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# Effect of oxidative stress on expression and function of human and rat organic anion transporting polypeptides in the liver

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ABSTRACT

Reactive oxygen species (ROS) have physiological function and involve alteration of physical state. However, it is not clear effect of oxidative stress on pharmacokinetics. Organic anion transporting polypeptides (human: OATPs, rodent: Oatps) are important for uptake of endogenous and exogenous compounds into hepatocytes. Thus, alteration of OATPs/Oatps expression level may affect pharmacokinetics of various drugs. In this study, we investigated the alteration of OATPs/Oatps expression levels and function by oxidative stress, and the effect of alteration of those on pharmacokinetics of a typical OATPs/Oatps substrate pravastatin. OATPs/Oatps expression levels and function were altered by $H_2O_2$-induced oxidative stress in *in vitro* experiments. The alteration of Oatps expression by oxidative stress also occurred in *in vivo* experiments. Oatp1a1, Oatp1a4 and Oatp1b2 expression in the liver were decreased in rats fed powdery diet containing 2% inosine, which induces oxidative stress through activation of xanthine oxidase, for 1 day. The decrease in Oatps expression levels by oxidative stress caused the suppression of pravastatin uptake to the liver, and resulted in high plasma concentration of pravastatin and low biliary excretion. In conclusion, oxidative stress induces alteration of OATPs/Oatps expression and function in hepatocytes, resulting in alteration of pharmacokinetics of their substrates.



## 1. Introduction

Oxidative stress is involved several types of cancer, diabetes mellitus, ischemic diseases, hepatitis and other diseases (Afanas'ev, 2011; Wu et al., 2004; Itagaki et al., 2010; Loguercio and Federico, 2003). It is well known that reactive oxygen species (ROS) cause oxidative stress. In the body, $O_2$ produces energy required for multiple cellular functions. ROS including hydroxyl radicals (•OH), superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$) accumulate as a by-products of the energy production process (Rojkind et al., 2002). $H_2O_2$ is more stable than other ROS, less reactive and acts as a second intracellular messenger that plays roles in the regulation of immunostimulation, cell growth and apoptosis (Murray and Cohn, 1980; Varela et al., 2004). On the other hand, excessive ROS cause cell death by induction of lipid peroxidation and alteration of protein conformation after oxidation of cysteine and methionine residues (Rojkind et al., 2002). In addition, severe oxidative stress causes several diseases as described above, and various drugs are used to treat oxidative stress-induced diseases. Therefore, it is important to clarify the effect of oxidative stress on pharmacokinetics.

Organic anion transporting polypeptides (humans: OATPs, rodents: Oatps) transport of a wide range of organic compounds such as bile acids, thyroid hormones, conjugated steroids and drugs (Takikawa, 2002; Fujiwara et al., 2001; Kanai et al., 1996). Moreover, there are some subtypes that recognize not only organic anion compounds but also organic cation compounds (Bossuyt et al., 1996; Franke et al., 2009). OATPs/Oatps are expressed at high levels in the liver and transport endogenous and exogenous compounds into hepatocytes (Kullak-Ublick et al., 2001; Kalliokoski and Niemi, 2009). The relationship between pharmacokinetics of statins and transport activity of OATPs/Oatps has been studied extensively. It has been shown that SLCO1B1 (coding for OATP1B1) polymorphism affects the pharmacokinetics of pravastatin and pitavastatin (Niemi et al., 2006; Wen and Xiong, 2010). In Oatp1b2 knockout mice, liver-to-plasma concentration ratios of lovastatin and cerivastatin were shown to be lower than those in wild-type mice (Chen et al., 2008). Moreover, it is well known that various drugs such as angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) (Kalliokoski and Niemi, 2009) are substrates of OATPs/Oatps. These findings indicate that elucidation of the expression levels and function of OATPs/Oatps are important for understanding of pharmacokinetics of various drugs such as statins.

It has been shown that expression levels of ATP-binding cassette (ABC) transporters, such as P-glycoprotein (P-gp) and multidrug resistance-associated protein 2 (MRP2), are altered by oxidative

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stress (Ziemann et al., 1999; Payen et al., 2001). Although alteration in the expression of OATPs/Oatps in oxidative stress-related diseases has been demonstrated (Tanaka et al., 2006; Obaidat et al., 2012), the direct effect of ROS on the expression of OATPs/Oatps has not been fully revealed. In this study, we investigated alterations of the expression and function of OATPs/Oatps caused by oxidative stress directly and the effect of those alterations on pharmacokinetics of a typical substrate of OATPs/Oatps, pravastatin. We found that oxidative stress affects the expression levels of OATPs/Oatps and the pharmacokinetics of pravastatin.

## 2. Materials and methods

### 2.1. Chemicals

Hydrogen peroxide ($H_2O_2$) and inosine were purchased from Wako Pure Chemical Industries (Osaka, Japan). [$^{14}$C]Cholic acid (CA) (specific activity: 48.6 mCi/mmol), [$^3$H]taurocholic acid (TCA) (specific activity: 5.0 Ci/mmol) and [$^3$H]estrone-3-sulfate (E3S) (specific activity: 57.3 Ci/mmol) were purchased from Perkin Elmer (Boston, MA). Pravastatin sodium was kindly donated by Daiichi Sankyo (Tokyo, Japan). All other reagents were of the highest grade available and used without further purification.

### 2.2. Cell culture

Human hepatocellular carcinoma cells (HLE cells) obtained from JCRB Cell Bank (Osaka, Japan) were maintained in plastic culture flasks (Corning Costar Corp., Cambridge, MA). The cells were kept in Dulbecco's modified Eagle's medium (Sigma Aldrich Japan, Tokyo, Japan) supplemented by 10% fetal bovine serum (Thermo Fisher Scientific K.K., Yokohama, Japan) and 100 IU/mL penicillin–100 μg/mL streptomycin (Sigma Aldrich Japan) at 37 °C under 5% $CO_2$-95% air. Rat primary hepatocytes were isolated by the collagenase perfusion technique as described previously with some modifications (Miyazaki et al., 1998). Collagen-coated 6-well and 24-well plates (Corning Costar Corp.) were prepared by using 1.4 mL/well and 0.7 mL/well of 50 μg/mL collagen solution, respectively. The plates were allowed to dry in a laminar flow cabinet for 16 h. Isolated primary hepatocytes were plated onto the collagen-coated plates in William's E medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific K.K.) and 100 IU/mL penicillin–100 μg/mL streptomycin (Sigma Aldrich Japan) and left to attach for 6 h in an incubator at 37 °C under 5% $CO_2$–95% air.

### 2.3. Treatment of HLE cells and rat primary hepatocytes with $H_2O_2$

HLE cells were seeded ($5.0 \times 10^5$ cells/mL) and cultured for 24 h, and rat primary hepatocytes were seeded ($1.0 \times 10^6$ cells/mL) and cultured for 6 h. Following cell attachment, 1 mM $H_2O_2$ was added for 6 h or 48 h in HLE cells and 12 h or 48 h in rat primary hepatocytes.

### 2.4. Animals

Male Wistar rats, aged 6 weeks, were obtained from Jla (Tokyo, Japan). The rats were housed for at least 1 day (190–350 g in body weight). The housing conditions were the same as those described previously (Ogura et al., 2012). During the period of acclimatization, the rats were allowed free access to food and water. The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the "Guide for Care and Use of Laboratory Animals".

### 2.5. Rats receiving powdery diet containing 2% inosine

Rodent labo diet 5L37 was ground into powder. Inosine at 2% was mixed with the powdery diet (Stirpe and Dellacorte, 1965). Rats were fed powdery diet containing 2% inosine for 1 day, 2 days or 5 days.

### 2.6. Quantitative real-time PCR

Real-time PCR was performed as described previously (Ogura et al., 2008). Total RNA was prepared from HLE cells, rat primary hepatocytes and rat liver using an ISOGEN (Nippon Gene, Tokyo, Japan) and an RNase-Free DNase Set (QIAGEN, Tokyo, Japan). Single-stranded cDNA was made from 1 μg total RNA by reverse transcription (RT) using a ReverTraAce (TOYOBO, Osaka, Japan). Gene-specific primers for hOATP1A2, hOATP1B1, hOATP1B3, hOATP2B1, hGAPDH, rOatp1a1, rOatp1a4, rOatp1b2, rOatp2b1 and rGapdh are shown in Table 1. Quantitative real-time PCR was performed using an Mx3000$^{TM}$ Real-time PCR System (STRATAGENE, Tokyo, Japan) with GoTaq® qPCR Master Mix (Promega, Tokyo, Japan) following the manufacturer's protocol and through 40 cycles of 95 °C for 15 s, 58 °C (for OATP1A2, OATP1B1, OATP1B3 and OATP2B1), 56 °C (for Oatp1b2) or 50 °C (Oatp1a1, Oatp1a4 and Oatp2b1) for 30 s and 72 °C for 30 s. The PCR products were normalized to amplified GAPDH, which was the internal reference.

### 2.7. Protein extraction from HLE cells and rat primary cells

All steps were performed on ice or at 4 °C. The growth medium was removed and cells were washed twice with ice-cold phosphate buffered saline (PBS). The cells were suspended in lysis buffer containing 1.0% Triton-X100, 0.1% sodium dodecyl sulphate (SDS) and 4.5 M urea. The suspension was left to stand for 5 min and sonicated for 15 min at 4 °C. Then it was centrifuged at 12,000 × *g* for 15 min at 4 °C.

### 2.8. Protein extraction from rat liver

All steps were performed on ice or at 4 °C. The liver was washed with ice-cold saline and cut. The pieces were homogenized in lysis buffer. The homogenate was left to stand for 5 min and sonicated for 15 min at 4 °C. Then it was centrifuged at 12,000 × *g* for 15 min at 4 °C.

### 2.9. Western blot analysis

The samples for Western blot analysis were protein extracts from HLE cells, rat primary hepatocytes and rat liver. Protein concentration in the clear supernatant was determined by the method of Lowry et al. (1951). The samples were denatured at 100 °C for 3 min in a loading buffer containing 50 mM Tris (hydroxymethyl)-aminomethane (Tris)-HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue (BPB) and 3.6 M urea and separated on 4.5% stacking and 10% SDS polyacrylamide gels. Proteins were transferred electrophoretically onto nitrocellulose membranes (for OATP1A2, OATP1B3, Oatp1a4 and actin) or polyvinylidene difluoride membranes (for OATP1B1, OATP2B1, Oatp1a1, Oatp1b2 and Oatp2b1) at 15 V for 90 min. The membranes were blocked with PBS containing 0.05% Tween 20 (PBS/T) and 10% or 1% non-fat dry milk for 1 h at room temperature. After being washed with PBS/T, the membranes were incubated with rabbit anti-OATP1 polyclonal antibody (H-55) (Santa Cruz Biotechnology, Santa Cruz, CA) (diluted 1:250), goat anti-OATP-C polyclonal antibody (N-16) (Santa Cruz Biotechnology) (diluted 1:250), rabbit anti-OATP8 polyclonal antibody (H-52) (Santa Cruz