一様となるように設計した 23 塩基長のオリゴ DNA である。SNP を含む SNP 特異的配列上で、5'クエリープローブと 3'クエリープローブが隣接してハイブリダイゼーションしたときにライゲース酵素により 2 つのプローブが連結される。エンコード反応により、各 SNP の遺伝子型は ED と D1 の組み合わせで表現されることとなる。続くラベリングの工程では、蛍光標識 EDs プライマー（Cy3-ED-1、Cy5-ED-2）と D1s プライマー（解析に使用した D1 すべてを混合したプライマー）を用いてライゲーション産物をテンプレートとした PCR を行う。最後に、蛍光導入された PCR 産物を DNA マイクロアレイへ直接ハイブリダイゼーションする。DNA マイクロアレイには D1 と相補的なオリゴ DNA がプローブとして固定されており、蛍光導入された PCR 産物内の D1 領域がプローブとハイブリダイゼーションすることにより各 SNP の対立遺伝子型が判定される。

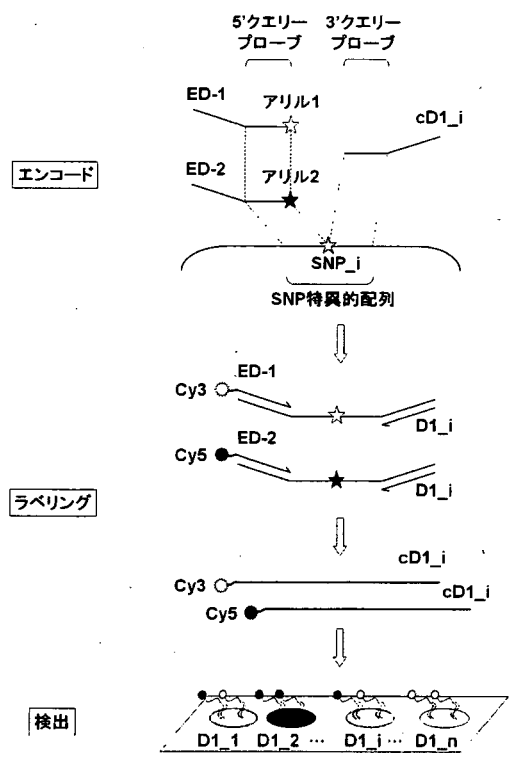

図1 DigiTag2法の概要図

薬剤応答性遺伝子群の機能 SNP 選択

東京大学大学院薬学系研究科分子薬物動態学教室の協力のもと、薬剤代謝酵素（CYP：20 種類、CYP 以外：59 種類）・薬剤トランスポーター（101 種類）に関して、遺伝子多型による機能変化をもたらす SNP を合計 180 種類選択した。これらの中から日本人におけるアリル頻度が 5%を超える SNP を優先的に選択し、最終的に計 47SNPs を解析対象に決定した。ただし、SNP019 および SNP029 は SNP ではなく遺伝子欠損であるため、今回は解析対象から外すこととした。

今回の解析対象として選択した 45 種類の SNP は 18 種類の遺伝子上に存在し、各遺伝子上に存在する SNP をひと組としてタイピングすることにより、薬物代謝や薬物応答性の診断を行うことができる。

DigiTag2 法による 45-plex SNP タイピング

SNPタイピング成功率を上げるために、45種類のSNPに対してクエリープローブをセンス鎖側とアンチセンス鎖側の両方に設計することとした。ゲノムDNAをテンプレートとした45-plex PCRを行った後、センス鎖側のクエリープローブを用いたエンコード反応とアンチセンス側のクエリープローブを用いたエンコード反応をそれぞれ独立に行い、エンコード反応産物を混合してからラベリング反応を行った。DigiTag2 法による45-plex SNPタイピングは以下の通りである。

10 ngのゲノムDNAをテンプレートとしたマルチプレックスPCRにより、45箇所のSNP部位をゲノムDNAから切り出す反応を行った。全量10 μlの反応溶液（QIAGEN Multiplex PCR Kit, QIAGEN）に各SNP部位特異的なプライマーペア0.3 pmolを加え、さらにゲノムDNA 10 ngと2×QIAGEN Multiplex PCR master mix 5 μlを加えた。マルチプレックスPCRは95 ℃、15分の熱変性後、95 ℃で30秒、68 ℃で6分を1サイクルとして40サイクル行った。続くエンコード反応はセンス側およびアンチセンス側に設計したクエリープローブを用いてそれぞれ独立に行い、反応条件は以下の通りとした。全量15 μlのライゲーション反応溶液（*Taq* DNA ligase、New England BioLabs）に45箇所のSNPに対応する5'クエリープローブと3'クエリープローブを5 fmolずつ加え、さらにマルチプレックスPCR産物を1 μlと5 Uの酵素を加えた。ライゲーション反応は、95 ℃、5分の熱変性後、50 ℃で1分間アニーリングした後に58 ℃で60分間の反応を行った。ラベリング反応では蛍光修飾EDsプライマーとD1sプライマー（90種類のD1を混合したプライマー）を用いてアシンメトリックPCRを行った。反応溶液は全量12 μlとし、60 fmolのD1プライマー90種類、6 pmolのCy3-ED-1とCy5-ED-2を加え、さらに0.6 Uの酵素（Ex*Taq* polymerase、TaKaRa）とセンス側およびアンチセンス側のライゲーション産物1 μlずつを加えた。PCRは95 ℃1分の熱変性後、95 ℃30秒、55 ℃6分、72 ℃30秒を1サイクルとして30サイクル行った。最後に蛍光導入されたPCR産物を回収し、DNAマイクロアレイへのハイブリダイゼーションを行った。全量12 μlのハイブリダイゼーション溶液（0.5×SSC、0.1% SDS、15 % ホルムアミド、1 mM EDTA）に、蛍光導入されたPCR産物5 μlを加えた。各サンプルについてハイブリダイゼーション溶液8 μlをDNAマイクロアレイ上の別個のエリアに散布した後、90 ℃で2分間熱変性をした後、37 ℃のハイブリオーブンに1時間静置してハイブリダイゼーションを行った。ハイブリダイゼーションの結果はDNAチップスキャナー（GenePix4000A、Axon Instruments）を用いて画像データとして読み取り、画像解析ソフト（GenePix Pro4.0、Axon Instruments）により数値データの解析を行った。

使用したゲノム DNA サンプルおよび研究倫理面への配慮

本研究は、すでにヒトゲノム・遺伝子解析研究倫理審査委員会より承認を得た疾患関連遺伝子多型解析研究に含まれる。また、本研究は技術開発研究であり、技術の評価に用いるヒトゲノム試料は、すべて公的財団（ヒューマンサイエンス振興財団）より得た連結不可能匿名化済みの試料（PSC 細胞株の精製 DNA サンプル）である。本研究では、PSC 細胞株の精製 DNA サンプルを 192 検体分購入して使用した。

C. 研究結果

日本人健常者 192 検体を用いて 45-plex SNP タイピングを行った結果、39 種類の SNP でタイピングが可能であることが明

らかとなった。図 2 には、センス側もしくはアンチセンス側のタイピング結果のうち、遺伝子型クラスターの分離状態が良い方を選択して、タイピングに成功したいくつかの SNP についてその散布図を示した。散布図には 96 検体分のタイピング結果をプロットし、青色と赤色がホモクラスター、緑色がヘテロクラスターを示す。

薬剤トランスポーターに関しては、薬剤の初期スクリーニングにおける肝毒性のマーカーとして有用な ABCC2(MRP2)遺伝子に存在する 3SNPs や血中コレステロール低下作用に影響を及ぼすと考えられる OATP1B1(SLCO1B1)遺伝子の 2SNPs などのタイピングに成功した。また、薬剤代謝酵素については、ワルファリンの治療効果に影響を及ぼすことが知られる VKORC1 遺伝子および CYP2D9 遺伝子に存在する SNP や、結核治療薬イソニアジド（INH）などの臨床上重要な薬剤の代謝に関与することが知られる NAT2 遺伝子の SNP などのタイピングに成功した。解析対象とした全 18 遺伝子（薬剤トランスポーター4 遺伝子、薬剤代謝酵素 14 遺伝子）のうち、薬剤トランスポーターの 3 遺伝子と薬剤代謝酵素の 10 遺伝子は今回用意したタイピングキットで解析できることが明らかとなった。

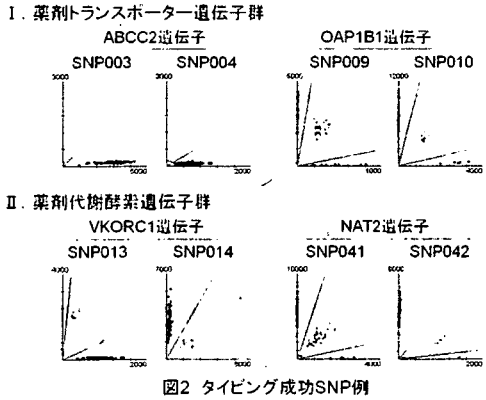

図2 タイピング成功SNP例

D. 考察

我々が独自開発した DigiTag2 法は、高価な自動装置を用いないで数十種から数百種の SNP を同時並列タイピングできるという大きな利点を持つ方法である。本研究では、薬剤応答性に関与する主要な遺伝子多型を対象としてタイピングキットを開発し、有望な成果を得ることができた。

今回、SNP タイピングに失敗した 6 種類の遺伝子多型のうちの 4 種類は、アリルが 3 種類存在する SNP（SNP002、SNP047）、繰り返し配列中に存在する SNP（SNP012）、一塩基挿入（SNP025）とこれまでに解析対象としてきた 2 対立遺伝子（Biallelic）の SNP とは異なるタイプの遺伝子多型であった。また、2 対立遺伝子型の SNP であった 2 種類の SNP（SNP028、SNP036）では、マルチプレックス PCR の段階で十分な増幅が得られなかったことが原因で、シグナル強度が弱くなり解析不能になったと考えられる。この 2 種類の SNP については、マルチプレックス PCR 用のプライマーを再デザインすることでタイピングが可能になる可能性がある。しかし、2 対立遺伝子型の SNP とは異なる遺伝子型多型に対しては、新たなプライマー・プローブデザインパラメータを検討する必要性のあることが示唆された。また、向精神薬、循環器系用剤、呼吸器系用剤など様々な薬剤の代謝に関与する CYP2D6 遺伝子は重要な薬剤代謝酵素であるが、遺伝子欠損を含むため現段階では同遺伝子上のすべての機能的多型は解析できていない。今後は、遺伝子欠損も含めて同時にタイピングできる DigiTag2 法キットを確立する必要性があ

る。

E. 結論

DigiTag2 法は、各 SNP の遺伝子型を ED および D1 の組み合わせに変換して解析を進めるため、異なる SNP セットに対して同一のマイクロアレイを用いた遺伝子型判定を行えるという特長を持っている。今回タイピングに成功した 13 遺伝子 28SNPs のほかに、その他の臨床的意義をもつ遺伝子多型を加えたタイピングキットを用意すれば、同一の実験条件・マイクロアレイを用いた SNP タイピングを行うことができる。今回タイピングに失敗した遺伝子多型に対して再デザインしたプローブの検討を進めると共に、新たな薬剤応答性遺伝子を加えた SNP セットを準備し評価・検討を進めていく予定である。

F. 健康危険情報

該当なし

G. 研究発表

1. 論文発表

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H.知的財産権の出願・登録状況

該当なし

III. 研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
西田奈央、徳永勝士	大規模SNPタイピングによる多因子疾患遺伝子の探索	榊　佳之、伊藤隆司、辻　省次、小原雄治（編）	ゲノム情報と生命現象の統合的理解2007	羊土社	東京	2007	178-184

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Aoki K, Nakajima M, Hoshi Y, Saso N, Kato S, Sugiyama Y and Sato H	Effect of aminoguanidine on lipopolysaccharide-induced changes in rat liver transporters and transcription factors	Biol Pharm Bull	31(3)	412-420	2008
Ishiguro N, Maeda K, Saito A, Kishimoto W, Matsushima S, Ebner T, Roth W, Igarashi T and Sugiyama Y	Establishment of a set of double transfectants coexpressing organic anion transporting polypeptide 1B3 and hepatic efflux transporters for the characterization of the hepatobiliary transport of telmisartan acyl glucuronide	Drug Metab Dispos	36(4)	796-805	2008
Matsushima S, Maeda K, Hayashi H, Debori Y, Schinkel AH, Schuetz JD, Kusuhara H and Sugiyama Y	Involvement of multiple efflux transporters in hepatic disposition of fexofenadine	Mol Pharmacol	In press		2008
Saji T, Kikuchi R, Kusuhara H, Kim I, Gonzalez FJ and Sugiyama Y	Transcriptional regulation of human and mouse organic anion transporter 1 by hepatocyte nuclear factor 1 alpha/beta	J Pharmacol Exp Ther	324(2)	784-790	2007

Enokizono J, Kusuhara H and Sugiyama Y	Effect of breast cancer resistance protein (Bcrp/Abcg2) on the disposition of phytoestrogens	Mol Pharmacol	72 (4)	967-975	2007
Kikuchi R, Kusuhara H, Hattori N, Kim I, Shiota K, Gonzalez FJ, Sugiyama Y	Regulation of tissue-specific expression of the human and mouse urate transporter 1 gene by hepatocyte nuclear factor 1 alpha/beta and DNA methylation	Mol Pharmacol	72 (6)	1619-1625	2007
Yamasaki Y, Ieiri I, Kusuhara H, Sasaki T, Kimura M, Tabuchi H, Ando Y, Irie S, Ware J, Nakai Y, Higuchi S and Sugiyama Y	Pharmacogenetic Characterization of Sulfasalazine Disposition Based on NAT2 and ABCG2 BCRP) Gene Polymorphisms in Humans.	Clin Pharmacol Ther	In press		2008
Ieiri I, Suwannakul S, Maeda K, Uchimaru H, Hashimoto K, Kimura M, Fujino H, Hirano M, Kusuhara H, Irie S, Higuchi S and Sugiyama Y	SLCO1B1 (OATP1B1, an uptake transporter) and ABCG2 (BCRP, an efflux transporter) variant alleles and pharmacokinetics of pitavastatin in healthy volunteers.	Clin Pharmacol Ther	82 (5)	541-547	2007
Takane H, Miyata M, Burioka N, Kurai J, Fukuoka Y, Suyama H, Shigeoka Y, Otsubo K, Ieiri I and Shimizu E	Severe toxicities after irinotecan-based chemotherapy in a patient with lung cancer: a homozygote for the SLCO1B1*15 allele.	Ther Drug Monit	29 (5)	666-668	2007

IV. 研究成果の刊行物・別刷

別刷は、以下の順序に掲載をしている。

(1) Aoki K, Nakajima M, Hoshi Y, Saso N, Kato S, Sugiyama Y and Sato H. Effect of aminoguanidine on lipopolysaccharide-induced changes in rat liver transporters and transcription factors. Biol Pharm Bull, 31(3), 412-420 (2008)

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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).

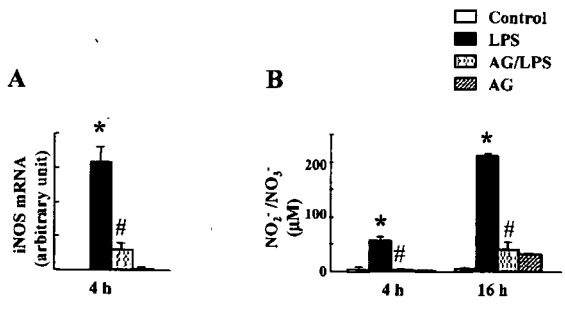

Fig. 1. Effect of AG on LPS-Induced Liver iNOS mRNA and Plasma NO_2^-/NO_3^-

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.

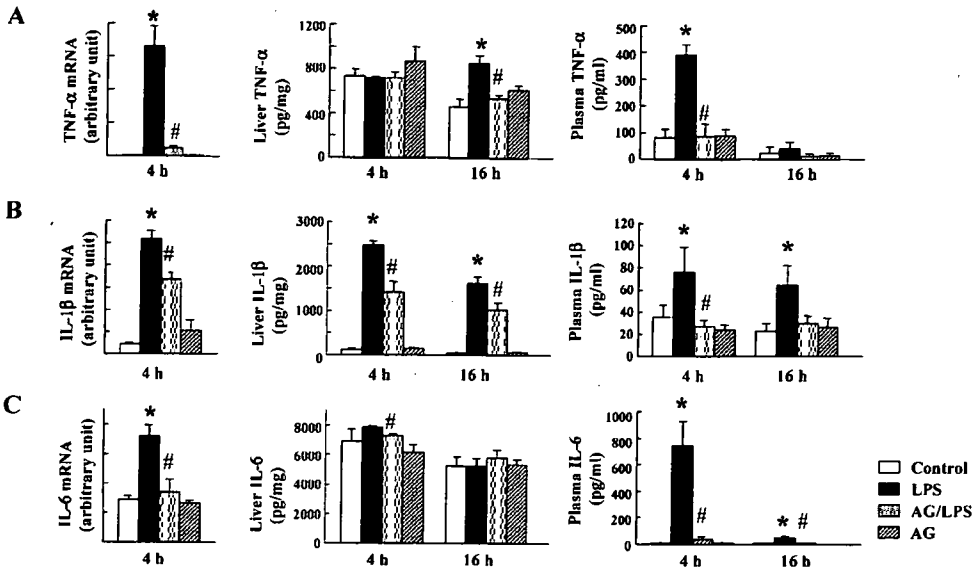

Fig. 2. Effect of AG on LPS-Induced Cytokine mRNA Levels and Protein Levels of Liver and Plasma

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

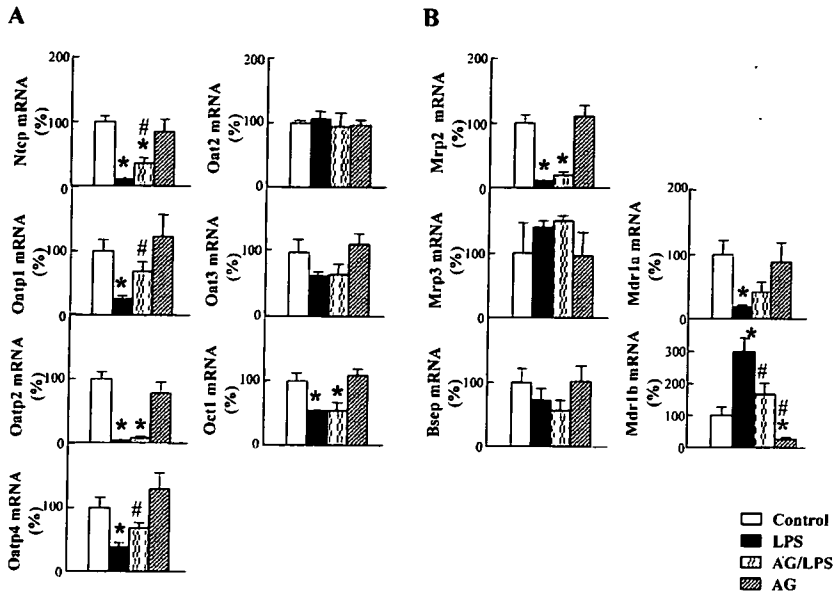

Fig. 3. Effect of AG on LPS-Induced Changes in Transporter mRNA Levels

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.

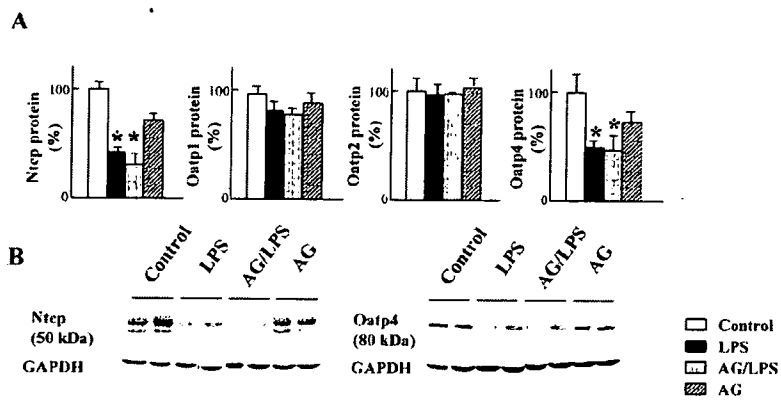

Fig. 4. Effect of AG on LPS-Induced Changes in Liver Protein Levels for Influx Transporter

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

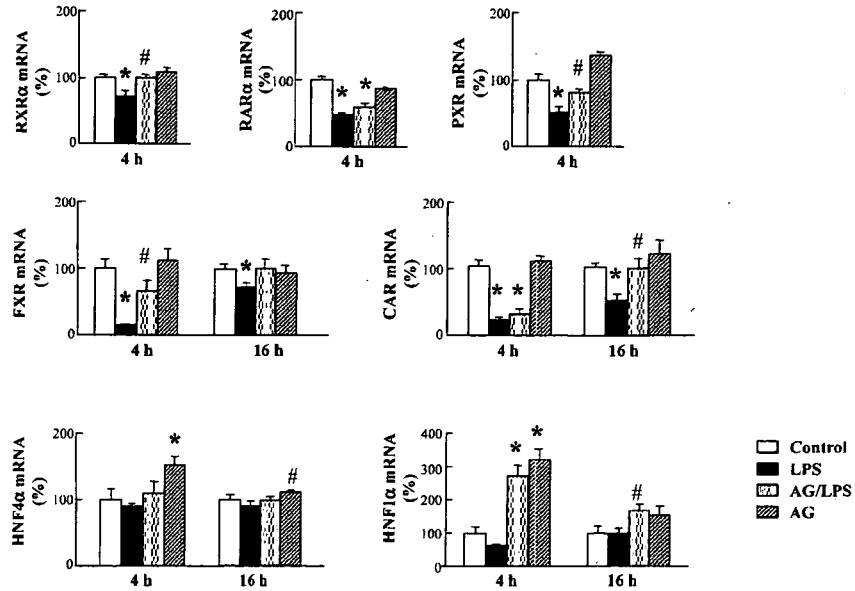

Fig. 5. Effect of AG on LPS-Induced Changes in mRNA Levels for Transcription Factor

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.

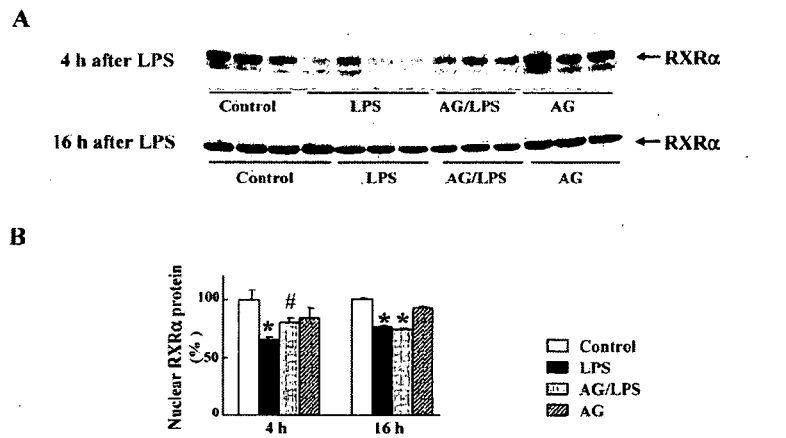

Fig. 6. Effect of AG on LPS-Induced Decrease in RXRα Protein Levels

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-

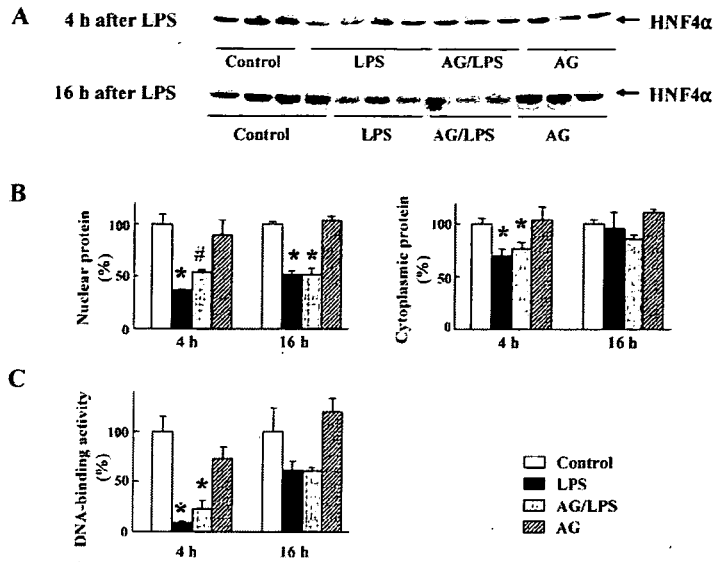

Fig. 7. Effect of AG on LPS-Induced Decrease in HNF4α Protein Levels and HNF4α DNA-Binding Activities

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 and cytoplasmic protein of liver isolated 4 h or 16 h after LPS. (A) Representative immunoblots of nuclear protein. (B) Relative nuclear and cytosolic HNF4α protein levels. The protein data from nuclear and cytoplasmic fractions after densitometric analysis are expressed as a percentage of the controls. (C) Relative HNF4α DNA-binding activity. DNA-binding activities measured with TransAM™ 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.

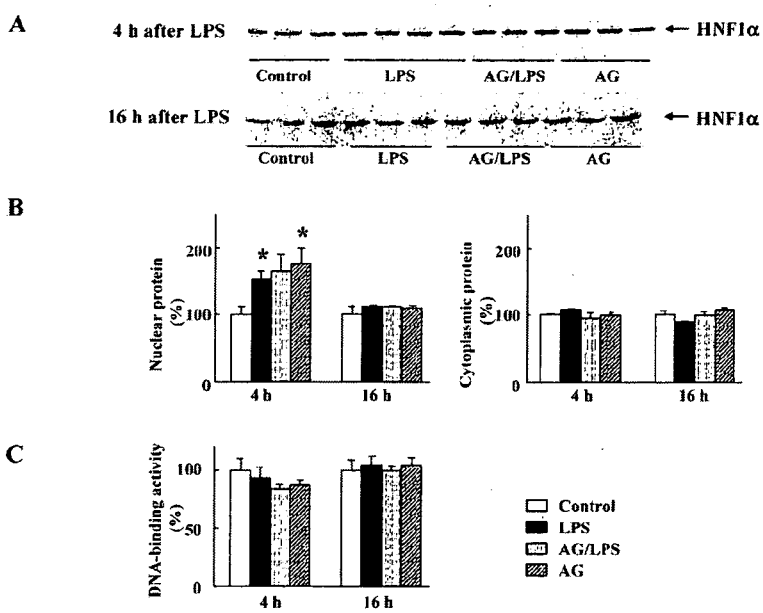

Fig. 8. Effect of AG on LPS-Induced Changes in HNF1α Protein Levels and HNF1 DNA-Binding Activities

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 and cytoplasmic protein of liver isolated 4 h and 16 h after LPS. (A) Representative immunoblots of nuclear protein. (B) Relative nuclear and cytosolic HNF1α protein levels. The protein data from nuclear and cytoplasmic fractions after densitometric analysis are expressed as a percentage of the controls. (C) Relative HNF1 DNA-binding activity. DNA-binding activities measured with TransAM™ are expressed as a percentage of the controls. HNF1 DNA-binding activities are expressed as a percentage of the controls. Each bar represents mean±S.E.M. (n=3—4). *p<0.05 compared with LPS groups.

tion. Antioxidant drugs such as *N*-acetylcysteine, α-tocopherol and selenium are known to attenuate LPS-induced NF-κB activation and thereby reduce mRNA levels for TNF-α, IL-1β and iNOS.[37—39] Further, *N*-acetylcysteine attenuates IL-1β release by inhibiting LPS-induced ICE activity.[36]

Protein kinase inhibitors also inhibit LPS-induced NF-κB-dependent gene transcription. Of three major MAPK subfamilies (p38MAPK, extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun-N-terminal kinase (JNK)), p38MAPK and ERK1/2 are thought to play crucial roles in LPS-induced cytokine production. p38MAPK and ERK1/2 inhibitors decrease the transcription of IL-1β, IL-6 and iNOS[40] and release of TNF-α and IL-1β.[41] AG has been reported to exert antioxidant activity by preventing ROS formation *in vivo*[42] and by directly scavenging H_2O_2, hydroxyl radical and peroxynitrite (a ROS formed from NO and superoxide) *in vitro*.[43]

Taken together, these findings indicate that the suppressive effects of AG on LPS-induced NF-κB activation result from several of its antioxidant properties, namely ROS quenching, inhibition of regulatory pathways distal to NF-κB activation such as p38MAPK and ERK1/2, or both.

Here, LPS induced a number of changes in mRNA expression of transporters in hepatocytes, namely the down-regulation of Ntcp, Oatp1, Oatp2, Oatp4, Oct1, Mrp2 and Mdr1a and up-regulation of Mdr1b. In addition to these changes, Cherrington *et al.*[2] reported that LPS also produced a significant decrease in mRNA levels for Oat3 and Bsep and an increase for Mrp3. These differences may have resulted from their use of a four-fold-higher dose of LPS than the 1 mg/kg used here.

Of interst, AG attenuated the up-regulated and constitutive Mdr1b transcription. Mdr1b is mainly regulated through NF-κB signaling (Table 5). The increase in Mdr1b mRNA expression by ROS generated in response to various stimuli, including LPS as well as extracellulary applied H_2O_2, is attenuated by antioxidant drugs.[44] Since cytokines produced in KCs stimulate hepatocytes, thereby leading to NF-κB activation, this finding indicates that AG reduces the transcription of NF-κB-dependent genes in hepatocytes *via* a decrease in LPS-induced KCs activation.

Transcription factors (RXRα, HNF1α, HNF4α) are known to be involved in the down-regulation of Ntcp, Oatp1, Oatp2, Oatp4, Mrp2 and Mdr1a transcription in response to LPS (Table 5). In the present study, LPS resulted in a decrease in nuclear RXRα protein levels with an associated decrease in mRNA levels, but also in a decrease in HNF4α DNA-binding activities and nuclear protein levels without any change in mRNA levels. These results are consistent with those of Beigneux *et al.*[45] and Wang *et al.*[46] However, our finding that HNF1α DNA-binding activities were not reduced by LPS is inconsistent with previous reports.[46—49] Despite the presence of an HNF4α binding site on the HNF1α promoter, the decrease in HNF4α DNA-binding activities did not decrease HNF1α mRNA levels or protein levels. The rapid decrease in DNA-binding activities of HNF1α and HNF4α within 1 h after LPS[48] may suggest that HNF1α DNA-binding activities are independent of HNF4α. However, further experiments are needed to examine whether LPS reduces HNF1α DNA-binding activities at an earlier time than the 4 h in our rat model, and also to clarify the meaning of the increase in HNF1α and HNF4α mRNA expression by AG alone.

RXRα and HNF4α protein are reported to be degraded *via* the ubiquitin/proteasome pathway.[46,50] In the case of RXRα, a decrease in mRNA also participates in the decrease in RXRα protein levels but, given the lack of the difference in reduced RXRα protein levels at 16 h between the LPS and AG/LPS groups in spite of the attenuation of LPS-reduced RXRα mRNA levels by AG, presumably to a lesser extent than the protein degradation. IL-1β, produced in KCs in response to LPS, is reported to be the main regulator of RXRα and HNF4α. In IL-1β-induced cell signaling, the JNK pathway produces a decrease in nuclear RXRα levels by inducing the export of phosphorylated RXRα from the nucleus and the degradation of RXRα protein in cytosol,[51] and a decrease in RXRα DNA-binding activity.[52] IL-1β also reduces HNF4α DNA-binding activity by decreasing nuclear protein levels[46]; and HNF4α phosphorylated by various protein kinases, including JNK, loses its DNA-binding activity.[53] Our results indicate that HNF4α DNA-binding activities were decreased to a greater degree than would be expected from the decreased nuclear protein levels at 4 h after LPS as reported by Cheng *et al.*[48] The transcriptional activity of RXRα and HNF4α is presumed to be regulated by IL-1β-induced cell signaling pathways, which lead to a decrease in nuclear protein levels *via* the proteasome pathway and to a change in phosphorylation states.

Hepatocytes are known to be stimulated by mediators from KCs activated by LPS. AG inhibited IL-1β release from KCs, but had little effect on the prolonged LPS-induced decrease in RXRα and HNF4α transcriptional activity. We wondered why the transcriptional activities of HNF4α, mainly in hepatocytes,[46] and RXRα, in both hepatocytes and nonparenchymal cells,[54] were down-regulated in the AG/LPS and LPS groups to almost the same extent. As a possible answer, it was recently demonstrated that hepatocytes express the components needed to respond to LPS, such as TLR4 and CD14, and can therefore respond to LPS by activating NF-κB and MAPKs in the absence of cytokines from KCs.[55] Our results also suggested that hepatocytes were stimulated directly by LPS, which lead to the activation of NF-κB and the reduction of RXRα and HNF4α transcriptional activities as rapid acute-phase responses. These rapid responses are thought to be mediated by cell signaling pathways common to LPS and IL-1β, but independent of IL-1β and NO released from KCs by LPS, the production of which requires time. This suggestion is supported by the results of Geier *et al.*, who found that even complete IL-1β inactivation cannot attenuate an LPS-induced decrease in RXRα protein levels.[49]

Our findings show that LPS stimulates hepatocytes directly to reduce either or both RXRα- and HNF4α-dependent gene transcription, such as Ntcp, Oatp1, Oatp2, Oatp4, Mrp2 and Mdr1a, and to induce NF-κB-dependent gene transcription such as Mdr1b, as early acute responses. As a longer-term response, cytokines and NO produced in KCs *via* LPS-induced activation of NF-κB enhanced the changes in Ntcp, Oatp1, Oatp4 and Mdr1b transcription in hepatocytes. Therefore, AG suppressed LPS-induced decreases in Ntcp, Oatp1 and Oatp4 transcription and increase in Mdr1b transcription by decreasing NF-κB activation in both hepatocytes and KCs, as well as cytokines and NO release from NF-κB-activated KCs.

In conclusion, NF-κB, cytokines and NO produced *via* NF-κB activation play a role in the regulation of Ntcp, Oatp1, Oatp4 and Mdr1b transcription. In contrast, RXRα and HNF4α, which participate in the down-regulation of transporters, are independent of cytokines and NO. It was impossible to discriminate the roles of cytokines and NO in this *in vivo* study using AG. The possible role of NO in the regulation of liver transporters deserves further *in vitro* investigation.

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Establishment of a Set of Double Transfectants Coexpressing Organic Anion Transporting Polypeptide 1B3 and Hepatic Efflux Transporters for the Characterization of the Hepatobiliary Transport of Telmisartan Acylglucuronide

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ABSTRACT:

In the hepatic uptake of organic anions, organic anion transporting polypeptide (OATP) 1B1 is believed to be mainly involved. We have constructed a set of double-transfected cells coexpressing OATP1B1 and hepatic efflux transporters and characterized the transcellular transport of several anions. Recent reports have also suggested the importance of OATP1B3 in the hepatic uptake of some compounds. However, there is little information about OATP1B3-selective substrate and no good tool for the evaluation of efflux transporters of OATP1B3 substrates. In the present study, we found an OATP1B3-selective substrate and established a novel set of double transfectants expressing OATP1B3. Telmisartan acylglucuronide (tel-glu) is a main metabolite of telmisartan, an angiotensin II receptor antagonist. Tel-glu is recognized by hepatobiliary transport systems and efficiently distributed to liver. Several studies using rat and human hepatocytes and transporter-expressing cells revealed that OATP1B3 was responsible for the hepatic uptake of tel-glu in humans. By using double transfectants expressing OATP1B3, we investigated the transcellular transport of tel-glu as well as estradiol 17β-D-glucuronide ($E_2$17βG) and cholecystokinin octapeptide (CCK-8) to identify the responsible efflux transporters in their biliary excretion. Vectorial basal-to-apical transport of tel-glu was observed in all kinds of double transfectants expressing OATP1B3. In contrast, basal-to-apical transport of $E_2$17βG and CCK-8 was seen only in the OATP1B3/MRP2 double transfectant compared with OATP1B3-expressing cells. Therefore, the newly established set of double transfectants expressing OATP1B3 combined with OATP1B1-expressing double transfectants can be used as a powerful tool for the rapid identification of hepatic uptake and efflux transporters of organic anions.

The hepatobiliary transport of xenobiotics is coordinated by uptake and efflux processes. Accumulating evidence has shown that OATP1B1 (Abe et al., 1999; Hsiang et al., 1999; König et al., 2000a) and OATP1B3 (König et al., 2000b) are responsible for the hepatocellular uptake of organic anions and that several kinds of ATP-binding cassette (ABC) transporters such as multidrug resistance 1 (MDR1), multidrug resistance-associated protein 2 (MRP2), and breast cancer resistance protein (BCRP) play an important role in the biliary excretion of many compounds including clinically important drugs.

The hepatic export of organic anions is generally believed to be mediated predominantly by MRP2 (Evers et al., 1998; Cui et al., 1999; König et al., 1999). However, recent reports suggested that some anionic compounds such as morphine 6-glucuronide (Huwyler et al., 1996) and fexofenadine (Cvetkovic et al., 1999) could be transported by MDR1. Hirano et al. (2005) showed that biliary excretion of pitavastatin is accounted for largely by BCRP in mice. Matsushima et al. (2005) established a set of double-transfected MDCKII cells coexpressing OATP1B1 and MRP2, MDR1, or BCRP and demonstrated that not only MRP2 but also BCRP and MDR1 are involved in the biliary excretion of several organic anions.

In the uptake process of anions, the broad substrate specificity of OATP1B3 commonly overlaps that of OATP1B1, so several compounds can be bisubstrates of both OATP1B1 and OATP1B3 (Vavricka

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ABBREVIATIONS: OATP/Oatp, organic anion transporting polypeptide; ABC, ATP-binding cassette; MDR1, multidrug resistance 1; MRP2, multidrug resistance-associated protein 2; BCRP, breast cancer resistance protein; CCK-8, cholecystokinin octapeptide; tel-glu, telmisartan acylglucuronide; $E_2$17βG, estradiol 17β-D-glucuronide; E-sul, estrone-3-sulfate; TCA, taurocholate; TEA, tetraethylammonium; MDCKII, Madin-Darby canine kidney strain II; HPLC, high-performance liquid chromatography; HSA, human serum albumin; PBS, phosphate-buffered saline.

et al., 2002; Hirano et al., 2004). It has recently been reported that some anions such as cholecystokinin octapeptide (CCK-8), telmisartan, and fexofenadine are taken up into hepatocytes mainly by OATP1B3 rather than OATP1B1 (Ismair et al., 2001; Shimizu et al., 2005; Ishiguro et al., 2006). Thus, OATP1B3 and OATP1B1 could be important transporters for the hepatocellular uptake of anionic compounds, and experimental methods to distinguish their contributions have been established (Hirano et al., 2004; Ishiguro et al., 2006).

For the characterization of efflux transport, there are several methods such as transcellular transport study using single ABC transporter-expressing cells and double transfectants in which uptake and efflux transporters are expressed and uptake study into membrane vesicles prepared from transporter-expressing cells. Using single ABC transporter-expressing cells, we can evaluate the transcellular transport of lipophilic compounds with high membrane permeability but not that of hydrophilic compounds including several organic anions such as pravastatin because of their limited access to efflux transporters from intracellular compartments (Sasaki et al., 2002, 2004). For the low membrane-permeable compounds, a membrane vesicle study may be a useful alternative. However, this experiment cannot be applied to compounds that easily adsorb to the membrane filter because it is difficult to distinguish between transporter-mediated uptake into membrane vesicles and nonspecific adsorption. In the transcellular transport assay using double transfectants, we measure the drug concentration in the buffer, so the effect of nonspecific adsorption to the cells and labware is basically negligible in the quantification of ligand concentration. We can also detect the efflux more sensitively because of the intracellular accumulation of ligands by a basolateral uptake transporter. To date, several kinds of double transfectants have been constructed (Cui et al., 2001; Sasaki et al., 2002, 2004; Letschert et al., 2004, 2005; Kopplow et al., 2005; Matsushima et al., 2005). However, there was no good tool for the identification of transporters involved in the biliary excretion of OATP1B3-specific substrates. Thus, this study was performed to demonstrate the usefulness of double transfectants expressing OATP1B3 for the investigation of the transport of tel-glu.

After oral administration of telmisartan, a part of the oral dose is conjugated with glucuronate in intestine and accumulated selectively in liver. Telmisartan is extensively glucuronidated in liver and excreted into bile as tel-glu. (Wienen et al., 2000; Stangier et al., 2000a,b). Thus, the identification of transporters to the hepatobiliary transport of telmisartan and tel-glu is important for predicting the pharmacokinetics and subsequent pharmacological effect of telmisartan. We have reported that telmisartan is taken up into liver mainly via OATP1B3, and the efflux of tel-glu into bile in rats is mediated by MRP2 and another unknown transporter(s) (Nishino et al., 2000; Ishiguro et al., 2006).

In the present study, we show that tel-glu is recognized mainly by OATP1B3 rather than by OATP1B1 using transporter expression systems and human hepatocytes. We also establish a novel set of double transfectants coexpressing OATP1B3 and MDR1, MRP2, or BCRP and characterize the transcellular transport of estradiol 17β-D-glucuronide ($E_2$17βG), CCK-8, and tel-glu, which are substrates of OATP1B3, to identify the efflux transporters responsible for their biliary excretion.

Materials and Methods

Chemicals. [^{3}H]Telmisartan (762 GBq/mmol, radiochemical purity >98%), 4′-[[4-methyl-6-(1-methyl-2-benzimidazolyl)-2-propyl-1-benzimidazolyl]methyl]-2-biphenyl carboxylic acid, and unlabeled telmisartan were synthesized by Boehringer Ingelheim Pharma KG (Biberach, Germany). Unlabeled telmisartan 1-*O*-acylglucuronide (purity >98%) was isolated from rat bile after i.v. dosing of telmisartan by Charles River (Tranent, Scotland, UK). [^{3}H]$E_2$17βG and [^{3}H]estrone-3-sulfate (E-sul), and [^{3}H]taurocholate (TCA) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA), and [^{3}H]CCK-8 was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Unlabeled $E_2$17βG, E-sul, TCA, CCK-8, tetraethylammonium (TEA), and digoxin were purchased from Sigma-Aldrich (St. Louis, MO). Parental MDCKII cells and MDCKII cells expressing human MRP2 (Evers et al., 1998) and MDR1 were kindly provided by Dr. Piet Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). All other chemicals and reagents were commercial products of reagent grade.

Cell Culture of Transporter-Expressing HEK293 Cells. OATP1B1-, OATP1B3-, and OATP2B1-expressing or vector-transfected HEK293 cells were established previously (Hirano et al., 2004; Shimizu et al., 2005). HEK293 cells were grown in Dulbecco's modified Eagle's medium (low glucose) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% antibiotic-antimycotic solution (Invitrogen) at 37°C with 5% CO_2 and 95% humidity. Cells were then seeded in 12-well plates at a density of 1.5×10^5 cells/well. For the transport study, the cell culture medium was replaced with culture medium supplemented with 5 mM sodium butyrate for 24 h before the transport assay to induce the expression of OATP1B1, 1B3, and 2B1.

Transport Study Using Transporter-Expressing HEK293 Cells. The transport study was carried out as described previously (Hirano et al., 2004). Uptake was initiated by adding Krebs-Henseleit buffer containing radiolabeled and unlabeled compounds after cells had been washed twice and preincubated with Krebs-Henseleit buffer at 37°C for 15 min. The uptake was terminated at a designated time by adding ice-cold Krebs-Henseleit buffer after removal of the incubation buffer. Then cells were washed twice with 1 ml of ice-cold Krebs-Henseleit buffer, solubilized in 1 N NaOH, and kept for 1 h at 37°C. During this incubation period, tel-glu was completely converted to telmisartan. Aliquots were transferred to scintillation vials or sample tubes after adding ½ volume of 2 N HCl. The radioactivity associated with the cells and incubation buffer was measured by a liquid scintillation counter (Tri-Carb 2500TR, PerkinElmer Life and Analytical Sciences) after transfer to 2-ml scintillation vials. The aliquots for tel-glu were stored at −20°C until HPLC analysis. The remaining 50 μl of cell lysate was used to determine the protein concentration by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Preparation of Rat and Human Hepatocytes before Transport Assay. Isolated rat hepatocytes were prepared from Sprague-Dawley rats weighing 200 to 300 g by the collagenase perfusion method described previously (Yamazaki et al., 1993). Isolated hepatocytes (viability >80%) were suspended in Krebs-Henseleit buffer, adjusted to 2.0×10^6 cells/ml, and stored on ice before the uptake experiment. Cryopreserved human hepatocytes (lots HH-OCF, HH-094, and HH-TDH) were purchased from Celsis In Vitro Technologies (Baltimore, MD). The hepatocytes were treated as described previously (Shitara et al., 2003). The cryopreserved human hepatocytes were resuspended in Krebs-Henseleit buffer to give a final cell density of 1.0×10^6 viable cells/ml for the uptake study. The number of viable cells was determined by trypan blue staining. To measure the uptake in the absence of Na^+, sodium chloride and sodium bicarbonate in Krebs-Henseleit buffer were replaced with choline chloride and choline bicarbonate, respectively.

Transport Study Using Isolated Hepatocytes. Before the uptake studies, cell suspensions were prewarmed in an incubator at 37°C for 3 min. The uptake studies were initiated by adding an equal volume of buffer containing radiolabeled and unlabeled compounds to the cell suspension. After incubation at 37°C for 0.5, 2, or 5 min, the reaction was terminated by separating the cells from the substrate solution. For this purpose, an aliquot of 80 μl of incubation mixture was collected and placed in a centrifuge tube (450 μl) containing 50 μl of 2 N NaOH under a layer of 100 μl of oil (density of 1.015, a mixture of silicone oil and mineral oil; Sigma-Aldrich), and subsequently the sample tube was centrifuged for 15 s in a centrifuge (15,000 rpm, MX-100; Tomy Seiko, Tokyo, Japan). During this process, hepatocytes passed through the oil layer into the alkaline solution. After an overnight incubation in alkali to dissolve the hepatocytes and to allow the conversion of tel-glu into telmisartan, the centrifuge tube was cut, and each compartment was transferred to a scintillation vial or experimental tube. The compartment containing the dissolved cells was neutralized with 50 μl of 2 N HCl. The aliquots for the radioactivity determination were mixed with scintillation cocktail, and the radioactivity was

measured by a liquid scintillation counter. The aliquots for tel-glu were stored at −20°C until HPLC analysis.

Estimation of Protein Unbound Concentration of Tel-Glu in the Presence of Human Serum Albumin. The protein unbound concentration of tel-glu in the presence of human serum albumin (0, 0.1, 0.3, 1, 3, and 5%) was determined after a 2-h incubation at 37°C by equilibrium dialysis (DIANORM; Dainippon Pharmaceutical Co. Ltd., Osaka, Japan).

Determination of Tel-Glu Concentration by HPLC. Telmisartan and tel-glu concentrations were determined by HPLC. A Waters alliance HPLC system combined with a fluorescence detector (Waters 474; Waters, Milford, MA) was used. After adding ¼ volume of acetonitrile, 10-μl aliquots were separated on an XTerra RP18 column (3.5 μm, 4.6 × 150 mm; Waters) using a mobile phase (acetonitrile-water-pyridine, 234:800:0.16) at a flow rate of 1.0 ml/min. The analyte peaks (telmisartan 9 min and tel-glu 15 min) were detected by fluorescence (excitation 300 nm and emission 385 nm). The stability of telmisartan and tel-glu in the Krebs-Henseleit buffer supplemented with 0.3% HSA was confirmed up to 1 h of incubation by this HPLC analysis.

Construction of Stably Transporter-Transfected MDCKII Cell Lines. MDCKII cells expressing MDR1 and MRP2 and parental MDCKII cells were kindly provided by Dr. Piet Borst (The Netherlands Cancer Institute) (Evers et al., 1998; Tang et al., 2002). MDCKII cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (low glucose) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (Invitrogen). For construction of the BCRP-expressing MDCKII cells, parental MDCKII cells were transfected with the expression vector pcDNA3.1(+) containing human BCRP cDNA (Kondo et al., 2004) by using FuGENE6 reagent (Roche Diagnostics, Indianapolis, IN). At 50% confluence, cells on six-well plates were exposed to serum-free Opti-MEM I (Invitrogen) containing plasmid and FuGENE6 according to the manufacturer's instructions. At 6 h after the initiation of transfection, the plasmid-FuGENE6 solution was replaced with the normal culture medium. The transfected MDCKII cells were selected with neomycin (500 μg/ml; Invitrogen). Expression of BCRP was screened by the detection of BCRP mRNA in cells of each clone. For construction of double-transfected MDCKII cells, MRP2-, BCRP-, and MDR1-expressing MDCKII cells were transfected with the expression vector pcDNA3.1/Zeo(+) containing SLCO1B3 cDNA (Hirano et al., 2004; Iwai et al., 2004), and selection was performed with Zeocin (700 μg/ml; Invitrogen).

Western Blot Analysis. For Western blot analysis, a crude membrane was prepared from MDCKII cells according to the method described in a previous report (Gant et al., 1991). After the crude membrane was suspended in PBS, it was frozen in liquid N_2 and stored at −80°C until use. The protein concentrations in the crude membranes prepared from MDCKII cells were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. The membrane fraction was dissolved in 3× SDS sample buffer (New England Biolabs, Beverly, MA) with 0.125 M dithiothreitol and loaded onto a 7.5% SDS-polyacrylamide electrophoresis gel (Daiichi Pure Chemical Co. Ltd., Tokyo, Japan). The molecular weight was determined using a prestained protein marker (New England Biolabs). Proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (Pall, East Hills, NY) using a blotter (Trans-blot; Bio-Rad, Hercules, CA) at 15 V for 1 h. The membrane was blocked with Tris-buffered saline with 0.05% Tween 20 (TTBS) and 5% skimmed milk overnight at 4°C. After washing with TTBS, the membrane was incubated at room temperature in TTBS with 1000-fold diluted anti-OATP1B3 polyclonal antiserum, which was raised in rabbits against the 21 amino acids at the carboxyl terminus of the deduced OATP1B3 sequence (Hirano et al., 2004), for 1 h, 125-fold diluted monoclonal antibody against MRP2 (M_2III-6; Biochemicals, Gruenberg, Germany) for 2 h, 100-fold diluted monoclonal antibody against MDR1 (C219; Signet Laboratories, Inc., Dedhem, MA) for 1 h, or 200-fold diluted monoclonal antibody against BCRP (BXP-21; Kamiya Biomedical Company, Seattle, WA) for 2 h. For the detection of each transporter, the membrane was placed in contact with 2500-fold diluted donkey anti-rabbit (OATP1B3) or anti-mouse IgG (MRP2, MDR1, and BCRP) conjugated with horseradish peroxidase (GE Healthcare) for 1 h in TTBS. The band was detected using an ECL Plus Western blotting starter kit (GE Healthcare).

Immunocytochemical Staining. For immunocytochemical staining, transfectants were plated at a density of 5.4 × 10^5 cells in 12-well plates 96 h before the experiments. Sodium butyrate (5 mM) was added to the culture medium 1 day before the experiments. After fixation with methanol at −20°C for 10 min and permeabilization with 1% Triton X-100 in PBS at room temperature for 5 min, cells were incubated for 1 h at room temperature with 50-fold diluted anti-OATP1B3 antiserum, 40-fold diluted monoclonal antibody against MRP2 (M_2III-6), 40-fold diluted monoclonal antibody against MDR1 (C219), or 40-fold diluted monoclonal antibody against BCRP (BXP-21). Then the cells were washed with PBS three times and incubated for 1 h at room temperature with 250-fold diluted goat anti-rabbit IgG Alexa 488 (Invitrogen) for OATP1B3 or 250-fold diluted goat anti-mouse IgG Alexa 568 (Invitrogen) for MRP2, MDR1, and BCRP. Nuclei were stained with 250-fold-diluted TO-PRO-3 iodide (Invitrogen). The localization was visualized by confocal laser microscopy (Zeiss LSM-510; Carl Zeiss Inc., Thornwood, NY).

Transcelluar Transport Study Using Double-Transfected MDCKII Cells. MDCKII cells coexpressing OATP1B1 and each hepatic efflux transporter (MDR1, MRP2, and BCRP) (Evers et al., 1998; Tang et al., 2002; Matsushima et al., 2005) and coexpressing OATP1B3 and each hepatic efflux transporter (MDR1, MRP2, and BCRP) were used for transcellular transport studies. The transcellular transport study was performed as reported previously (Sasaki et al., 2002). Briefly, MDCKII cells were seeded on the Transwell (6.5-mm diameter, 0.4 μm pore size; Corning Coster, Bodenheim, Germany) at a cell density of 1.4 × 10^5 cells/well and grown on Transwell membrane inserts at confluence for 7 days, and the expression of transporters was induced by replacing the culture medium with medium containing 5 mM sodium butyrate for 48 h before the transport study. Cells were first washed with Krebs-Henseleit buffer (118 mM NaCl, 23.8 mM $NaHCO_3$, 4.8 mM KCl, 1.0 mM KH_2PO_4, 1.2 mM $MgSO_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.5 mM $CaCl_2$ adjusted to pH 7.4) and preincubated with Krebs-Henseleit buffer at 37°C for 20 min. Subsequently, the transport study was initiated by adding Krebs-Henseleit buffer containing test compounds to either the apical compartments (250 μl) or the basolateral compartments (1 ml) after removal of Krebs-Henseleit buffer used for preincubation. In the case of tel-glu, the incubation volume was changed to 100 μl for the apical compartments and to 600 μl for the basolateral compartments, and 0.3% HSA was supplemented in Krebs-Henseleit buffer. After a designated period, for the radioactive compounds, the radioactivity in 100 μl of medium in the opposite compartments was measured by a liquid scintillation counter (Tri-Carb 2500TR; PerkinElmer Life and Analytical Sciences). For the measurement of tel-glu, a sample of 50 μl was removed from the opposite compartments, and 10-μl aliquots were mixed with an equal volume of 1 N NaOH and stored for 1 h at 37°C for the complete conversion from tel-glu to telmisartan. The mixtures were neutralized with 5 μl of 2 N HCl, and then the tel-glu concentration in the medium was measured by HPLC fluorescence as described above after addition of 6.25 μl of acetonitrile. Additionally, the tel-glu concentration in a 20-μl sample was measured after adding 30 μl of distilled water and 12.5 μl of acetonitrile to assess the decomposition from tel-glu to telmisartan during the incubation period. At the end of the experiments, the cells were washed three times with 1.5 ml of ice-cold Krebs-Henseleit buffer and solubilized in 150 μl of 1N NaOH. After addition of 75 μl of 2 N HCl and 56 μl of acetonitrile, the tel-glu concentration in the cells was determined by HPLC fluorescence. Twenty-microliter aliquots of cell lysate were used to determine protein concentrations by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Kinetic Analysis. Ligand uptake was expressed as the uptake volume (microliters per milligram of protein), given as the radioactivity associated with the cells (disintegrations per minute per milligram of protein) divided by its initial concentration in the incubation medium (disintegrations per minute per microliter). Specific uptake was obtained by subtracting the uptake into vector-transfected cells from the uptake into cDNA-transfected cells. Kinetic parameters were obtained using eq. 1:

$$v = \frac{V_{max} \times S}{K_m + S} \quad (1)$$

where v is the uptake velocity of the substrate (picomoles per minute per milligram of protein), S is the substrate concentration in the medium (micromolar), K_m is the Michaelis constant (micromolar concentration), and V_{max} is the maximum uptake rate (picomoles per minute per milligram of protein). Fitting was performed by the nonlinear least-squares method using a MULTI program (Yamaoka et al., 1981). The half-inhibitory concentration (IC_{50}) of