function leads to Tangier disease and familial HDL deficiency, which are genetic disorders characterized by the marked reductions in plasma HDL concentrations and increased risk of cardiovascular disease (Singaraja et al., 2003). Functional defect of ABCA1 in mice has also been reported to induce the absence of HDL (Christiansen-Weber et al., 2000; McNeish et al., 2000), and the inactivation of ABCA1 in macrophages has been shown to increase the susceptibility to atherosclerosis (Aiello et al., 2002; van Eck et al., 2002). Cells isolated from patients with Tangier disease are defective in the process of apolipoprotein-mediated removal of cholesterol and phospholipids (Walter et al., 1994; Francis et al., 1995; Rogler et al., 1995; Remaley et al., 1997). Therefore, it is assumed that ABCA1 transports intracellular cholesterol and phospholipids to cell surface-bound apolipoproteins and forms nascent HDL. Thus, ABCA1 seems to be essential for the first and rate-controlling step in the reverse cholesterol transport pathway.

Investigations on the regulation of ABCA1 gene expression have revealed that ABCA1 is induced in cholesterol-loaded cells as a result of the activation of the nuclear receptors liver X receptor (LXR)/retinoid X receptor heterodimer (Costet et al., 2000; Repa et al., 2000; Venkateswaran et al., 2000a). At least under such conditions, oxysterols seem to serve as the ligands for LXR (Forman et al., 1997; Venkateswaran et al., 2000a). Synthetic LXR agonists can elevate plasma HDL concentrations in experimental animals (Schultz et al., 2000; Cao et al., 2002; Grefhorst et al., 2002). Deficiency of LXR leads to exacerbation of atherosclerosis (Schuster et al., 2002; Tangirala et al., 2002), whereas a synthetic LXR agonist

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.104.068213.

**ABBREVIATIONS:** HDL, high-density lipoprotein; ABC, ATP-binding cassette transporter; LXR, liver X receptor; LDL, low-density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; DMEM, Dulbecco's modified Eagle's medium; LPDS, lipoprotein-deficient bovine calf serum; OHC, hydroxycholesterol; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

exerts antiatherogenic properties probably due to an induction of ABCA1 expression (Joseph et al., 2002). Thus, LXR is potentially a central mediator of ABCA1 action. LXR has two subtypes, LXR$\alpha$ and LXR$\beta$, which are considered to regulate ABCA1 expression (Lund et al., 2003).

It has become common knowledge that the management of low-density lipoprotein (LDL) cholesterol concentrations is important for the prevention of coronary heart disease, and if necessary, treatment with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors for lowering LDL is recommended (National Cholesterol Education Program, 2001). HMG-CoA reductase inhibitors reduce LDL cholesterol concentrations through the blockade of mevalonate pathway and consequent increment of LDL receptor expression in the liver (Goldstein and Brown, 1990). Interestingly, Forman et al. (1997) reported that HMG-CoA reductase inhibitors could inhibit constitutive activity of the LXR$\alpha$-retinoid X receptor heterodimer by the reduction of mevalonic acid in vitro. Although ABCA1 is a notable target of these transcription factors, it remains to be determined whether HMG-CoA reductase inhibitors reduce expression levels of ABCA1. If they suppress ABCA1 activity, combination therapy with such an agent as LXR agonist, which increases ABCA1 activity, might be more effective for the prevention of atherosclerosis. To address this issue, we examined the effects of an HMG-CoA reducatse inhibitor pravastatin on ABCA1 expression in vitro and in vivo.

## Materials and Methods

**Chemicals.** Pravastatin sodium was kindly provided by Sankyo Co. (Tokyo, Japan). Cholesterol was obtained form Wako Pure Chemicals (Osaka, Japan). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

**Cell Culture.** The murine macrophage cell line RAW264.7 and human hepatocyte cell line HepG2 were originally obtained from American Type Culture Collection (Manassas, VA). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or DMEM/Ham's F-12 mixture (Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated fetal bovine serum (Dainippon Pharmaceutical, Osaka, Japan), 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin (Invitrogen) at 37°C in an atmosphere of 5% $CO_2$, 95% air. For the present experiments, cells were seeded in six-well plates and grown to confluence within 2 to 4 days. When the cells became nearly confluent, the medium was replaced by DMEM with 10% lipoprotein-deficient bovine calf serum (LPDS; Biomedical Technologies, Stoughton, MA) and mevalonic acid (0.1 mM). After 24 h, RAW264.7 and HepG2 cells were then incubated for 8 h in DMEM supplemented with 10% LPDS ± pravastatin (1 or 10 $\mu$M) in the presence of either vehicle (dimethyl sulfoxide), mavalonic acid (1 mM), cholesterol (25 $\mu$M), or 22(*R*)-hydroxycholesterol (OHC) (25 $\mu$M), and total RNA was purified.

**Mice and Treatments.** Male C57BL/6 mice were obtained from Japan SLC (Hamamatsu, Japan) at 7 weeks of age and maintained under a specific pathogen-free condition with controlled temperature and humidity and a 12-h light/12-h dark cycle. Mice were given a standard laboratory diet (CE-2; CLEA Japan, Tokyo, Japan) and water ad libitum. After a 7-day acclimation period, animals were divided into three groups and given drinking water with or without pravastatin (0.01 or 0.1%) for 2 weeks. Blood and liver samples were obtained from mice after an 8-h fast (from 6:00 AM to 2:00 PM). All animal procedures were preformed in accordance with the Guideline for Animal Research at Jichi Medical School.

**Serum Cholesterol Measurement.** Serum total cholesterol was determined enzymatically using kits purchased from Wako Pure Chemicals.

**RNA Extraction and Real-Time Quantitative PCR.** The isolation of total RNA was achieved using the RNeasy Mini kit or the QIAamp RNA Blood Mini kit according to the manufacturer's instructions (QIAGEN, Valencia, CA). Reverse transcription was done by 1.2 $\mu$g of total RNA, random hexamer primer, and RevertAid M-MuLV reverse transcriptase (Fermentas, Hanover, MD). The resulting cDNA equivalent to 60 ng of RNA was used for the real-time quantitative PCR in the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). All of specific sets of primers and TaqMan probes in the present study were obtained from Applied Biosystems (Assays-on-Demand Gene Expression Products and TaqMan Rodent GAPDH Control Reagents). All primer sets but that of TaqMan Rodent GAPDH Control Reagents were designed to be located in two exons to avoid the amplification of potentially contaminating genomic DNA. To control for the variation in the amount of DNA available for PCR in the different samples, gene expressions of the target sequence were normalized in relation to the expression of an endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In the present study, no agent affected the mRNA expression of GAPDH: the coefficients of variation of its Ct values were all <3%. Because the efficiency of the target amplification was approximately equal to that of the GAPDH amplification, data were analyzed using comparative threshold cycle method (Su et al., 2002). Because the intra- and interassay coefficients of variation of the relative expression values were <20%, we considered the mean relative values of less than 0.8 or more than 1.2 to be significant in this study.

**Western Blot Analysis.** Cells and tissue samples were lysed in CelLytic MT reagent supplemented with 1% protease inhibitor cocktail. The supernatant was collected after centrifugation and the protein concentrations were determined using the RC DC Protein Assay kit (Bio-Rad, Herclues, CA). Proteins (40 $\mu$g/lane) were separated by SDS-polyacrylamide gel electrophoresis (6% Tris-glycine gel; Invitrogen) and then transferred to polyvinylidene difluoride membranes (Invitrogen). Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20. Membranes were subsequently incubated for 1 h at room temperature with anti-human ABCA1 rabbit polyclonal antibody (Novus Biologicals, Littleton, CO). The antibody, which reacts with both human and mouse ABCA1, was diluted 1000-fold in Tris-buffered saline containing 0.1% Tween 20 and 3% skim milk. After incubation with a secondary horseradish peroxidase-labeled antibody, the blots were visualized using ECL Plus Western Blotting Detection Reagents (Amersham Biosciences Inc., Piscataway, NJ). Quantification of the signals was performed by densitometry using the 1D Image Analysis Software (Eastman Kodak, Rochester, NY).

**Statistics.** Data were analyzed using the Mann-Whitney *U* test or one-way analysis of variance with a post-test of Fisher's protected least significant differences. Values are presented as the means ± S.E., and a *P* value of less than 0.05 was considered significant. All calculations were performed using the computer program StatView, version 5.0 (SAS Institute, Cary, NC).

## Results

**Effects of Pravastatin on Abca1 mRNA Expression Levels in RAW264.7 Cells.** To investigate whether an HMG-CoA reductase inhibitor reduces mRNA expression levels of ABCA1 in vitro, RAW264.7 murine macrophages were cultured for 8 h in DMEM supplemented with 10% LPDS with or without pravastatin. Ten but not 1 $\mu$M pravastatin significantly increased transcript levels of HMG-CoA synthase 1, one of sterol regulatory genes (Goldstein and Brown, 1990), suggesting that it was enough to reduce the metabo-

lites of mevalonate pathway (Fig. 1A). As shown in Fig. 1B, 10 μM pravastatin reduced the transcript levels of murine ABCA1 gene Abca1 by 35%. The inhibition was due to the decreased mevalonic acid production because the addition of exogenous mevalonic acid could restore Abca1 mRNA levels (Fig. 1B). In addition, mevalonic acid itself significantly increased its mRNA levels. Cholesterol also significantly increased Abca1 mRNA levels and compensated for the inhibition by pravastatin. Moreover, the transcript levels of Abca1 were induced >100-fold in response to the LXR agonist 22(*R*)-OHC, and pravastatin did not blunt the effect of 22(*R*)-OHC (Fig. 1B). Figure 1C shows the changes in mRNA expression levels of Abcg1, the gene of another member of ABC transporter superfamily, which is also known to be regulated by LXR (Venkateswaran et al., 2000a,b). In concordance with the results of Abca1, 10 μM pravastatin suppressed Abcg1 mRNA expression by 26% and did not diminish its 6-fold increase by 22(*R*)-OHC. On the other hand, pravastatin did not affect mRNA levels of Abca5, another member of ABCA subclass (data not shown). Because macrophages strongly express LXRα subtype (Lund et al., 2003), we also examined the changes in LXRα expression levels. Whereas pravastatin did not obviously alter LXRα expression itself, 22(*R*)-OHC induced a 1.3-fold increase (Fig. 1D). These results suggest that inhibition of HMG-CoA reductase decreases ABCA1 mRNA expression levels without affecting LXRα transcript levels in the absence, but not in the presence of LXR agonists.

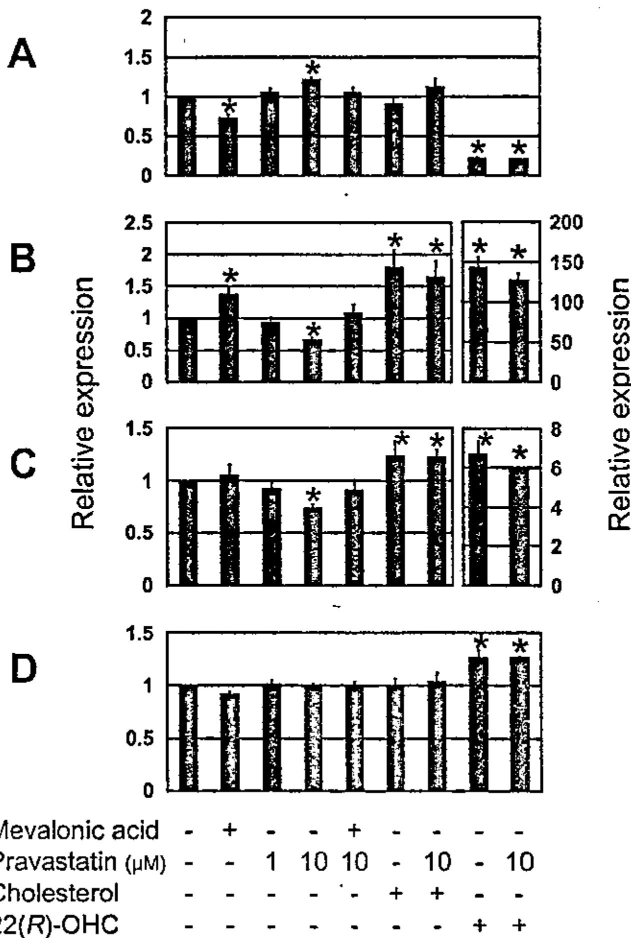


**Fig. 1.** Effects of pravastatin on mRNA expression levels of HMG-CoA synthase 1 (A), Abca1 (B), Abcg1 (C), and LXRα (D) in RAW264.7 macrophages. Nearly confluent RAW264.7 cells were cultured for 24 h in DMEM supplemented with 10% LPDS and mevalonic acid (0.1 mM). The cells were then incubated for 8 h in DMEM supplemented with 10% LPDS ± pravastatin (1 or 10 μM) in the presence of either vehicle (dimethyl sulfoxide), mavalonic acid (1 mM), cholesterol (25 μM), or 22(*R*)-OHC (25 μM), and total RNA was purified. The mRNA expression levels of the target genes were determined by the real-time quantitative reverse transcription-PCR and normalized with GAPDH mRNA levels. Data are means + S.E. of three independent experiments and expressed as relative value to control. *, $P < 0.05$ versus control.

**Effects of Pravastatin on ABCA1 mRNA Expression Levels in HepG2 Cells.** Whereas macrophage ABCA1 may be important for the prevention of atherosclerosis (Aiello et al., 2002; van Eck et al., 2002), hepatic ABCA1 contributes to overall plasma HDL concentrations (Haghpassand et al., 2001; Basso et al., 2003). Therefore, we next investigated the effect of pravastatin in human hepatocytes, HepG2. As shown in Fig. 2A, treatment with pravastatin resulted in a concentration-dependent increase in HMG-CoA reductase mRNA levels. However, contrary to the results in RAW264.7 cells, the treatment did not induce any change of ABCA1 mRNA levels (Fig. 2B). In addition, pravastatin did not affect LXRα mRNA expression levels in HepG2 cells (Fig. 2C).

**In Vivo Effects of Pravastatin on Abca1 mRNA Expression Levels in Mice.** To confirm the above-mentioned findings in vivo, pravastatin was given to mice at 0.01 or 0.1% concentration in drinking water for 2 weeks. Total pravastatin dosages estimated by water consumption were about 20 and 200 mg/kg body weight/day in 0.01 and 0.1% pravastatin groups, respectively. In mice, the lower dosage of pravastatin is high enough to have various pleiotropic effects, although it has no effects on plasma lipid profile (Narisawa et al., 1994; Kwak et al., 2003; Sasaki et al., 2003). Under our condition, neither pravastatin treatment significantly reduced serum total cholesterol concentrations (control, 86.2 ±

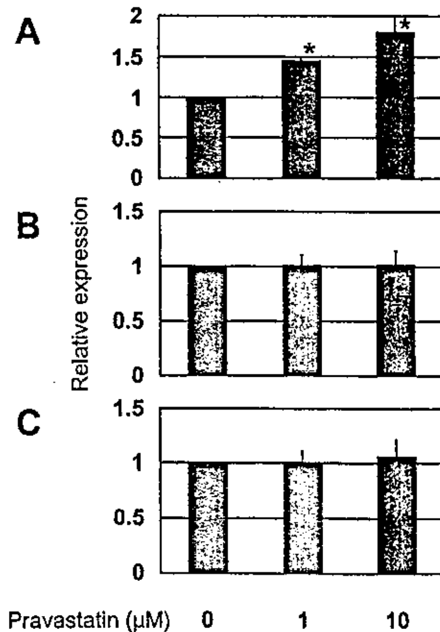


**Fig. 2.** Effects of pravastatin on mRNA expression levels of HMG-CoA reductase (A), ABCA1 (B), and LXRα (C). Nearly confluent HepG2 cells were cultured for 24 h in DMEM supplemented with 10% LPDS and mevalonic acid (0.1 mM). The cells were then incubated for 8 h in DMEM supplemented with 10% LPDS ± pravastatin (1 or 10 μM). Data are means + S.E. of three independent experiments and expressed as relative value to control. *, $P < 0.05$ versus control.