Table 11
Pharmacokinetic alterations of statins following coadministration of CsA or gemfibrozil

	AUC fold increase	C_{max} fold increase
Coadministration of CsA		
Simvastatin	2.6	2.1
	8.0	7.6
Lovastatin	20	–
Pravastatin	7.9	22.8
	5–7	–
Fluvastatin	1.9	1.3
	3.1–3.5	4.1–6.0
Cerivastatin	3.8	5
Atorvastatin	7.4	6.7
Pitavastatin	4.5	6.5
Rosuvastatin	7.1	10.6
Coadministration of gemfibrozil		
Simvastatin	2.5	2.1
Lovastatin	2.8	2.8
Pravastatin	2	1.8
Fluvastatin	1.1	1.1
Cerivastatin	4.2	1.8
	4.4	2.5
Pitavastatin	1.3	1.1
Rosuvastatin	1.9	2.2

–, no report.
References: Arnadottir et al. (1993), Regazzi et al. (1993), Spence et al. (1995), Goldberg and Roth (1996), Olbricht et al. (1997), Muck et al. (1999), Backman et al. (2000), Asberg et al. (2001), Ichimaru et al. (2001), Kyrklund et al. (2001), Mueck et al. (2001), Park et al. (2001), Hasunuma et al. (2003), Kyrklund et al. (2003), Mathew et al. (2004) and Schneck et al. (2004).

gemfibrozil, as shown in Fig. 13(b), gemfibrozil altered $t_{1/2}$ of cerivastain and it is different from the interaction between cerivastatin and CsA. The increase in $t_{1/2}$ can be explained if the hepatic clearance is decreased to a higher extent than the distribution volume (V_d). It suggests that the pharmacokinetic alteration of cerivastatin caused by gemfibrozil is not mainly due to the reduction in the hepatic uptake although it may be involved to a limited extent.

5.2. CYP2C8 inhibitor

Cerivastatin is the only statin which is metabolized by CYP2C8. Our group has shown that the interaction between gemfibrozil and cerivastatin is due to CYP2C8 inhibition by gemfibrozil glucuronide (Shitara et al., 2004). Although gemfibrozil itself also inhibits CYP2C8-mediated metabolism, the IC_{50} values obtained were around 80 μM. This is much higher than its plasma unbound concentration in clinical situations, suggesting that clinically relevant drug–drug interactions are unlikely. On the other hand, gemfibrozil glucuronide is a more potent inhibitor of CYP2C8 whose IC_{50} value is 4 μM. However, this IC_{50} value is still higher than the plasma unbound concentration of gemfibrozil glucuronide in clinical situations (2 μM). We hypothesized that gemfibrozil glucuronide is actively taken up into the liver and concentrated there to high concentration enough to inhibit the CYP2C8-mediated metabolism. More recently, Ogilvie et al. reported that gemfibrozil glucuronide is a metabolism-dependent inhibitor of CYP2C8 (Ogilvie et al., 2006). Its inhibitory effect was enhanced by preincubation in the presence of NADPH with human liver microsomes in an incubation time dependent manner. Their data support it as an irreversible or quasi- irreversible inactivator of CYP2C8. The results by Ogilvie et al. have important implications for the mechanism of the clinically relevant interaction between gemfibrozil and CYP2C8 substrates such as cerivastatin, in addition to the higher concentration of gemfibrozil glucuronide in the liver compared to the plasma. The IC_{50} value for the inhibition of CYP2C8-mediated metabolism by gemfibrozil glucuronide

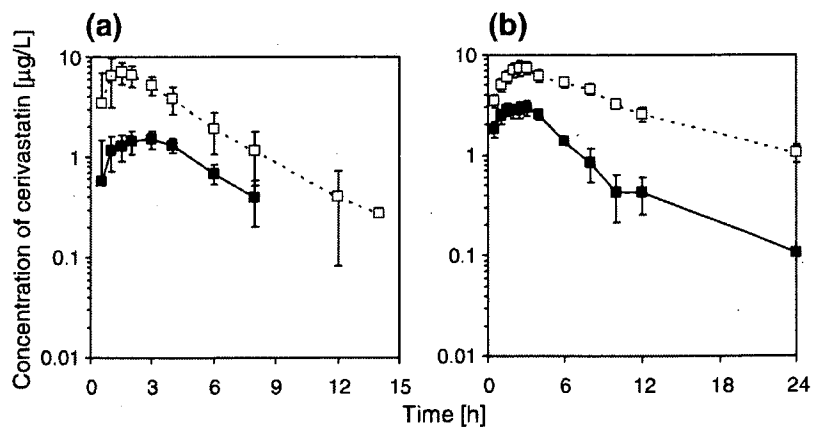

Fig. 13. Altered pharmacokinetics of cerivastatin by coadministrations of CsA (a) or gemfibrozil (b). (a) Plasma concentrations of cerivastatin in kidney transplant recipients receiving CsA treatment (□) or healthy subjects not receiving CsA(■) are shown. In kidney transplant recipients taking CsA, the AUC of cerivastatin was increased 3.8-fold: the C_{max} and $t_{1/2}$ were increased 5.0- and 1.2-fold, respectively. (b) Plasma concentrations of cerivastatin in healthy volunteers concomitantly taking gemfibrozil (□) or placebo (■) are shown. Concomitant administration of gemfibrozil increased the plasma concentration of cerivastatin. The AUC was increased 4.4-fold: the C_{max} and $t_{1/2}$ were increased 2.5- and 2.4-fold, respectively. Reprint from "Increase in cerivastatin systemic exposure after single and multiple dosing in cyclosporine-treated kidney transplant recipients" by Muck et al., 1999, and "Gemfibrozil greatly increases plasma concentrations of cerivastatin" by Backman et al., 2002, with permissions from the American Society for Clinical Pharmacology & Therapeutics.

decreased from 24 to 1.8 μM after 30 min preincubation with human liver microsomes and NADPH. In this case, the rate of inactivation (λ) of CYP2C8 can be described by the following Eq. (34):

$$\lambda = \frac{k_{inact} \cdot [I]}{K_{i,app} + [I]} \quad (34)$$

where k_{inact}, $[I]$ and $K_{i,app}$ are the maximal rate of inactivation, inhibitor concentration and the inhibitor concentration which produces half the maximal rate of inactivation, respectively. They estimated k_{inact} to be 0.21 min^{-1} and $K_{i,app}$ to be 20 and 52 μM with human liver microsomes at the concentration of 0.1 and 1.0 mg/mL, respectively. The fold increase in the oral AUC by coadministration of gemfibrozil was estimated by using the following Eq. (35), which was reported by Lu et al. (2003).

$$\frac{AUC_{po(i)}}{AUC_{po(c)}} = \frac{1}{\left(\frac{f_m \cdot f_{m,P450}}{1+[k_{inact}/K_{i,app} \cdot [I]/k_e]}\right) + \left[1-\left(f_m \cdot f_{m,P450}\right)\right]} \quad (35)$$

where $AUC_{po(i)}$ and $AUC_{po(c)}$ represent oral AUC with or without coadministration of metabolism-dependent inhibitors, and $f_m \cdot f_{m,P450}$ and k_e are fraction of the concomitantly administered drug metabolized by a P450 isoform in interest and the rate constant for enzyme degradation (0.0008 min^{-1}), respectively. For cerivastatin, the $f_m \cdot f_{m,P450}$ for CYP2C8 is 0.61 (Shitara et al., 2004). Thus, the fold increase in the oral AUC of cerivastatin by the metabolic alteration caused by gemfibrozil glucuronide is calculated to be 2.2–2.4 using the maximum plasma concentration (C_{max}) of gemfibrozil glucuronide not bound to plasma protein (2.3 M). It is less than the clinically observed increase in the oral AUC of cerivastatin, that is, 4.4-fold. Thus, this increase may not be explained only by the inhibition of CYP2C8. However, it should be kept in mind that the f_m;$f_{m,P450}$ of CYP2C8 to the total clearance might exhibit interindividual differenceand for some individuals, the inhibition of CYP2C8 mediated metabolism may play more important role. In addition, the inhibition of transporter mediated transporter (hepatobiliary transport, renal uptake and secretion, and intestinal efflux) should be also taken into consideration in understanding the interactions between statins and gemfirozil. Table 11 shows the pharmacokinetic changes in statins produced by coadministration of gemfibrozil. Except for cerivastatin, the effect of gemfibrozil on other statins is small (Table 11). In the cases of simvastatin and lovastatin, the plasma concentration of the open acid form of the statin is affected while that of the lactone form is not. This may be explained by the inhibition of UGT-mediated lactonization (Prueksaritanont et al., 2002b). But, the reported IC_{50} values for the lactonization are also much higher than the therapeutic unbound concentration.

5.3. CYP3A4 inhibitor

As most statins are substrates of CYP3A4, many interactions involving CYP3A4-mediated metabolism have been reported. Table 12 shows the pharmacokinetic changes of statins following coadministration of CYP3A4 inhibitors.

The coadministration of itraconazole changed the plasma concentrations of statins (Fig. 14). It drastically increases the plasma concentration of simvastatin and lovastatin (Neuvonen & Jalava, 1996; Kivisto et al., 1998; Neuvonen et al., 1998). Itraconazole also increases the plasma concentration of atorvastatin (Kantola et al., 1998a; Mazzu et al., 2000). But the effect of itraconazole on the plasma concentration of atorvastatin is smaller than that of simvastatin and lovastatin although the dosing regimens and dosage periods were similar in these different studies and, thus, the different effects could not be attributed to the inhibitor concentrations. This difference will be explained below. On the other hand, itraconazole did not interact with statins, which are not substrates of CYP3A4 (i.e. pravastatin, fluvastatin and rosuvastatin) (Kivisto et al., 1998; Mazzu et al., 2000; Cooper et al., 2003a). In addition, there was no interaction with cerivastatin although it is metabolized by CYP3A4 (Kantola et al., 1999; Mazzu et al., 2000). This is due to the dual metabolic pathway involving CYP3A4 and 2C8 and the smaller contribution of CYP3A4 compared with 2C8 (Shitara et al., 2004). There have been some reports that coadministration of itraconazole causes a higher risk of myotoxicity as well as the pharmacokinetic changes (Vlahakos et al., 2002). This is due to the higher exposure of statins to muscle.

There are also many reports of interactions with macrolide antibiotics. Coadministration of erythromycin results in high plasma concentrations of simvastatin (Kantola et al., 1998b). Erythromycin and clarithromycin increase the plasma concentration of atorvastatin while azithromycin does not (Siedlik et al., 1999; Amsden et al., 2002). This observation is matched by the report that the inhibitory effect of azithromycin is less than erythromycin and clarithromycin (Ito et al., 2003). There are some reports that coadministration of macrolide antibiotics, including erythromycin and clarithromycin, and statins, including simvastatin and lovstatin, results in rhabdomyolysis and, thus, care should be taken when using these combinations (Spach et al., 1991; Grunden & Fisher, 1997; Lee & Maddix, 2001; Kahri et al., 2004; Trieu et al., 2004).

Human immunodeficiency virus (HIV) protease inhibitors also interact with statins. The plasma concentrations of simvastatin and atorvastatin are increased by coadministration of nelfinavir and combined therapy with ritonavir and saquinavir (Hsyu et al., 2001; Fichtenbaum et al., 2002). On the other hand, Fichtenbaum et al. reported that combination therapy involving ritonavir, saquinavir and pravastatin resulted in a reduction in the plasma concentration of pravastatin (58% of control in C_{max}; Fichtenbaum et al., 2002). These HIV protease inhibitors inhibit OATP1A2-mediated transport and this statin may be taken up into the intestine via OATP family transporter (s) (Cvetkovic et al., 1999; Kobayashi et al., 2003). Thus, the HIV protease inhibitors may inhibit the intestinal absorption of pravastatin although the mechanism of intestinal absorption of pravastatin in humans in vivo is unknown. There have been some reports of severe myotoxicity caused by the coadministrations of HIV protease inhibitors and statins, which are

Table 12
Clinically relevant pharmacokinetic alterations of statins following coadministration of CYP3A4 inhibitors

Inhibitor	Substrate	AUC fold increase	C_{max} fold increase	$t_{1/2}$ increase
Itraconazole	Simvastatin	10<	10<	
	Simvastatin acid	19	17	+25%
	Lovastatin	20<	20<	
	Lovastatin acid	20	13	
	Lovastatin	15<	15	
	Lovastatin acid	15	12	
	Pravastatin	<2	<2	–
	Pravastatin	1.51	1.24	+23%
	Fluvastatin	NS	NS	+17%
	Atorvastatin	2.5	1.38	+30%
	Atorvastatin	3	NS	× 3
	Cerivastatin	1.27	1.25	+19%
	Rosuvastatin (10 mg)	1.39	1.36	
	Rosuvastatin (80 mg)	1.28	1.15	
Ketoconazole	Rosuvastatin	1.02	0.954	
Fluconazole	Pravastatin	NS	NS	
	Fluvastatin	1.84	1.44	+80%
	Rosuvastatin	1.14	1.09	
Erythromycin	Simvastatin acid	3.9	5	
	Atorvastatin	1.33	1.38	
	Cerivastatin	NS	NS	
	Rosuvastatin	NS	NS	NS
Clarithromycin	Atorvastatin	1.82	1.56	
Azithromycin	Atorvastatin	NS	NS	
Nelfinavir	Simvastatin acid	6.05	6.17	
	Atorvastatin	1.74	2.22	
Ritonavir+ Saquinavir	Simvastatin acid	30.59	31	
	Atorvastatin	3.47	4.31	
	Pravastatin	0.5	0.58	
Verapamil	Simvastatin acid	2.8	3.4	
Grapefruit juice	Simvastatin	3.6	3.9	
	Simvastatin acid	3.3	4.3	
	Simvastatin	13.4	12	
	Simvastatin acid	4.51	5.03	
	Simvastatin	16	9	
	Simvastatin acid	7	7	
	Lovastatin	15	12	
	Lovastatin acid	5	4	
	Lovastatin	1.94	2.26	
	Lovastatin acid	1.57	1.65	
	Pravastatin	NS	NS	
	Pravastatin	0.92	0.94	
	Atorvastatin	1.4		
	Atorvastatin	2.46	1.06	

NS, not significantly increased.
References: Neuvonen and Jalava (1996), Kantola et al. (1998a, 1998b, 1998c), Kivisto et al. (1998), Lilja et al. (1998), Neuvonen et al. (1998), Kantola et al. (1999), Lilja et al. (1999), Rogers et al. (1999), Siedlik et al. (1999), Lilja et al. (2000), Mazzu et al. (2000), Hsyu et al. (2001), Amsden et al. (2002), Fichtenbaum et al. (2002), Cooper et al. (2003a), Fukazawa et al. (2004) and Lilja et al. (2004).

metabolized by CYP3A4 and, thus, concomitant use of these drugs should be carefully monitored (Cheng et al., 2002; Hare et al., 2002).

The concomitant intake of grapefruit juice also causes an interaction with statins. The plasma concentrations of simvastatin, lovastatin and atorvastatin have been reported to be increased by the intake of grapefruit juice although its effect differs from one statin to another (Kantola et al., 1998c; Lilja et al., 1998, 1999; Rogers et al., 1999; Lilja et al., 2000; Fukazawa et al., 2004; Lilja et al., 2004).

Table 12 shows many CYP3A4 inhibitors increased the AUC and C_{max} of atorvastatin to a lesser extent than those of simvastatin and lovastatin although they are all CYP3A4 substrates (also refer to Fig. 14). This gap may be explained taking the involvement of hepatic uptake transporter(s) into consideration. For atorvastatin, a hydrophilic statin with an open acid form, specific transporter(s) including OATP1B1 are involved in its hepatic uptake (Kameyama et al., 2005; Lau et al., 2006). In this case, the $CL_{int,all}$ can be described by the Eq. (6). Although the change of $PS_{u,influx}$ correlates with $CL_{int,all}$, the change of CL_{int} which includes metabolic clearance does not necessarily correlate with it. On the other hand, for simvastatin and lovastatin, liopophilic statins with lactone forms, their membrane permeabilities are mainly mediated by passive diffusion and not by the transporter-mediated mechanism. In this case, $CL_{int,all}$ can be described by the Eq. (9) and those of simvastatin and lovastatin are directly affected by CYP3A4 inhibitors. Thus, the change of the metabolic rate drastically alters the pharmacokinetics of simvastatin and lovastatin while it makes a smaller alteration in that of atorvastatin.

5.4. CYP2C9 inhibitor

As fluvastatin is mainly metabolized by CYP2C9 and eliminated by this pathway, the effect of CYP2C9 inhibitors could be important. However, there have been few reports of any clinically relevant drug–drug interactions involving fluvastatin and CYP2C9 inhibitors, including omeprazole and tolbutamide, and multiple P450 inhibitors, including cimetidine, fluvoxamine and azole antifungals. On the other hand, there are some reports that fluvastatin affects the pharmacokinetics of other drugs which are metabolized by CYP2C9. For example, fluvastatin produces a slight reduction in the clearance of glyburide, tolbutamide and diclofenac (Appel et al., 1995; Transon et al., 1995). However, it does not affect the plasma concentration of losartan (Meadowcroft et al., 1999). Although fluvastatin affects the plasma concentrations of glyburide and tolbutamide, it has no effects on their hypoglycemic action, which suggests that the pharmacokinetic changes are not great enough to influence their pharmacological effects (Appel et al., 1995).

5.5. Effect of enzyme induction

There are also some reports of drug–drug interactions associated with enzyme induction. When coadministerered with drugs that are enzyme inducers, the plasma concentrations of

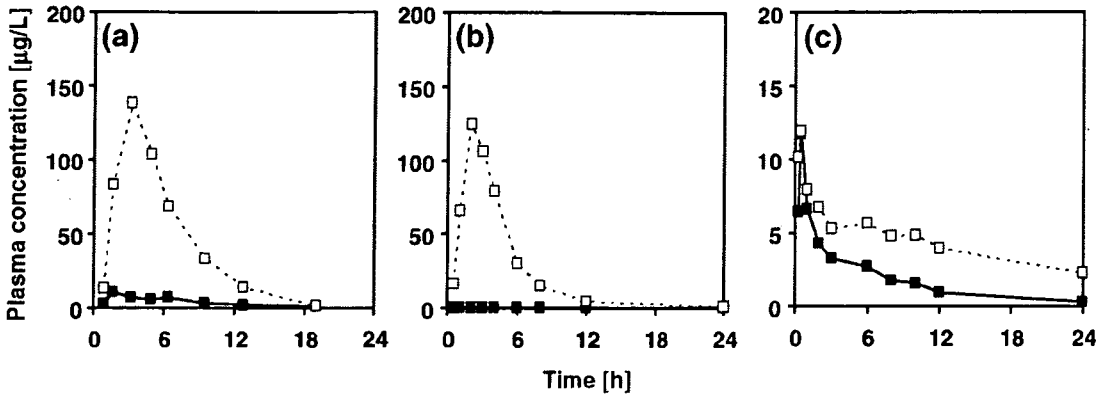

Fig. 14. Plasma concentrations of simvastatin (a), lovastatin (b), atorvastatin (c) with or without coadministration of itraconazole. The plasma concentrations of different statins in humans with (□) or without (■) itraconazole, a potent inhibitor of CYP3A4, are shown. While the plasma concentrations of simvastatin and lovastatin were drastically altered by itraconazole, that of atorvastatin was altered to a much smaller extent. It can be explained by the involvement of active transport system for atorvastatin though no involvement for simvastatin and lovastatin. Reprint from "Itraconazole drastically increases plasma concentrations of lovastatin and lovastatin acid" by Neuvonen and Jalava, 1996, and "Simvastatin but not pravastatin is very susceptible to interaction with the CYP3A4 inhibitor itraconazole" by Neuvonen et al., 1998, with permissions from the American Society for Clinical Pharmacology & Therapeutics.

statins may be reduced, leading to the decrease in their cholesterol-lowering effects.

The effects of rifampicin have been reported. When coadministered with rifampicin, the plasma AUC of simvastatin and simvastatin acid are reduced to 23% and 7% of the controls, respectively (Kyrklund et al., 2000; Niemi et al., 2003). This pharmacokinetic change may be caused by the induction of CYP3A4. In addition, rifampicin reduces the AUC of fluvastatin, a substrate of CYP2C9, to 50% of the control (Jokubaitis, 1994). Interestingly, it also reduces the AUC of pravastatin to 69% of the control although pravastatin is not metabolized by P450 (Kyrklund et al., 2004). It may suggest that rifampicin also induces drug transporters as well as metabolizing enzymes. As rifampicin induces MRP2, the plasma concentration of pravastatin may be affected by the induction of intestinal MRP2, leading to suppression of intestinal absorption (Fromm et al., 2000).

Carbamazepine reduces the plasma concentration of simvastatin by enzyme induction (Ucar et al., 2004). It reduces the AUC of simvastatin and simvastatin acid to 25% and 18% of the controls, respectively.

There was a case report showing that phenytoin reduce s the pharmacological effect of simvastatin (Murphy & Dominiczak, 1999). The serum cholesterol level in patients with hypercholesterolemia taking simvastatin is increased after taking phenytoin although there have been no reports about its plasma concentration (Murphy & Dominiczak, 1999). This can be explained by the induction of CYP3A4, leading to an increase in the clearance of simvastatin.

St. John's wort also reduces the plasma concentration of statins by the induction of CYP3A4. Sugimoto et al. reported that the administration of St. John's wort reduces the plasma AUC of simvastatin and simvastatin acid to 66% and 48% of control, respectively, although it has no effect on pravastatin concentrations (Sugimoto et al., 2001).

6. Interindividual variations in the pharmacokinetics of 3-hydroxy-3-methylglutaryl coenzyme A reducatase inhibitors and changes in their pharmacodynamics associated with pharmacokinetic alterations

6.1. OATP1B1

There have already been some reports of pharmacokinetic alterations of statins associated with the genetic polymorphism of OATP1B1 (Nishizato et al., 2003; Iwai et al., 2004; Morimoto et al., 2004; Mwinyi et al., 2004; Niemi et al., 2004; Tachibana-Iimori et al., 2004). Fig. 15 shows the reported genetic polymorphisms of this transporter and Table 13 shows the allelic frequencies of the variants found in the OATP1B1 gene (Tirona et al., 2001; Nozawa et al., 2002; Nishizato et al., 2003; Niemi et al., 2004). It should be noted that there exist great differences in the allelic frequencies of some SNPs, which may possibly lead to the ethnic differences observed in the pharmacokinetics of some statins. Nishizato et al. and Mwinyi et al. reported the pharmacokinetic alterations in pravastatin associated with the genetic polymorphism of OATP1B1 (Nishizato et al., 2003; Mwinyi et al., 2004; Niemi et al., 2004: Table 14). Nishizato et el. analyzed the relationship between the polymorphism in OATP1B1 and the pharmacokinetics of pravastatin in Japanese subjects (Nishizato et al., 2003; Fig. 16(a)). In the Japanese population, subjects with OATP1B1*1b/*1b are at the most common (Nishizato et al., 2003). Fig. 16(a) shows the plasma concentration of pravastatin in Japanese subjects with OATP1B1*1b/*1b, *1b/*15 and *15/*15. It was higher in subjects having the OATP1B1*15 allele (Fig. 16(a)). The clearance of pravastatin in subjects with *1b/*15 is significantly lower than that in subjects with *1b/*1b. Also, in subjects with *15/*15, the clearance is reduced and the plasma concentration of pravastatin is higher although the difference was not statistically significant due to the small

	217	245	388	411	455	463	467	521	571	578	597	721	1058	1294	1385	1463	1929	1964	2000	
	T	T	A	G	G	C	A	T	T	T	C	G	T	A	A	C	A	A	A	
*1a																				
*1b			G																	*1F, *1G, *1H
*1c					A							A								
*1J, *1K, *1L									C											
*2	C																			
*3		C					G													
*4						A														
*5								C												
*5B								C			T									
*6													C							
*7														G						
*8															G					
*9																C				
*10																		G		
*11																			G	
*12	C																	G		
*13		C					G												G	
*14			G			A														
*15			G					C												
*15B			G					C			T									
*16, *17			G					C			T									
*18			G	A		A			C		T									
*19									C								C			
*20, *21			G								T						C			

Fig. 15. Reported alignment of OATP1B1 haplotypes. The genetic polymorphism of OATP1B1 is summarized.

sample size. They also examined the correlation with the SNPs in OAT3, which is involved in the renal excretion of pravastatin, but they were unable to find any relationship. Mwinyi et al. examined the pharmacokinetics of pravastatin in comparison with the subjects with OATP1B1*1a/*1a (Mwinyi et al., 2004). They found that the plasma AUC of pravastatin in subjects with *1a/*5 was significantly higher than that in those with *1a/*1a. As *15 and *5 commonly have the same variation in T521C, this variation may be involved in the reduced clearance, that is, reduced hepatic uptake of pravastatin. Mwinyi et al. also

Table 13
Allelic frequencies of variations found in the OATP1B1 gene

	European American[1] (n=98)	African American[1] (n=44)	Japanese[2] (n=534)	Japanese[3] (n=240)	Finnish[4] (n=82)
T217C	0.02	0.00	–	–	–
T245C	0.02	0.00	–	–	–
A388G	0.30	0.74	0.534	0.458	0.447
(A388G/T521C)			0.103	0.150	
G411A	–	–	–	–	0.066
A452G	–	–	–	0.038	–
G455A	–	–	0.00	–	–
C463A	0.16	0.02	–	–	0.066
A467G	0.02	0.00		–	
T521C	0.14	0.02	0.007	0.000	0.183
(A388G/T521C)			0.103	0.150	
T571C				–	0.512
T578G				–	0.000
C597T			–	–	0.415
G721A		–		–	–
T1058C	0.02	0.00	–	–	–
A1294G	0.01	0.00	–	–	–
A1385G	0.01	0.00	–	–	–
G1463C	0.00	0.09	–	–	–
A1929C	–	–	–	–	0.085
A1964G	0.02	0.00		–	–
A2000G	0.02	0.34	–	–	–

References: (1) Tirona et al. (2001), (2) Nozawa et al. (2002), (3) Nishizato et al. (2003), (4) Niemi et al. (2004).

Table 14
Pharmacokinetic alterations of pravastatin in subjects with a genetic polymorphism in OATP1B1

Variation	n	C_{max} [ng/mL]	AUC [ng h/mL]	$t_{1/2}$ [h]	k_{el} [h^{-1}]
11187GG	35	46±34	100±69.1	1.6±0.3	
11187GA	6	94±61	200±126.4	1.9±0.2	
521TT	28	40±29	90±64	1.7±0.1	
521TC	11	84±57	180±105	1.6±0.2	
521CC	2	52±19	140±39.3	1.8±0.4	
non-carriers of *15B	37	48±39	110±80	1.7±0.3	
heterozygous of *15B	4	92±57	210±120	1.7±0.3	
non-carriers of *17	38	47±37	110±80	1.6±0.3	
heterozygous of *17	3	120±50	250±70	2.0±0.2	
*1a/*1a	2		61		0.17
*1a/*1b	4		47±27		0.28±0.09
*1b/*1b	4		44±6		0.31±0.09
*1b/*1b	4		44±6		0.31±0.09
*1b/*15	9		62±22		0.26±0.09
*15/*15	1		110		0.21
*1b/*1b	4		44±6		0.31±0.09
*1b/*16	2		110		0.21
*1a/*1a	10	58±38	110±70		
*1a/*1b or *1b/*1b	10	37±21	75±36		
*1a/*1a	10	58±38	110±70		
*1a/*5	10	84±32	160±60		

References: Nishizato et al. (2003); Niemi et al. (2004); Mwinyi et al. (2004).

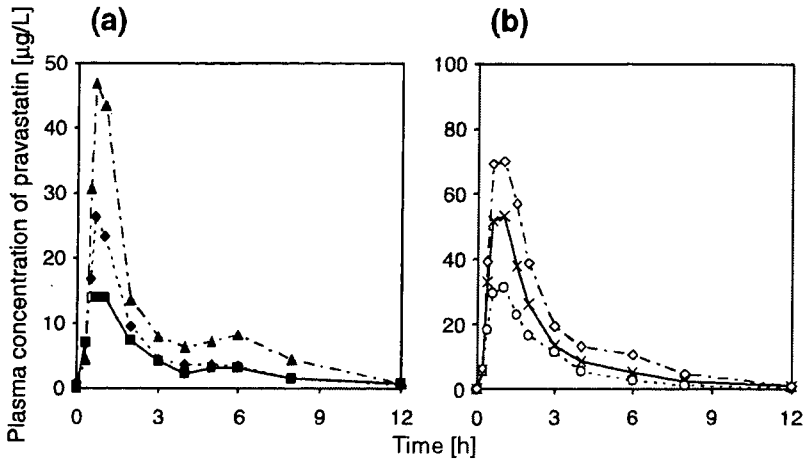

Fig. 16. Plasma concentration of pravastatin in subjects with genetic polymorphism in OATP1B1. (a) The plasma concentration of pravastatin in 23 Japanese male subjects with OATP1B1*1b/*1b (■, $n=4$), *1b/*15 (◆, $n=9$) and *15/*15 (▲, $n=1$) are shown. In subjects having *15 allele, the plasma concentration of pravastatin was increased. (b) The plasma concentration of pravastatin in 30 white male subjects in Germany with OATP1B1*1a/1a (X, $n=10$), *1a/*1b or *1b/*1b (○, $n=8$ for *1a/*1b, $n=2$ for *1b/*1b) and *1a/*5 (◇, $n=10$). In subjects having *5 allele, the plasma concentration was increased comparing to the subjects with *1a/*1a. On the other hand, in subjects having *1b, it was decreased. Reprint from "Polymorphisms of OATP-C (SLC21A6) and OAT3 (SLC22A8) genes: consequences for pravastatin pharmacokinetics" by Nishizato et al., 2003, and "Evidence for inverse effects of OATP-C (SLC21A6) 5 and 1b haplotypes on pravastatin kinetics" by Mwinyi et al., 2004, with permissions from the American Society for Clinical Pharmacology & Therapeutics.

compared the plasma concentration of pravastatin in patients with OATP1B1*1a/*1a and those with *1a/*1b or *1b/*1b. As shown in Fig. 16(b), in patients having *1b, the plasma concentration of pravastatin seems to be lower than that in patients with *1a/*1a. Thus *1b seems to increase the plasma clearance of pravastatin although a statistically significant difference was not observed. Tirona et al. reported that in HeLa cells transfected with cDNA encoding OATP1B1*5, the amount of OATP1B1 protein on the cell surface is reduced although total amount remains unchanged (Tirona et al., 2001). In addition, the transport activity in OATP1B1*5 is significantly reduced in the case of estradiol 17β-D-glucuronide and estrone 3-sulfate. In the case of estrone 3-sulfate, the maximum uptake rate, reflecting the transport capacity, is significantly reduced. On the other hand, Iwai et al. reported that the total protein expression and transport activity in *15 are markedly lower although the corresponding values in *5 are not altered in cDNA transfected HEK293 cells (Iwai et al., 2004). However, they did not determine the transporter expression level on the cell surface. These results suggest that allelic variations *5 and *15 are associated with reduced intrinsic transport acitivity and/or protein expression level on the cell surface, which would explain the in vivo phenomenon. Michalski et al. also showed that there is altered transporter expression on the plasma membrane associated with genetic polymorphism in human liver tissue samples (Michalski et al., 2002).

Tachibana-Iimori et al. carried out a retrospective cohort study of the pharmacological effect of statins in subjects with different OATP1B1 alleles (Tachibana-Iimori et al., 2004; Table 15). They found that the reduction in the total serum cholesterol was reduced in patients with the T521C variation. This variation was associated with higher plasma concentrations of pravastatin as reported by Nishizato et al. and Mwinyi et al., suggesting higher exposure (Nishizato et al., 2003; Mwinyi et al., 2004). Thus, in patients with higher plasma concentration of statins, the cholesterol lowering effect was decreased. It can be explained by our estimation that, as shown in the Section 4, the AUC of protein unbound statins in the liver is decreased by the reduction in the hepatic uptake rate when the renal excretion is not negligible (Fig. 12; also see Eq. (32)). Tachibana-Iimori et al. investigated patients taking statins including pravastatin, atorvastatin and simvastatin (Tachibana-Iimori et al., 2004). Among them, the renal excretion of pravastatin is not negligible, allowing them to observe different pharmacological effects of statins associated with SNPs in OATP1B1. In addition, even for statins with a very low renal clearance, the hepatic uptake rate affects the liver concentration–time profile of statins (Fig. 12). This change may affect the pharmacological effect.

Genetic polymorphism in the hepatic uptake transporter(s) may also lead to a change in the frequency of the side effects of

Table 15
Association of lipid-lowering effect by statins and OATP1B1 polymorphism

	T521C	n	Pre (mg/dL)	Post (mg/dL)	% Change	p
TC	TT	44	260±40	200±30	−22	<0.05
	TC	20	260±30	210±30	−17	0.094
LDL-C	TT	39	170±40	120±30	−29	0.745
	TC	20	160±50	120±20	−12	0.492
HDL-C	TT	38	56±15	57±14	1.2	
	TC	20	63±26	65±17	11	
TG	TT	40	170±90	130±70	−11	
	TC	19	150±100	130±60	3.4	

TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; CI, confidence interval; UL, upper limit; LL, lower limit.
Reference: Tachibana-Iimori et al. (2004).

statins. Morimoto et al. found a novel polymorphism in OATP1B1 (T1628G; L543W) in patients with myopathy who were taking pravastatin (Morimoto et al., 2004). They also reported an association between OATP1B1*15 and pravastatin- or atorvastatin-induced myopathy (Morimoto et al., 2005a, 2005b). This may be explained by the different exposure to pravastatin due to the altered pharmacokinetics associated with the polymorphism in OATP1B1.

6.2. CYP2C8

CYP2C8 is involved in the pharmacokinetics of cerivastatin (Muck, 2000; Shitara et al., 2004). Ishikawa et al. analyzed genetic polymorphisms in OATP1B1, CYP2C8 and CYP3A4, which are determinants of cerivastatin pharmacokinetics, in patients with rhabdomyolysis taking cerivastatin and found variations in CYP2C8 (Ishikawa et al., 2004). They identified 475delA, G874C and T1551C and, among them, 475delA was found to be homozygous. As the 475delA alteration generally completely deletes the function of CYP2C8, this should result in a drastic reduction in the cerivastatin elimination rate although there have not been clinical data showing the change in pharmacokinetics and/or pharmacodynamics associated with such genetic polymorphisms. As the contribution of CYP2C8 to the metabolism of cerivastatin is relatively high, the altered function of CYP2C8 should lead to severe side effects. Hichiya et al. described other SNPs and, among them, R186X and R186G were associated with a change in the amino acid sequence, resulting in altered function (Hichiya et al., 2005).

6.3. CYP3A4/3A5

CYP3A4 is involved in the metabolism of many statins. Thus, a genetic polymorphism in CYP3A4 can be associated with the pharmacokinetic, pharmacodynamic and toxic effects of many statins. To date, there have been no reports of pharmacoikinetic changes in statins produced by SNPs in CYP3A4. However, there are some reports of a changed pharmacological effect associated with SNPs in CYP3A4 (Kajinami et al., 2004a; Wang et al., 2005). Table 16 shows these reports. Wang et al. reported stimulation of the pharmacological effect of simvastatin by the genetic variation of I118V in CYP3A4, which resulted in reduced metabolism (Wang et al., 2005). In Section 4, we showed that reduced metabolism (CL_{met}) results in an increase in the plasma concentration and the AUC of statins and enhancement of the pharmacological effect. Thus, the clinical report by Wang et al. agrees with our simulation analysis. Kajinami et al. examined the effects of a variation in the promoter region, A-290G, and amino acid substitution, M455T, on the pharmacological effect of atorvastatin (Kajinami et al., 2004a). They found a significant reduction in the pharmacological effect only in patients with A-290G (Table 16). The variation A-290G might alter the transcription of CYP3A4. However, they did not measure the enzyme activity. Their report was not confirmed by our simulation showing that a reduction in the metabolic rate should be associated with enhancement of

Table 16
Association of lipid-lowering effect by statins and CYP3A4/3A5 polymorphism

(a) Association of the effect of simvastatin and CYP3A4 Ile118Val

	Ile118Val	n	Pre (mmol/L)	Post (mmol/L)	% Change	p
TC	Ile/Ile		5.6±0.8	4.4±0.7	−22±20	0.034
	Ile/Val		5.1±0.8	3.1±0.7	−36±10	
LDL-C	Ile/Ile		3.5±0.7	2.5±±0.6	−29±7	0.072
	Ile/Val		3.2±0.9	2.0±.04	−37±9	
TG	Ile/Ile		2.3±1.0	1.9±0.6	·25±8	0.002
	Ile/Val		2.0±0.8	1.4±0.4	·38±8	

(b) Association of the effect of simvastatin and CYP3A4 A-290G

	A-290G	n	Pre (mg/dL)	Post (mg/dL)	% Change	p AA vs. AG	p (AA+AG) vs. GG
TC	AA	305	272±26	199±26	−27±8	0.604	0.385
	AG	27	262±22	190±24	−29±9		
	GG	8	269±19	203±29	−24±12		
LDL-C	AA	305	187±22	117±19	−37±10	0.372	0.113
	AG	27	190±23	116±18	−38±10		
	GG	8	193±15	131±21	−31±14		
HDL-C	AA	305	50±11	54±12	+8±12	0.182	0.672
	AG	27	47±10	49±10	+6±15		
	GG	8	47±6	51±7	+9±16		
TG	AA	305	175±72	139±59	16±31	0.096	0.34
	AG	27	159±63	125±53	19±19		
	GG	8	147±46	103±38	·27±24		

(c) Association of the effect of atorvastatin and CYP3A4 Met445Thr

	Met445Thr	n	Pre (mg/dL)	Post (mg/dL)	% Change	p
TC	Met/Met	335	270±30	200±30	−27±8	0.542
	Met/Thr	5	260±20	180±20	−29±6	
LDL-C	Met/Met	335	190±20	120±20	−37±10	0.469
	Met/Thr	5	170±30	100±20	−40±14	
HDL-C	Met/Met	335	50±11	53±12	+8±13	0.662
	Met/Thr	5	50±11	56±16	+10±22	
TG	Met/Met	335	170±70	140±60	−17±30	0.856
	Met/Thr	5	200±130	150±80	−15±43	

(d) Association of the effect of lovastatin, simvastatin or atorvastatin and CYP3A5 polymorphisms

	Variation	n	Pre (mmol/L)	Post (mmol/L)	% Change	p
TC	*1/*3	7	6.2±0.9	5.1±1.0	17±20	0.026
	*3/*3	39	6.0±0.8	4.1±0.6	·31±13	
LDL-C	*1/*3	7	3.9±0.6	2.5±0.8	−31±26	0.083
	*3/*3	39	3.9±0.6	2.1±0.5	−46±18	

(e) Association of the effect of fluvastatin or pravastatin and CYP3A5 polymorphisms

	Variation	n	Pre (mmol/L)	Post (mmol/L)	% Change	p
TC	*1/*3	6	5.5±0.2	4.3±0.5	−22±7	0.99
	*3/*3	17	5.8±1.0	4.5±0.5	·21±8	
LDL-C	*1/*3	6	3.2±0.4	2.1±0.4	−35±8	0.90
	*3/*3	17	3.6±0.8	2.4±0.5	·34±14	

TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol.
References: Kajinami et al. (2004a), Kivisto et al. (2004b) and Wang et al. (2005).

the pharmacological effect. However, in patients with SNPs in CYP3A4, the serum cholesterol level may be different due to the altered metabolism of endogenous hormones metabolized by CYP3A4. This may account for the discrepancy between our simulation and the clinical data.

Interestingly, Kivisto et al. have described the effect of a polymorphism in CYP3A5 (Kivisto et al., 2004b). Due to a small sample size, they analyzed the effect of polymorphism in CYP3A5 on total cholesterol and LDL-cholesterol lowering effects of statins in patients dividing into 2 groups: lovastatin, simvastatin or atorvastatin taking group and fluvastatin or pravastatin taking group; although the effect may be different between lovastatin or simvastatin group and atorvastatin group due to the involvement of the hepatic uptake transporter(s) as described in the Section 5.3. In patients with reduced activity of CYP3A5 produced by the *3 variant, enhanced pharmacological effects of lovastatin, simvastatin and atorvastatin were reported. On the other hand, the effects of pravastatin and fluvastatin were unchanged because they are not metaobolized by CYP3A4. The enhancement of pharmacological effects associated with the reduced metabolism was matched by the report by Wang et al. (2005) and is fully explained by our analysis given in the Section 4.

6.4. CYP2C9

CYP2C9 is involved in the metabolism of fluvastatin and, thus, interindividual differences in this enzyme may result in changes in the pharmacokinetics of this statin. To date, there has been only one report by Kirchheiner et al. (2003) concerning the effect of a genetic polymorphism in CYP2C9 on the pharmacokinetics and pharmacodynamics of fluvastatin. Table 17 shows the results. In patients with CYP2C9*3/*3, the plasma concentration is increased whereas the pharmacological effect is reduced. The pharmacokinetic and pharmacodynamic changes conflicted and the mechanism is unknown.

7. Conclusion

In this review, we have described the pharmacokinetic properties and physicochemical features of the statins. In addition, we have performed a model-based analysis and shown how sensitively the pharmacokinetic alterations are caused by a change in the metabolizing enzymes and/or transporters. In the case of combination therapy with statins, their elimination pathways and mechanism, which includes the metabolizing enzyme(s) and transporter(s) involved in their

Table 17
Association of lipid-lowering effect and pharmacokinetics of fluvastatin, and CYP2C9 or CYP2D6 polymorphisms

(a) Association of the pharmacokinetics of fluvastatin and CYP2C9 or CYP2D6 polymorphisms

	n	(−)-3*S*,5*R*-fluvastatin				(+)-3*R*,5*S*-fluvastatin			
		AUC (μg h/L)	C_{max} (μg h/L)	$t_{1/2}$ (h)	t_{max} (h)	AUC (μg h/L)	C_{max} (μg/L)	$t_{1/2}$ (h)	t_{max} (h)
CYP2C9*1/*1	5	230±130	200±130	0.6±0.3	1.0±0.3	170±90	120±80	0.8±0.4	1.1±0.4
CYP2C9*1/*2	4	210±40	160±70	0.6±0.3	1.1±0.3	200±40	110±40	1.3±0.8	1.3±0.3
CYP2C9*2/*2	3	290±110	150±110	1.0±0.4	1.8±1.0	180±100	81±54	1.3±0.6	1.8±1.0
CYP2C9*1/*3	5	360±100	300±90	0.5±0.1	1.1±0.3	230±90	170±60	0.6±0.1	1.1±0.3
CYP2C9*2/*3	4	520±190	200±90	1.5±0.7	1.4±0.5	390±120	120±50	2.0±0.4	1.3±0.6
CYP2C9*3/*3	3	1100±300	490±110	1.4±0.6	1.0±0.1	530±120	250±40	0.9±0.3	1.0±0.1
CYP2C9*1/*1 and CYP2D6*4/*4	2	300±220	350±220	0.4±0.2	0.5±0.0	210±170	220±160	0.4±0.1	0.5±0.0

(b) Association of pharmacodynamic effect of fluvastatin and CYP2C9 or CYP2D6 polymorphisms

		n	Baseline (mg/dL)	Difference (mg/dL)	% Change
TC	CYP2C9*1/*1, *1/*2 and *2/*2	12	210±40	−35±25	16
	CYP2C9*1/*3 and *2/*3	9	200±40	−36±22	18
	CYP2C9*3/*3	3	180±20	2±19	+1.1
	CYP2D6*4/*4 (CYP2C9*1/*1)	2	200±20	3±16	+1.5
TG	CYP2C9*1/*1, *1/*2 and *2/*2	12	93±26	15±40	+16
	CYP2C9*1/*3 and *2/*3	9	110±70	18±41	+16
	CYP2C9*3/*3	3	57±13	20±19	+35
	CYP2D6*4/*4 (CYP2C9*1/*1)	2	110±70	3±41	+2.8
HDL−C	CYP2C9*1/*1, *1/*2 and *2/*2	12	63±21	0±7	0
	CYP2C9*1/*3 and *2/*3	9	64±14	1±6	+1.6
	CYP2C9*3/*3	3	68±12	4±2	+5.9
	CYP2D6*4/*4 (CYP2C9*1/*1)	2	55±6	7±4	+13
LDL-C	CYP2C9*1/*1, *1/*2 and *2/*2	12	130±30	−38±24	−29
	CYP2C9*1/*3 and *2/*3	9	110±30	−34±21	−30
	CYP2C9*3/*3	3	100±10	−6±18	−6.1
	CYP2D6*4/*4 (CYP2C9*1/*1)	2	120±0	−4±12	−3.3

Reference: Kirchheiner et al. (2003).

elimination, need to be known in order to predict and avoid any drug–drug interactions. The contribution of each enzyme or transporter in the liver, intestine and kidney to the total elimination and absorption also needs to be considered. Prior to the coadministration with other drugs, a consideration should be given to their effects on these enzymes or transporters. The inhibitory effects of other drugs must be predicted by comparison of K_i and therapeutic unbound concentrations. If the unbound concentration of the inhibitor drug(s) in clinical situations is much lower than K_i, a drug–drug interaction is unlikely to occur. The concentration of statins in the tissue is also important for the estimation of the extent of changes in pharmacological and toxicological effect caused by drug–drug interactions because the tissue concentration is their determinant. For the development of new statins, the balance of safety versus toxicity is also important. In other words, the pharmacokinetic properties, that is, selective distribution to the pharmacological target organs (liver for statins) but not to the toxic organs (muscle and brain), as well as the intrinsic potency of the pharmacological effect, that is, the affinity for the HMG-CoA reductase, need to be taken into consideration.

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Effect of Aminoguanidine on Lipopolysaccharide-Induced Changes in Rat Liver Transporters and Transcription Factors

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To determine the role of nitric oxide (NO) in rat liver transporter regulation, we investigated whether NO mediates lipopolysaccharide (LPS)-induced changes in transporters and their transcription factor expression using aminoguanidine (AG), an inhibitor of induced nitric oxide synthase (iNOS). We confirmed that LPS decreased mRNA levels for Ntcp, Oatp1, Oatp2, Oatp4, Oct1, Mrp2, Mdr1a and increased those for Mdr1b at 16 h after administration. AG attenuated these decreases for Ntcp, Oatp1 and Oatp4 (retinoid X receptor (RXR)α- and hepatocyte nuclear factor (HNF)4α-dependent genes) and increase for Mdr1b (nuclear factor (NF)-κB-dependent gene). Concomitantly, it suppressed LPS-induced NF-κB-dependent gene transcription, such as those for proinflammatory cytokines (cytokines; tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6) and iNOS, and also suppressed IL-1β release from Kupffer cells (KCs) at post-translational levels, but had little effect on the LPS-induced decreases in RXRα and HNF4α transcriptional activities. These findings indicate that hepatocytes were stimulated directly by LPS, which lead to the activation of NF-κB and reduction of RXRα and HNF4α transcriptional activities as early responses, and indirectly by cytokines and NO released from KCs *via* activation of NF-κB by LPS as delayed responses. We conclude that AG, which suppresses LPS-induced NF-κB activation in both hepatocytes and KCs and then the release of cytokines and NO from KCs, attenuates LPS-induced changes of Ntcp, Oatp1, Oatp4 and Mdr1b transcription in hepatocytes. The roles of cytokines and NO could not be distinguished, however. Further *in vitro* study is needed to clarify the role of NO in transporter regulation.

Key words rat liver transporter regulation; lipopolysaccharide; aminoguanidine; nuclear factor κB; nuclear receptor

Lipopolysaccharide (LPS) administration in the rat produces a sepsis model of cholestasis which is directly associated with changes in the expression of several liver transporters.[2)] Among the various constitutively expressed influx (Ntcp, Oatp1, Oatp2, Oatp4, Oct1, Oat2, Oat3) and efflux transporters (Mrp2, Mrp3, Bsep, Mdr1a, Mdr1b) in rat liver, LPS down-regulates most of the influx and efflux transporters, but up-regulates some efflux transporters, including Mrp3 and Mdr1b. These changes are thought to represent a defense mechanism which protects the liver from the accumulation of endogenous compounds such as bile acid and bilirubin, as well as exogenous toxic compounds.

With regard to mechanism, LPS induces the production of proinflammatory cytokines (cytokines), such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 in Kupffer cells (KCs), and nitric oxide (NO) *via* the induction of NO synthase (iNOS) in KCs and hepatocytes. These cytokines are thought to be major mediators of the down-regulation of mRNA of transporters such as Ntcp, Oatp1, Oatp2, Mrp2, Mrp3, Bsep and Mdr1a, and of the up-regulation of Mdr1b.[3—9)] Extensive studies of the molecular mechanisms of transporter regulation show that the LPS-induced down-regulation of transporters is strongly affected by the preceding decrease in the quantity or function of nuclear transcription factors such as hepatocytes nuclear factors (HNF1α, HNF4α) and nuclear receptor heterodimers with retinoid X receptor (RXR)α (retinoic acid receptor (RAR))α, pregnane X receptor (PXR), farnesoid X receptor (FXR), constitutive ardrostane receptor (CAR)).[10)]

In contrast, relatively few studies have investigated the role of NO derived from iNOS in the regulation of hepatic transporters. Among studies to date, a regulatory role of NO on the mRNA of Oat2 was identified in *in vivo* experiments with LPS (1 mg/kg) and aminoguanidine (AG, 20 mg/kg, an iNOS inhibitor).[11)] On the contrary, AG (100 mg/kg) had no effect on LPS (4 mg/kg)-induced changes in rat liver transporter mRNA levels.[2)] Nevertheless, NO's effect in decreasing transcriptional activities of RXRα[12)] and HNF4α[13)] indicate its possible role as a mediator of transporter genes.

Here, we investigated the role of iNOS-derived NO in the regulation of mRNA expression of rat liver transporters (Table 1; Ntcp, Oatp1, Oatp2, Oatp4, Oat2, Oat3, Oct1, Mrp2, Mrp3, Bsep, Mdr1a, Mdr1b) by examining the *in vivo* effect of a high dose of AG (400 mg/kg) on LPS-induced changes in liver transporters. Further, we also examined

Table 1. Nomenclature for Rat Liver Transporters

		Gene symbol
Influx transporters		
Ntcp	Na^+-dependent taurocholate transporter	Slc10a1
Oatp1 (Oatp1a1)	Organic anion transporting peptide 1	Slc21a1
Oatp2 (Oatp1a4)	Organic anion transporting peptide 2	Slc21a5
Oatp4 (Oatp1b2)	Organic anion transporting peptide 4	Slc21a10
Oat2	Organic anion transporter 2	Slc22a7
Oat3	Organic anion transporter 3	Slc22a8
Oct1	Organic cation transporter 1	Slc22a1
Efflux transporters		
Mrp2	Multidrug resistance protein 2	Abcc2
Mrp3	Multidrug resistance protein 3	Abcc3
Bsep	Bile salt export pump	Abcb11
Mdr1a	Multiple drug resistance protein 1a	Abcb1a
Mdr1b	Multiple drug resistance protein 1b	Abcb1b

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whether the speculated transcription factors participate in NO-related regulation of transporters.

MATERIALS AND METHODS

Animals and Treatment Four groups of male Sprague-Dawley rats weighing 200—300 g (Saitama Experimental Animal Supply; Saitama, Japan) were treated by i.p. injection with AG (400 mg/kg; Sigma-Aldrich, MO, U.S.A.) or saline, followed 30 min later by i.p. injection of LPS (1 mg/kg, *Escherichia coli*, serotype 0111:B4, Sigma-Aldrich) or saline to provide control, LPS, AG/LPS and AG groups. The animals were bled by direct cardiac puncture under ether anesthesia 4 or 16 h after LPS or saline and the plasma was stored at −80 °C. The liver was excised, perfused with cold saline, snap-frozen in liquid nitrogen and stored at −80 °C until use. The present study was approved by the Animal Research Committee of Showa University.

Plasma Analysis Total NO_2^-/NO_3^- levels (stable NO oxidative metabolites) were determined by Griess assay after the reduction of NO_3^- to NO_2^- with nitrate reductase.[14] Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and total bile acid levels were assayed using the Transaminase CII-test Wako and total bile acids-test Wako, respectively (Wako Pure Chemical Industries, Osaka, Japan).

Cytokine Analysis TNF-α, IL-1β and IL-6 levels in plasma and the cytoplasmic fraction of liver were analyzed using enzyme-linked immunosorbent assay (ELISA) kits (BioSource International Inc., CA, U.S.A.).

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis Total liver RNA was subjected to reverse transcription followed by semi-quantitative PCR[15] or real-time PCR using the gene-specific primers shown in Tables 2 and 3, respectively. Real-time PCR was conducted with qPCR Master Mix Plus for SYBR green I (Nippon Gene, Tokyo, Japan) and human β-actin cDNA as a standard template. Thermal cycling conditions were 10 min at 95 °C, 40 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s on an ABI Prism 7000 sequence detection system (Applied Biosystem, Tokyo, Japan). mRNA levels were expressed as a ratio to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western Blot Analysis Liver protein was extracted to measure transporters according to Cao *et al.*[24] Nuclear and cytoplasmic proteins of the liver to measure transcription factors and cytokines were prepared using a CelLytic™ NuCLEAR™ Extraction kit (Sigma-Aldrich) by a slight modification of the manufacturer's protocol with a buffer containing 10 μM MG132 (Sigma-Aldrich), which was added to protect RXRα and HNF4α from degradation by proteasome during extraction. Protein concentration was determined according to the method of Bradford.[25]

After mixing with 2×loading buffer, liver protein (50—100 μg) was left at room temperature while nuclear (20 μg) and cytoplasmic protein (30 μg) were heated at 95 °C for 5 min. Proteins were separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking in phosphate buffered saline (PBS) containing 0.1% Tween 20 and 5% nonfat dry milk, the membranes were probed with a primary antibody. Blots were then incubated in a peroxidase-conjugated second antibody and visualized with an enhanced chemiluminescence (ECL) Advance Western Blotting Detection kit (Amersham Biosciences, Buckinghamshire, U.K.). Signals were detected with an ATTO Cool Saver, and the band intensities were determined using an ATTO Lane Analyzer (ATTO, Tokyo, Japan).

The primary antibodies used were RXRα, HNF1α and HNF4α (Santa Cruz Biotechnology, CA, U.S.A.) and GAPDH (Chemicon International, CA, U.S.A.). Anti-transporter polyclonal antibody was prepared by immunizing rabbits with the peptide-keyhole limpet hemocyanin conjugate (Hokkaido System Science, Hokkaido, Japan). The peptides were 14 amino acids (CLGEKESEHTDVHG) for Oatp1,[26] 12 amino acids (CTEVLRSKVTED) for Oatp2,[27] 17 amino acids (CNGYYCVPYDEQSNETPL) for Oatp4[28] and 15 amino acids (CLQPGPGTHNGNIPP) for Ntcp.[29]

Analysis of DNA-Binding Activity DNA-binding activities of HNF1 and HNF4α were measured with nuclear protein (10 μg) using a TransAM™ HNF family kit from Active Motif (CA, U.S.A.).

Statistics Statistical analyses were conducted using Student's *t*-test, with differences considered significant at $p<0.05$. Results are expressed as mean±S.E.M.

RESULTS

Effect of AG on LPS-Induced NO Production AG (400 mg/kg) was selected based on its competitive inhibition of an LPS-induced increase in NO production and ability to decrease iNOS mRNA levels.[30] As expected, AG attenuated the LPS-induced increases in plasma NO levels (16 h) and liver iNOS mRNA levels (4 h) to 19.2% and 18.7% of that in the LPS groups, respectively (Fig. 1). Further, AG at 100 and 300 mg/kg reduced NO production to 76% and 61% of that in the LPS groups at 16 h, respectively.

Plasma ALT and AST activities were measured at 16 h after LPS to examine the liver toxicity of AG (400 mg/kg) and LPS (1 mg/kg). The LPS and AG/LPS groups showed slightly lower ALT activities and slightly higher AST activities than the controls (Table 4). These small changes in ALT and AST activities indicated that LPS and AG show no liver toxicity. Further, total bile acid plasma levels at 16 h tended to be lower in the AG/LPS than in the LPS groups (Table 4).

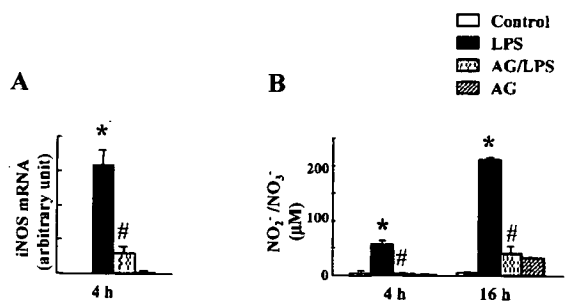

Fig. 1. Effect of AG on LPS-Induced Liver iNOS mRNA and Plasma NO_2^-/NO_3^-

Rats were treated with saline or AG (400 mg/kg) 0.5 h before saline or LPS (1 mg/kg). (A) Relative liver iNOS mRNA levels. mRNA data normalized to those of β-actin are expressed in arbitrary units. (B) Plasma total NO_2^-/NO_3^- levels. Each bar represents mean±S.E.M. (n=4—6). *$p<0.05$ compared with control groups. #$p<0.05$ compared with LPS groups.

Table 2. Primer Sequences for Semi-Quantitative PCR Assay

Target sequence (μl)[a]	Sequences (5′→3′)		
RXRα	324—343	CACTCGCCTATCAGCACCCT	NM_012805
(0.1)	1394—1374	GCAGTACGCTTCTAGTGATGC	
RARα	1117—1134	CAGATGCACAACGCTGGC	ref. 16
(0.2)	1513—1496	CCGACTGTCCGCTTAGAG	
CAR	377—396	TTTGCTGGGAGGTGTGAGGT	NM_022941
(0.2)	787—768	TAGGGAACGGAAAAGGGGCA	
PXR	208—228	AAACCTGGAGATGAGACCTGA	AF151377
(0.0375)	997—977	GTGGGATATGACTTTGGCGAA	
FXR	111—131	ATTTGAAGACCACCATCCCAG	NM_021745
(0.2)	740—720	AACATTCCCATCTCTCTGCAC	
HNF4α	185—206	AGTGCCCTGTGTGCCATCTGTG	ref. 17
(0.1)	896—874	AGATGATGGCTTTGAGGCAGGCG	
HNF1α	156—180	TTCTAAGCTGAGCCAGCTGCAGACG	ref. 18
(0.4)	429—405	GCTGAGGTTCTCCGGCTCTTTCAGA	
IL-1β	539—559	CTCCATGAGCTTTGTACAAGG	ref. 19
(0.05)	783—763	TGCTGATGTACCAGTTGGGG	
IL-6	93—113	AGCCAGTTGCCTTCTTGGGAC	ref. 20
(0.2)	716—696	GCTTAGGCATAGCACACTAGG	
iNOS	1490—1514	TCGAGCCCTGGAAGACCCACATCTG	ref. 21
(0.1)	1765—1736	GTTGTTCCTCTTCCAAGGTGTTTGCCTTAT	
β-Actin	926—951	TCATGAAGTGTGACGTTGACATCCGT	Promega
(0.0125)	1210—1184	CCTAGAAGCATTTGCATTTGCGGTGCACGATG	

a) As a volume of the reverse transcription reaction mixture used for semi-quantitative PCR.

Table 3. Primer Sequences for Real-Time PCR Assay

Target sequence	Sequences (5′→3′)		
Ntcp	194—212	AAGGCGCTTAGCATCATCC	NM_017047
	320—302	CCACCAAGGCAACGATCAC	
Oatp1	2022—2042	GAGAAGGAAAGCGAGCACACA	L19031
	2093—2072	CTTCGTTTTCAGTTCTCCGTCA	
Oatp2	1295—1313	CTCTGCCTGTCTGAGTACC	U88036
	1469—1450	GATCCCATGTGTTCGTTGAG	
Oatp4	495—513	ACGACATTGGCTCTCTAGG	AJ271682
	736—718	ACCTAGGTGCATGGAAGTG	
Oat2	1050—1073	CGTTGCAAAGACCCTCATACTTAG	NM_053537
	1124—1104	CCATCATGCAGCACAGTGAGA	
Oat3	1964—1985	CCCATACTCCTGCACTCATCCT	NM_031332
	2029—2008	TATGGCAAGGGTTGACAGAAGA	
Oct1	1488—1507	TGGTGTTCAGGCTGATGGAA	NM_012697
	1549—1531	GCCCAAAACCCCAAACAAA	
Mrp2	3668—3688	CTGGAGCTGGTTGGAAACTTG	X96393
	3746—3727	CGTCCCCGGTTAAGGTTTTT	
Mrp3	2979—2998	AAATGCCCTTGCTATCGGAG	NM_080581
	3069—3048	CCTTACGGAGGTGTTGTTCTGC	
Bsep	2351—2370	ATCCGGCAACGCTCCAAGTC	U69487
	2472—2449	TCAACTTCTTCCACAAGCACGTCA	
Mdr1a	2012—2036	GATGGAATTGATAATGTGGACA	AF257746[22]
	2138—2120	GTACGTCGTCATCCAGAGC	
Mdr1b	1942—1966	GAAATAATGCTTATGAATCCCAAAG	AY082609[22]
	2244—2267	GGTTTCATGGTCGTCGTCTCTTGA	
TNF-α	306—326	GGTGATCGGTCCCAACAAGGA	NM_012675[23]
	478—460	CACGCTGGCTCAGCCACTC	
GAPDH	1599—1623	TGCCAAGTATGATGACATCAAGAAG	NM_017008
	1669—1650	AGCCCAGGATGCCCTTTAGT	

Table 4. Plasma ALT and AST Activities and Total Bile Acid Levels after LPS Administration

	(h)[a]	Saline	LPS	AG/LPS	AG
ALT activity (IU/ml)	16	11.4±0.2	7.9±2.1	6.9±0.9*	10.8±0.7
AST activity (IU/ml)	16	30.6±1.2	40.1±8.4	34.9±3.4	27.7±4.5
Total bile acids (μM)	4	10.6±3.7	12.6±2.6	13.1±2.2	9.8±3.6
	16	11.6±3.2	30.5±5.9*	21.2±1.1*	20.5±6.0

a) Time after LPS administration. *$p<0.05$ compared with controls.

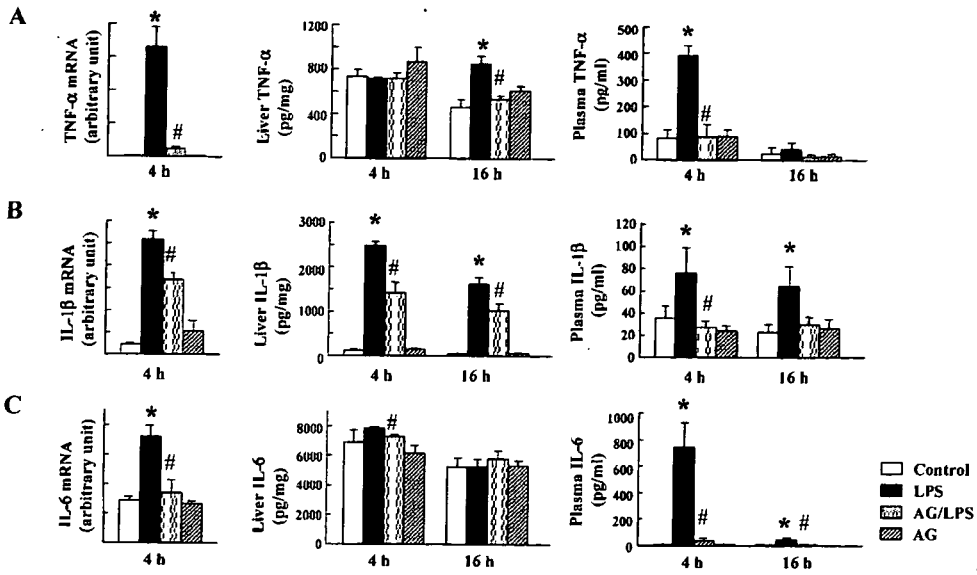

Fig. 2. Effect of AG on LPS-Induced Cytokine mRNA Levels and Protein Levels of Liver and Plasma

Rats were treated with saline or AG (400 mg/kg) 0.5 h before saline or LPS (1 mg/kg). (A) TNF-α. (B) IL-1β. (C) IL-6. Plasma and liver (cytosol) cytokine levels were determined with ELISA kits. mRNA data normalized to those of β-actin are expressed in arbitrary units. Each bar represents mean±S.E.M. (n=4—6). *p<0.05 compared with control groups. #p<0.05 compared with LPS groups.

Effect of AG on LPS-Induced Cytokine Production AG has been reported to show contradictory effects on cytokine production *in vivo*, with no effects seen on LPS-induced TNF-α or IL-1β mRNA levels[30] *versus* a reduction in carrageenan-induced exudates TNF-α levels.[31] In addition to mRNA levels, we therefore also measured plasma and liver protein levels of TNF-α, IL-1β and IL-6 by ELISA (Fig. 2).

As expected, administration of LPS increased mRNA as well as plasma and liver protein levels of TNF-α, IL-1β and IL-6. In particular, plasma and liver IL-1β levels were sustained up to 16 h. AG reduced mRNA levels (% of LPS groups) to 6.9% for TNF-α 12.7% for IL-6, and 63.1% for IL-1β and liver protein levels decreased accordingly. Consistent with the lower effect of AG against IL-β mRNA, liver IL-1β levels in the AG/LPS groups remained at 55—63% those of the LPS groups (4, 16 h). In contrast, plasma TNF-α IL-1β and IL-6 levels of the AG/LPS groups were near control levels. These results suggest that AG had a suppressive effect on TNF-α, L-1β and IL-6 expression at the transcriptional level, and further on IL-1β release from KCs at a post-translational level.

Effect of AG on LPS-Induced Changes in Transporter mRNA Levels In a preliminary experiment, LPS decreased transporter mRNA levels for Ntcp, Oatp1, Oatp2 and Oatp4 at 16 h but had no significant effect at 4 h. We therefore measured mRNA levels of major liver transporters (Table 1) at 16 h after LPS.

Results showed that LPS reduced mRNA levels (% of controls) for Ntcp (10.4%), Oatp1 (24.6%), Oatp2 (3.5%), Oatp4 (37.8%), Oct1 (54.8%), Mrp2 (10.4%) and Mdr1a (17.1%), but up-regulated those for Mdr1b (298.5%) (Fig. 3). AG attenuated mRNA levels for Ntcp from 10.4 to 35.3%, Oatp1 from 24.6 to 68.8%, Oatp4 from 37.8 to 67.3%, and Mdr1b from 298.5 to 167.6% of the controls. In addition, AG alone suppressed mRNA levels of Mdr1b to 25.7% of controls.

To assess the correlation between mRNA and protein expression, we measured liver protein levels of Ntcp, Oatp1, Oatp2 and Oatp4 by Western blot analysis. Despite the decrease in mRNA levels for these transporters, LPS reduced protein levels of Ntcp and Oatp4 only, to 42.5% and 49.2% of those of the controls, respectively (Fig. 4). Further, AG had no effect on the protein level of any transporter. Since these results suggested the necessity of measuring transporter protein levels on and after 16 h, we focused on mRNA levels of transporters in this study.

Effect of AG on LPS-Induced Changes in Transcription Factor Expressions We measured mRNA levels for RXRα PXR, FXR, CAR, RARα, HNF1α and HNF4α as transcription factors involved in LPS-induced down-regulation of transporter genes (Table 5). Results showed that LPS decreased mRNA levels (% of controls) for RXRα (70.7%), RARα (46.7%), PXR (51.1%), CAR (22.2%) and FXR (14.8%) at 4 h, preceding the decrease in transporter mRNA levels (Fig. 5). The decreased mRNAs for RXRα, RARα and PXR returned to control levels by 16 h, whereas those for CAR and FXR remained lower than the controls. AG attenuated mRNA levels for RXRα from 70.7 to 99.0%, PXR from 51.1 to 79.4% and FXR from 17.8 to 66.9% at 4 h, and that for CAR from 50.7 to 97.9% at 16 h. In contrast, LPS had no effect on mRNA levels for HNF1α or HNF4α. AG alone increased mRNA levels for HNF4α and HNF1α to 152% and 322% of those of controls at 4 h, respectively.

Given the role of RXRα as the essential heterodimer partner for type II nuclear receptors, including PXR, FXR, CAR and RARα we next focused on the protein levels of RXRα, HNF1α and HNF4α, and the DNA-binding activities of HNF1α and HNF4α. LPS decreased nuclear RXRα protein levels (% of controls) to 65.2% at 4 h and 76.8% at 16 h without the detection of cytoplasmic protein in any groups at both time points (Fig. 6). AG attenuated this LPS-induced decrease in RXRα protein at 4 h from 65.2 to 79.9%, but had no effect on that at 16 h.

In contrast, HNF4α and HNF1α protein were detected in both nuclear and cytoplasmic fractions of liver. LPS reduced

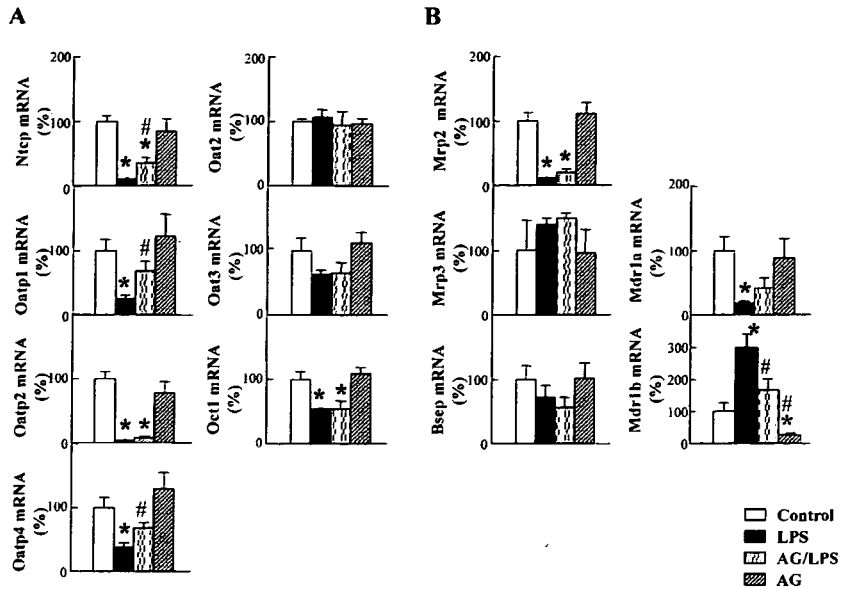

Fig. 3. Effect of AG on LPS-Induced Changes in Transporter mRNA Levels

Rats were treated with saline or AG (400 mg/kg) 0.5 h before saline or LPS (1 mg/kg). Total RNA was isolated from the liver 16 h after LPS. (A) Influx transporter. (B) Efflux transporter. mRNA data normalized to those of GAPDH are expressed as a percentage of the controls. Each bar represents mean±S.E.M. (n=4—6). *p<0.05 compared with control groups. #p<0.05 compared with LPS groups.

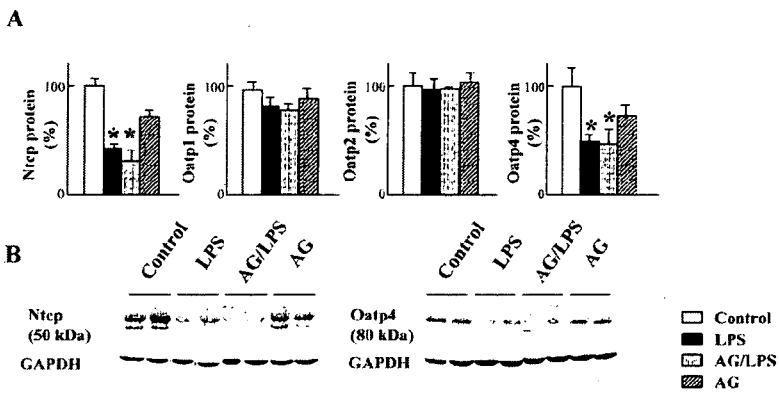

Fig. 4. Effect of AG on LPS-Induced Changes in Liver Protein Levels for Influx Transporter

Rats were treated with saline or AG (400 mg/kg) 0.5 h before saline or LPS (1 mg/kg). The liver was isolated 16 h after LPS administration. (A) Relative transporter protein levels. The protein data after densitometric analysis of Western blot are normalized to those of GAPDH and are expressed as a percentage of the controls. Each bar represents mean±S.E.M. (n=3). (B) Representative immunoblots. *p<0.05 compared with control groups. #p<0.05 compared with LPS groups.

Table 5. Transcription Factors that Regulate Transporter Genes

Transporter	Transcription factor
Ntcp[10]	RARα, RXRα, HNF4α, HNF1α
Oatp1[10]	HNF4α, HNF1α
Oatp2[10]	PXR, CAR, RXRα, HNF1α
Oatp4[32]	RARα, RXRα, HNF1α
Mrp2[10]	PXR, CAR, FXR, RARα, RXRα
Mdr1a[33]	PXR, RXRα
Mdr1b[10]	NF-κB

HNF4α protein levels (% of controls) to 36.8% (nuclear fraction) and 69.7% (cytoplasmic fraction) at 4 h and to 51.6% (nuclear fraction) at 16 h (Fig. 7). HNF4α DNA-binding activities were reduced to 8.5% and 61.8% of controls at 4 h and 16 h, respectively. This 8.5% decrease in HNF4α DNA-binding activity at 4 h was compared with a smaller decrease (36.8%) in nuclear protein levels, whereas the decrease in DNA-binding activities corresponded with that in nuclear protein levels at 16 h. AG attenuated the LPS-induced decrease in nuclear HNF4α levels from 36.8 to 54.0% of controls, but had no effect on the LPS-induced decrease in HNF4α DNA-binding activities at 4 h (Fig. 7c). Further, AG alone had no effect on protein levels at 4 h.

On the other hand, LPS did not induce a decrease in protein levels or DNA-binding activities of HNF1α (Fig. 8); on the contrary, it increased nuclear HNF1α levels to 153.3% of controls at 4 h. AG alone increased nuclear HNF1α levels to 176.1%, in accordance with the increase in mRNA levels, but produced no additional enhancement of the LPS-induced increase in nuclear HNF1α levels.

DISCUSSION

This study shows for the first time that AG attenuates changes in transporter mRNA levels induced by LPS, namely

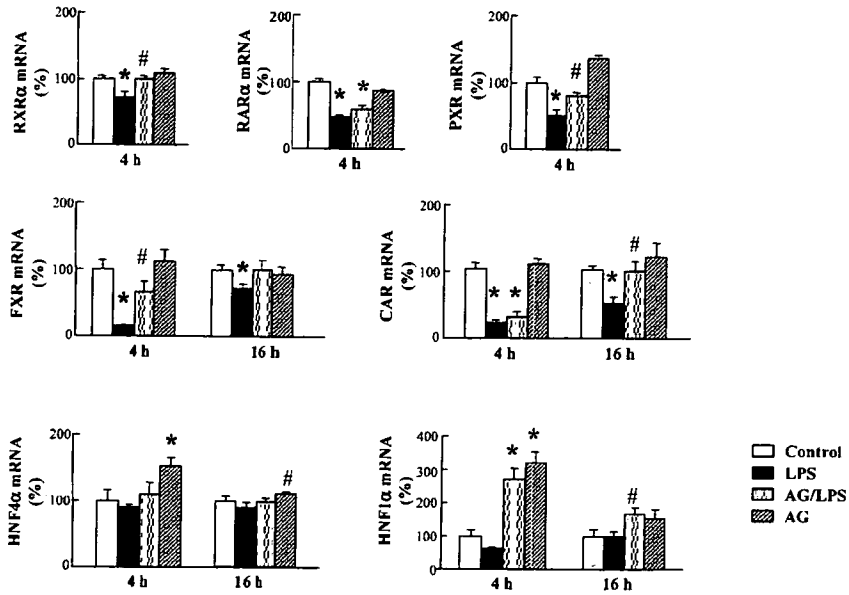

Fig. 5. Effect of AG on LPS-Induced Changes in mRNA Levels for Transcription Factor

Rats were treated with saline or AG (400 mg/kg) 0.5 h before saline or LPS (1 mg/kg). Total RNA was isolated from liver 4 h and 16 h after LPS. mRNA data normalized to those of β-actin are expressed as a percentage of the controls. Each bar represents mean±S.E.M. (n=4—6). *p<0.05 compared with control groups. #p<0.05 compared with LPS groups.

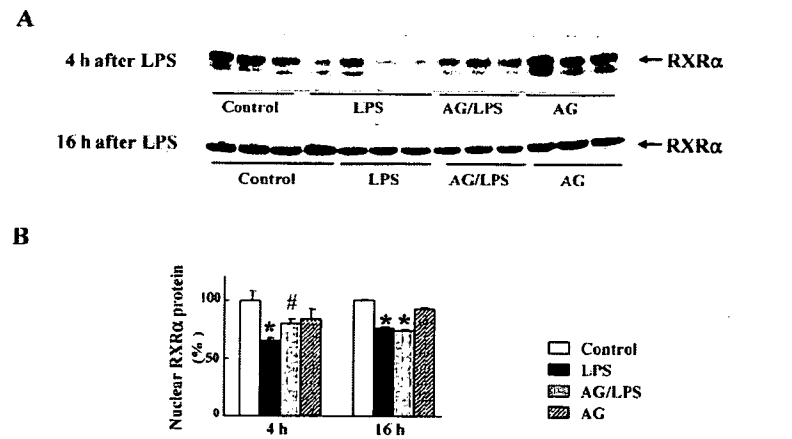

Fig. 6. Effect of AG on LPS-Induced Decrease in RXRα Protein Levels

Rats were treated with saline or AG (400 mg/kg) 0.5 h before saline or LPS (1 mg/kg). Western blot analysis was performed with nuclear protein of liver isolated 4 h and 16 h after LPS. (A) Representative immunoblots of nuclear protein. (B) Relative nuclear RXRα protein levels. The protein data after densitometric analysis are expressed as a percentage of the controls. Each bar represents mean±S.E.M. (n=3—4). *p<0.05 compared with control groups. #p<0.05 compared with LPS groups.

the down-regulation of RXRα- and HNF4α-dependent genes (Ntcp, Oatp1, Oatp4) and the up-regulation of NF-κB-dependent gene (Mdr1b). AG decreased LPS-induced increases in cytokines (TNF-α, IL-1β, IL-6) and NO production *via* NF-κB activation and in IL-1β release from KCs at post-translational levels, but had no effect on LPS-induced decreases in RXRα or HNF4α transcriptional activities. These data suggest that cytokines and NO from KCs stimulated by LPS play a role in the regulation of Ntcp, Oatp1, Oatp4 and Mdr1b.

Cytokines and iNOS-derived NO are released from KCs in response to LPS. Results showed that AG decreased the LPS-induced increase in mRNA levels for iNOS and cytokines (TNF-α, IL-1β, IL-6) and also inhibited NO production as an iNOS inhibitor in a competitive manner. Despite liver IL-1β protein levels in the AG/LPS groups were approximately 60% those of the LPS groups, plasma IL-1β levels were close to control levels. IL-1β is synthesized as a 33 kDa precursor (proIL-1β), cleaved into the active 17 kDa form by IL-1β-converting enzyme (ICE) or caspase 1 in cells and then released from cells.[34] TNF-α, IL-1β, IL-6 and iNOS are NF-κB-dependent genes[35] which are induced by LPS mainly in KCs. Our results therefore suggest that AG has a suppressive effect on LPS-induced NF-κB activation in KCs as well as on LPS-enhanced processing of proIL-1β and the release of IL-1β from KCs.

In KCs, LPS signals are transduced into cells with CD14 surface receptors *via* Toll-like receptor 4 (TLR4)[35] and Rac-1-dependent reactive oxygen species (ROS),[36] both of which then lead to NF-κB activation. Protein kinases, including the mitogen-activated kinases (MAPKs), play diverse roles in both pathways linking to LPS exposure and to NF-κB activa-