HOKKAIDO UNIVERSITY

Title	Effect of oxidative stress on expression and function of human and rat organic anion transporting polypeptides in the liver
Author(s)	Tsujimoto, Takashi; Ogura, Jiro; Kuwayama, Kaori et al.
Citation	International journal of pharmaceutics, 458(2), 262-271 https://doi.org/10.1016/j.ijpharm.2013.10.013
Issue Date	2013-12-31
Doc URL	https://hdl.handle.net/2115/54673
Type	journal article
File Information	WoS_63668_Iseki.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

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

Takashi Tsujimoto, Jiro Ogura, Kaori Kuwayama, Takahiro Koizumi, Shunichi Sasaki, Yusuke Terada, Masaki Kobayashi, Hiroaki Yamaguchi, Ken Iseki*

Laboratory of Clinical Pharmaceutics & Therapeutics, Division of Pharmasciences, Faculty of Pharmaceutical Sciences, Hokkaido University

Kita-12-jo, Nishi-6-chome, Kita-ku, Sapporo 060-0812, Japan

*To whom correspondence should be addressed.

Ken Iseki, Ph. D., Laboratory of Clinical Pharmaceutics & Therapeutics, Division of Pharmasciences, Faculty of Pharmaceutical Sciences, Hokkaido University

Kita-12-jo, Nishi-6-chome, Kita-ku, Sapporo 060-0812, Japan

Tel/Fax: +81-11-706-3770, e-mail: ken-i@pharm.hokudai.ac.jp

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.

Chemical compounds studied in this article

Hydrogen Peroxide (PubChem CID: 784); Inosine (PubChem CID: 6021); Pravastatin (PubChem CID: 16759173)

Keywords: organic anion transporting polypeptide, hydrogen peroxide, oxidative stress, pharmacokinetics

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 et al., 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 stress (Zieman 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

77 OATPs/Oatps caused by oxidative stress directly and the effect of those alterations on
78 pharmacokinetics of a typical substrate of OATPs/Oatps, pravastatin. We found that oxidative
79 stress affects the expression levels of OATPs/Oatps and the pharmacokinetics of pravastatin.
80

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 (5.0×10^5 cells/mL) and cultured for 24 h, and rat primary hepatocytes were seeded (1.0×10^6 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”.

117

118 2.5. Rats receiving powdery diet containing 2% inosine

119 Rodent labo diet 5L37 was ground into powder. Inosine at 2% was mixed with the powdery

120 diet (Stirpe and Dellacorte, 1965). Rats were fed powdery diet containing 2% inosine for 1 day, 2

121 days or 5 days.

122

123 2.6. Quantitative real-time PCR

124 Real-time PCR was performed as described previously (Ogura et al., 2008). Total RNA was

125 prepared from HLE cells, rat primary hepatocytes and rat liver using an ISOGEN (Nippon Gene,

126 Tokyo, Japan) and an RNase-Free DNase Set (QIAGEN, Tokyo, Japan). Single-stranded cDNA

127 was made from 1 μg total RNA by reverse transcription (RT) using a ReverTraAce (TOYOBO,

128 Osaka, Japan). Gene-specific primers for hOATP1A2, hOATP1B1, hOATP1B3, hOATP2B1,

129 hGAPDH, rOatp1a1, rOatp1a4, rOatp1b2, rOatp2b1 and rGapdh are shown in Table 1.

130 Quantitative real-time PCR was performed using an Mx3000^{TM} Real-time PCR System

131 (STRATAGENE, Tokyo, Japan) with GoTaq® qPCR Master Mix (Promega, Tokyo, Japan)

132 following the manufacturer's protocol and through 40 cycles of 95°C for 15 sec, 58°C (for

133 OATP1A2, OATP1B1, OATP1B3 and OATP2B1), 56°C (for Oatp1b2) or 50°C (Oatp1a1,

134 Oatp1a4 and Oatp2b1) for 30 sec and 72°C for 30 sec. 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

153 buffer containing 50 mM Tris (hydroxymethyl)-aminomethane (Tris)-HCl, 2% SDS, 5%
154 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue (BPB) and 3.6 M urea and
155 separated on 4.5% stacking and 10% SDS polyacrylamide gels. Proteins were transferred
156 electrophoretically onto nitrocellulose membranes (for OATP1A2, OATP1B3, Oatp1a4 and actin)
157 or polyvinylidene difluoride membranes (for OATP1B1, OATP2B1, Oatp1a1, Oatp1b2 and
158 Oatp2b1) at 15 V for 90 min. The membranes were blocked with PBS containing 0.05% Tween
159 20 (PBS/T) and 10% or 1% non-fat dry milk for 1 h at room temperature. After being washed
160 with PBS/T, the membranes were incubated with rabbit anti-OATP1 polyclonal antibody (H-55)
161 (Santa Cruz Biotechnology, Santa Cruz, CA) (diluted 1:250), goat anti-OATP-C polyclonal
162 antibody (N-16) (Santa Cruz Biotechnology) (diluted 1:250), rabbit anti-OATP8 polyclonal
163 antibody (H-52) (Santa Cruz Biotechnology) (diluted 1:250), rabbit anti-OATP-B polyclonal
164 antibody (M-153) (Santa Cruz Biotechnology) (diluted 1:250), rabbit anti-OATP1 affinity
165 purified polyclonal antibody (Millipore, Bedford, MA) (diluted 1:500), rabbit anti-OATP2
166 polyclonal antibody (M-50) (Santa Cruz Biotechnology) (diluted 1:500), goat anti-OATP4
167 polyclonal antibody (N-17) (Santa Cruz Biotechnology) (diluted 1:250) or mouse anti-actin
168 monoclonal antibody (Millipore) (diluted 1:500) overnight at room temperature and washed three
169 times with PBS/T for 10 min each time. The membranes were subsequently incubated for 1 h at
170 room temperature with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody

(Santa Cruz Biotechnology), horseradish peroxidase-conjugated donkey anti-goat secondary antibody (Santa Cruz Biotechnology) or horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:2,000 or 1:4,000 and washed three times with PBS/T for 10 min each time. The bands were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Bioscience Corp., Piscataway, NJ).

2.10. Uptake study in HLE cells and rat primary hepatocytes

The concentrations of substrates were 1 μM for [^{14}C]CA, 50 nM for [^{3}H]TCA and 5 nM for [^{3}H]E3S (Uptake times were 10 sec/2 min for [^{14}C]CA, 30 sec/3 min for [^{3}H]TCA and 1/3 min for [^{3}H]E3S in HLE cells/rat primary hepatocytes, respectively.). The incubation medium used for the uptake study was Hanks' Balanced Salt Solution (HBSS) buffer (pH 7.4) (25 mM $_{D}$-glucose, 137 mM NaCl, 5.37 mM KCl, 0.3 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.26 mM $CaCl_2$, 0.8 mM $MgCl_2$ and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)). After removal of the growth medium, cells were preincubated at 37°C for 10 min with 0.5 mL HBSS buffer (pH 7.4). After removal of the incubation medium, 0.5 mL of incubation medium containing radiolabeled compounds was added. The cells were incubated for the indicated time at 37°C. The cells were washed rapidly twice with ice-cold incubation medium at the end of the

189 incubation period. To quantify the radioactivity of radiolabeled compounds, the cells were
190 solubilized in 1% SDS/0.2 N NaOH. The remainder of the sample was mixed with 3 mL of
191 scintillation cocktail (Perkin Elmer) to measure the radioactivity. All of the uptake values were
192 corrected against the protein content. The protein content was determined by the method of BCA
193 protein assay.
194
195 2.11. Evaluation of oxidative stress
196 Evaluation of oxidative stress through determination of malondialdehyde (MDA) levels
197 was performed as described previously (Ogura et al., 2011). Thiobarbituric acid (TBA) solution
198 consisted of 2.6 mM TBA, 918 mM trichloroacetic acid, 0.3 mM HCl and 1.8 mM 2,
199 6-di-tert-butyl-4-metylphenol in 22% ethanol. The reaction mixture contained 0.2 mL of rat liver
200 homogenate, 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid buffer (pH 3.5), and 1.5 mL of
201 TBA solution. After vortexing, the mixture was heated at 95°C for 1 h. Subsequently, the mixture
202 was cooled in iced water for 3 min. After the cooling process, 1 mL of distilled water and 5 mL
203 of n-butanol were added, and the mixture was individually mixed by vortexing. Finally, the
204 mixture was centrifuged at 330×g for 5 min at 4°C. The colored upper layer was placed in a glass
205 cell for spectrophotometer reading at 535 nm with 1, 1, 3, 3-tetraethoxypropane (TEP) as a
206 standard. The amount of MDA was corrected by protein content. The protein content was

207 determined by the method of BCA protein assay.

208

209 2.12. Pharmacokinetic study

210 A pharmacokinetic study was carried out in male Wistar rats (7 weeks old) fed powdery
211 diet containing 2% inosine for 1 day or 5 days. The common bile duct of each rat was cannulated
212 with a polyethylene tube (SP10, NATSUME, Tokyo, Japan) to collect bile specimens. Pravastatin
213 sodium dissolved in saline (10 mg/mL) was administered as an intravenous injection at a dose of
214 10 mg/kg. Blood samples (each 0.4 mL) were collected from the jugular vein at designated time
215 points (1, 3, 5, 15, 30, 45 and 60 min after injection) into eppendorf tubes containing trace
216 heparin. Plasma was prepared by centrifugation (850×g for 5 min at 4°C) of blood samples. Bile
217 samples were collected from the bile duct at designated time points (0-15, 15-30, 30-45 and
218 45-60 min after injection). Liver samples were harvested at 60 min after injection and
219 homogenized in distilled water. All samples were stored at −30°C until analysis.

220

221 2.13. Sample preparation

222 To 100 μL of a plasma sample, 0.5 mL of methanol was added for de-proteination and
223 mixed by vortexing. After vortexing, the sample was centrifuged at 12,000×g for 5 min at room
224 temperature. The supernatant was filtrated using a Cosmonice filter W (pore size of 0.45 μm,

225 Nacalai Tesque, Kyoto, Japan). Bile samples were prepared by liquid–liquid extraction. To 100
226 μL of a bile sample, 1 mL of ethyl acetate was added and mixed by vortexing. After vortexing,
227 the sample was centrifuged at 12,000×g for 5 min at room temperature. After centrifugation, the
228 upper layer was evaporated to dryness at 40°C using a gentle stream of air. The residue was
229 reconstituted in 500 μL of distilled water. Liver samples were prepared by liquid–liquid
230 extraction and solid-phase extraction. Three milliliters of a sample was centrifuged at 12,000×g
231 for 5 min at room temperature. To 1.5 mL of the supernatant, 5 mL of ethyl acetate was added
232 and mixed by vortexing. After vortexing, the sample was centrifuged at 12,000×g for 5 min at
233 room temperature. After centrifugation, the upper layer was evaporated to dryness at 40°C using
234 a gentle stream of air. The residue was reconstituted in 150 μL of methanol and 150 μL of
235 distilled water. After reconstitution, the sample was purified by solid-phase extraction using
236 Bond-Elute C18 solid phase extraction cartridges (Agilent Technologies, Tokyo, Japan). The
237 cartridges were conditioned with 1 mL of methanol and 1 mL of distilled water before applying
238 the liver samples. The samples were transferred to solid-phase extraction cartridges and
239 centrifuged at 1,300×g for 5 min at room temperature to obtain a flow through the cartridges.
240 After centrifugation, the cartridges were eluted twice with 1 mL of methanol. The eluting solvent
241 was evaporated to dryness at 40°C using a gentle stream of air. The residue was reconstituted in
242 200 μL of distilled water.

243

244 2.14. Analytical procedures

245 Pravastatin concentration was determined using an HPLC system equipped with a
246 RHEODYINE 7125 valve, Hitachi L-7110 pump, Hitachi L-7450 UV detector, Hitachi D-2500
247 chromato-integrator and Hitachi L-7300 column oven. The column was a Mightysil RP-8 GP
248 (250 mm×4.6 mm in i.d., KANTO CHEMICAL, Tokyo, Japan) with a guard column of a
249 Mightysil RP-18 GP Aqua (5 mm×4.6 mm in i.d., KANTO CHEMICAL). A mobile phase
250 consisting of water and acetonitrile mixture (63:17 (v/v), containing CH_3COONH_3 at a final conc.
251 of 1.25 mM) was used. Column temperature and flow rate were 40 °C and 1.5 mL/min,
252 respectively. The wavelength of the detector was 238 nm. The samples were diluted appropriately,
253 and 50 μL of each sample was injected onto the HPLC column. The standard solution was diluted
254 with blood, bile and liver homogenate mixture to prepare calibration curves. The calibration
255 curves were constructed at 4-6 concentration levels for standard solutions of each analyte.

256 Statistical significance was evaluated by Student's *t*-test, and a value of $p<0.05$ was
257 considered significant.

258

259 **3. Results**

260 3.1. Effects of H_2O_2 on mRNA, protein expression levels and function of OATPs in HLE cells

261 Firstly, we examined alterations of mRNA and protein expression levels of OATPs
262 induced by H_2O_2 in HLE cells (Fig. 1). In HLE cells, the mRNA and protein expression levels of
263 OATP1A2 were significantly decreased by exposure to H_2O_2 for 6 h but were significantly
264 increased by exposure for 48 h (Fig. 1A). The mRNA and protein expression levels of OATP1B1
265 were significantly increased by exposure to H_2O_2 for 6 h, but only the mRNA level was increased
266 by exposure for 48 h (Fig. 1B). The mRNA and protein expression levels of OATP1B3 were
267 significantly increased by exposure to H_2O_2 for 48 h but were not altered by exposure for 6 h (Fig.
268 1C). The mRNA level of OATP2B1 was significantly decreased by exposure to H_2O_2 6 h, though
269 the protein expression level was not altered by exposure for either 6 h or 48 h (Fig. 1D). To
270 evaluate effects of oxidative stress on the function of OATPs, uptake of [^{14}C]CA, [^{3}H]TCA or
271 [^{3}H]E3S by H_2O_2-exposed HLE cells was assessed (Fig. 2). Before the investigation of the effect
272 of H_2O_2 on uptake of each compound, we determined the initial uptake rate of each compound in
273 HLE cells from time course experiments, and we evaluated the initial uptake for 10 sec for
274 [^{14}C]CA, 30 sec for [^{3}H]TCA and 1 min for [^{3}H]E3S in HLE cells, respectively (data not shown).
275 The uptake of [^{14}C]CA by HLE cells was found to be significantly decreased by exposure to
276 H_2O_2 for 6 h but significantly increased by exposure for 48 h (Fig. 2A). The uptake of [^{3}H]TCA

by HLE cells was significantly increased by exposure to H_2O_2 for 48 h but was not altered by exposure for 6 h (Fig. 2B). The uptake of [^{3}H]E3S by HLE cells was significantly increased by exposure to H_2O_2 for 6 h but was not altered by exposure for 48 h (Fig. 2C).

In rat primary hepatocytes, the mRNA levels of Oatp1a1 and Oatp1a4 were significantly decreased by exposure to H_2O_2 for 12 h and 48 h (Figs. 3A and 3B). However, the protein expression levels were decreased only by exposure to H_2O_2 for 12 h (Figs. 3A and 3B). The mRNA and protein expression levels of Oatp1b2 were not altered by exposure to H_2O_2 for either 12 h or 48 h (Fig. 3C). The mRNA level of Oatp2b1 was significantly decreased by exposure to H_2O_2 for 12 h, though the protein expression level was not altered by exposure for either 12 h or 48 h (Fig. 3D). The initial uptake rate of [^{14}C]CA, [^{3}H]TCA and [^{3}H]E3S in rat primary hepatocytes was determined by time course experiments, and we evaluated the initial uptake for 2 min for [^{14}C]CA, 3 min for [^{3}H]TCA and 3 min for [^{3}H]E3S in rat primary hepatocytes, respectively (data not shown). The uptake of [^{14}C]CA and that of [^{3}H]TCA by rat primary hepatocytes were significantly decreased by exposure to H_2O_2 for 12 h but were not altered by exposure for 48 h (Figs. 4A and 4B). The uptake of [^{3}H]E3S by rat primary hepatocytes was not altered by exposure to H_2O_2 for either 12 h or 48 h (Fig. 4C).

3.2. Effect of inosine-induced oxidative stress on lipid peroxidation in the liver

295 Activation of xanthine oxidase is one of the major pathways of oxidative stress in the body.
296 Inosine induces oxidative stress through activation of xanthine oxidase (Stirpe and Dellacorte,
297 1965). To confirm the generation of oxidative stress in rats fed powdery diet containing 2%
298 inosine, we assessed oxidative stress by the level of MDA, which is a marker of lipid
299 peroxidation, in the liver. During all periods, MDA levels in the livers of rats fed powdery diet
300 containing 2% inosine were significantly increased compared with those in control rats (Fig. 5).
301
302 3.3. Effect of inosine-induced oxidative stress on mRNA and protein expression levels of Oatps
303 in the rat liver
304 The mRNA and protein expression levels of Oatp1a1 and Oatp1a4 were significantly
305 decreased by feeding powdery diet containing 2% inosine for 1 day and 2 days (Figs. 6A and 6B).
306 The protein expression level of Oatp1b2 was significantly decreased at 1 day and 2 days, though
307 the mRNA level was not altered (Fig. 6C). The mRNA and protein expression levels of Oatp2b1
308 were not altered in any period (Fig. 6D).
309
310 3.4. Effect of inosine-induced oxidative stress on pharmacokinetics of pravastatin
311 The plasma concentration, biliary excretion and liver accumulation of pravastatin in rats
312 fed powdery diet containing 2% inosine are shown in Fig. 7. The plasma concentration of

pravastatin at 1-5 min after intravenous administration was significantly increased by feeding powdery diet containing 2% inosine for 1 day. Moreover, liver accumulation of pravastatin at 60 min after intravenous administration and biliary excretion of pravastatin at each time point were significantly decreased by feeding powdery diet containing 2% inosine for 1 day. The pharmacokinetic parameters calculated as a 2-compartment model are shown in Table 2. In rats fed powdery diet containing 2% inosine for 1 day, the area under the blood concentration-time curve ($AUC_{0-\infty}$) of pravastatin was significantly increased, and total body clearance (CL_{tot}) of pravastatin was significantly decreased, but the distribution volume (V_d) of pravastatin was not altered. The pharmacokinetics of pravastatin recovered in rats fed powdery diet containing 2% inosine for 5 days.

4. Discussion

It is well known that P-gp expression is increased in oxidative stress-induced diseases, such as cancer and ischemia disease, and that this increase in P-gp expression is caused by ROS, which mainly cause oxidative stress (Ghanem et al., 2004; Wu et al., 2009). Recently, alteration in the expression of OATPs/Oatps in oxidative stress-related diseases has been demonstrated (Tanaka et al., 2006; Obaidat et al., 2012), but the effect of ROS on the expression of OATPs/Oatps has not been fully revealed. Furthermore, the effect of oxidative stress on the pharmacokinetics of clinical drugs that are transported by OATPs has not been demonstrated. In this study, we characterized ROS-induced alteration of the expression of OATPs/Oatps and alteration of pharmacokinetics of the clinical drug pravastatin in an oxidative stress-exposed state.

An *in vitro* study showed that the oxidative stress was induced by H_2O_2 and H_2O_2 exposure times were decided on the basis of results of MTT assays (Supplementary Fig. 1). HLE cells were more susceptible to H_2O_2 than were rat primary hepatocytes. The viability in HLE cells exposed to H_2O_2 for 6 h was the same as that of rat primary hepatocytes exposed to H_2O_2 for 12 h. Thus, HLE cells and rat primary hepatocytes were exposed to H_2O_2 for 6 h and 12 h, respectively, in this study. Moreover, to determine the effect of exposure time on alterations in the expression and function of OATPs/Oatps, both HLE cells and rat primary hepatocytes were exposed to H_2O_2 for

342 48 h. The mRNA levels of OATPs/Oatps in HLE cells/rat primary hepatocytes were altered by 1
343 mM H_2O_2 (Figs. 1 and 3) but were not altered by 0-100 μM H_2O_2 (data not shown). Moreover,
344 H_2O_2 at the concentration of 1 mM was previously shown to up-regulate the rat multidrug
345 resistance mdr1b gene (Ziemann et al., 1999) in primary rat hepatocyte cultures. Thus, in this
346 study, effects of oxidative stress on the expression level and function of OATPs/Oatps were
347 evaluated by exposure to 1 mM H_2O_2. As a result, mRNA and protein expression levels of
348 OATPs/Oatps were altered by exposure to H_2O_2 (Figs. 1 and 3), and uptake of some organic
349 anion compounds was also altered (Figs. 2 and 4). Alterations in the uptake of [^{3}H]CA (Fig. 2A),
350 [^{3}H]TCA (Fig. 2B) and [^{3}H] E3S (Fig. 2C) in HLE cells were associated with alterations of
351 OATP1A2 (Fig. 1A), OATP1B3 (Fig. 1C) and OATP1B1 (Fig. 1B) protein expression levels,
352 respectively. In rat primary hepatocytes, [^{3}H]CA (Fig. 4A) and [^{3}H]TCA (Fig. 4B) uptake was
353 decreased by exposure to H_2O_2 for 12 h, being associated with decreasing Oatp1a1 (Fig. 3A) and
354 Oatp1a4 (Fig. 3B) protein expression levels. It has been shown that bile acids are mainly
355 transported by Oatp1a1 and Oatp1a4 in rats (Gartung and Matern, 1997). Thus, the decrease in
356 the uptake of [^{3}H]CA and [^{3}H]TCA might be caused by the decrease in expression levels of
357 Oatp1a1 and Oatp1a4 for hepatic uptake. The sodium-taurocholate cotransporting polypeptide
358 (NTCP/Ntcp) is known to be various bile acids uptake transporter. However, NTCP mRNA in
359 HLE cells was not detected RT-PCR (data not shown). Moreover, Ntcp mRNA level was not

altered by exposure to H_2O_2 for either 12 h or 48 h (data not shown). The alteration of OATPs/Oatps expression did not completely parallel between HLE cells and rat primary hepatocytes. There is no strict one-to-one relationship between human OATPs genes and rodent Oatps genes (Hagenbuch and Meier, 2004). This might be one of the reasons for the difference of alteration of OATPs/Oatps expression between HLE cells and rat primary hepatocytes.

To further determine the involvement H_2O_2-induced alterations in the expression and function of OATPs/Oatps, the effect of co-exposure to PEG-catalase, which is an H_2O_2 scavenger, was examined. The H_2O_2-induced alterations in expression and function of OATPs/Oatps were diminished by co-exposure to PEG-catalase (Supplementary Figs. 2-5). These results confirmed the effects of H_2O_2 on the expression of OATPs/Oatps and their transport activities. However, a part of the alterations in expression and function of OATPs/Oatps was different from the above results (Figs.1-4). PEG-catalase was stocked in 50% glycerol/water solution at 5,000 U/mL and diluted with a medium to 100 U/mL. Therefore, we used a medium containing 1% glycerol in the experiments with co-exposure to PEG-catalase. The difference between the alterations in expression and function of OATPs/Oatps in the experiments may have been caused by the presence or absence of 1% glycerol in the medium.

Alteration in expression of Oatps by oxidative stress also occurred in *in vivo* experiments. Oatp1a1, Oatp1a4 and Oatp1b2 expression levels in the livers of rats fed powdery diet containing

2% inosine for 1 day were decreased (Fig. 6). Although a decrease in Oatp1b2 protein expression level occurred only in the *in vivo* experiment, the decreases in Oatp1a1 and Oatp1a4 expression levels were similar to the results of *in vitro* experiments. Pravastatin is a substrate of Oatp1a1 and Oatp1b2 but not of Oatp1a4 (Hsiang et al., 1999; Chen et al., 2008). Thus, the decreases in Oatp1a1 (61%) and Oatp1b2 (66%) expression by oxidative stress caused suppression of pravastatin uptake in the liver (61%) and resulted in high plasma concentration and low biliary excretion of pravastatin (Fig. 7). In Oatp1b2 knockout mice, liver concentration of pravastatin at 2 h after s.c. injection was decreased to 76% than that in wild-type mice (Chen et a., 2008). Moreover, the uptake of pravastatin by rat primary hepatocytes was decreased to 73% of the control level by the exposure to H_2O_2 for 12 h (Supplementary Fig. 6B), and then Oatp1a1 (51%) expression decreased, but Oatp1b2 expression not altered (Fig. 3A and 3C). These findings suggested that the decrease in Oatp1a1 expression level had a greater contribution than the decrease in Oatp1b2 expression level to the decrease in accumulation of pravastatin in the liver. On the other hand, the uptake of pravastatin by HLE cells was increased by exposure to H_2O_2 for 48 h (Supplementary Fig. 6A), being associated with OATP1B3 expression (Fig. 1C). It has been shown that pravastatin uptake is mainly mediated by OATP1B1 in cryopreserved human hepatocytes (Kusuhara and Sugiyama, 2009), though it is also mediated by OATP1B3 and OATP2B1 (Kalliokoski and Niemi, 2009). However, the increase in OATP1B1 expression by

exposure to H_2O_2 for 6 h (Fig. 1B) did not affect the uptake of pravastatin by HLE cells (Supplementary Fig. 6A). Contribution of OATP1B1 to uptake of organic anion compounds might have been underestimated in the study using HLE cells. It is known that MRP2/Mrp2, which is expressed in the canalicular membrane, has a predominant influence on the efflux of pravastatin from hepatocytes to bile. Mrp2 mRNA level was increased by feeding powdery diet containing 2% inosine for 1 day and 2 days (Supplementary Fig. 7). An increase in MRP2/Mrp2 expression was induced by exposure to H_2O_2 for 6 h and 12 h in HLE cells and rat primary hepatocytes, respectively (Supplementary Fig. 8). These findings suggested that the low biliary excretion of pravastatin was caused by decrease in the expression of Oatps not by alteration of Mrp2 expression.

Despite the persistence of oxidative stress, Oatp1a1, Oatp1a4 and Oatp1b2 expression and function were recovered in rats fed powdery diet containing 2% inosine for 5 days (Fig. 5), resulting in recovery of the pharmacokinetics of pravastatin (Fig. 7). Recovery of the expression of Oatps with long-term exposure to H_2O_2 also occurred in *in vitro* experiments (Fig. 3). These results suggested that the effects of oxidative stress on the expression and function of OATPs/Oatps depended on oxidative stress loaded time. It has been shown that the effect of oxidative stress on physiological status in a body was changed by oxidative stress loaded time (Jendrach et al., 2008). On the other hand, Macias *et al*. (2011) reported that prolonged

obstructive cholestasis in rats for 8 weeks, which induces severely oxidative stress, Oatp1a1 and Oatp1a4 mRNA level in the liver were significantly decreased. These findings are not consisted our results in this study. However, their prolonged obstructive cholestasis induces high mortality (20%), suggesting that the rats have not function of the recovery of Oatps expression because the liver injury in prolonged obstructive cholestasis rats progresses to the irreversible stage. Indeed, Donner *et al*. (2007) reported that Oatp1a1, Oatp1a4 and Oatp1b2 expression levels were decreased as with the peak of 3 days followed by the gradual increased with time up to 7 days after obstructive cholestasis in rats. Response to oxidative stress in a body occurs for maintenance of homeostasis and/or recovery from oxidative damage. Since OATPs/Oatps transport biologically active substances, such as bile acids and thyroid hormones, they play an important role in homeostasis. Although details of the molecular mechanism remain unclear, our results suggested that recovery of the expression and function of OATPs/Oatps might be one mechanism for cellular protection when oxidative damage has occurred.

Recently, oxidative modifications of proteins and phospholipids are related with the alteration of protein function (Jacob et al., 2012; Ullery and Marnett, 2012). Unfortunately, to our knowledge, there are no reports describing the effect of oxidative modification on OATPs/Oatps but there is a possibility to modified OATPs/Oatps by oxidative stress. Although we could not elucidate the effect of oxidative modifications on OATPs/Oatps function in this study, the

comprehensive evaluation of the effect of oxidative modifications on the OATPs/Oatps function is the subject of future investigation.

In conclusion, oxidative stress induces alterations in the expression and function of OATPs/Oatps in hepatocytes, resulting in alteration in the pharmacokinetics of pravastatin. Moreover, the alterations in the expression and function of OATPs/Oatps were affected by oxidative stress loaded time. The newly found influence of oxidative stress on the expression and function of OATPs/Oatps may be a key to perform appropriate drug therapy.

References

Afanas'ev, I., 2011. Reactive oxygen species signaling in cancer: comparison with aging. Aging Dis. 2, 219-230.

Bossuyt, X., Müller, M., Hagenbuch, B., Meier, P.J., 1996. Polyspecific drug and steroid clearance by an organic anion transporter of mammalian liver. J. Pharmacol. Exp. Ther. 276, 891-896.

Chen, C., Stock, J.L., Liu, X., Shi, J., Van Deusen, J.W., DiMattia, D.A., Dullea, R.G., de Morais, S.M., 2008. Utility of a novel Oatp1b2 knockout mouse model for evaluating the role of Oatp1b2 in the hepatic uptake of model compounds. Drug Metab. Dispos. 36, 1840-1845.

Donner, M.G., Schumacher, S., Warskulat, U., Heinemann, J., Häussinger, D., 2007. Obstructive cholestasis induces TNF-alpha- and IL-1 -mediated periportal downregulation of Bsep and zonal regulation of Ntcp, Oatp1a4, and Oatp1b2. Am. J. Physiol. Gastrointest. Liver Physiol. 293, G1134-G1146.

Franke, R.M., Scherkenbach, L.A., Sparreboom, A., 2009. Pharmacogenetics of the organic

458 anion transporting polypeptide 1A2. Pharmacogenomics. 10, 339-344.

459

460 Fujiwara, K., Adachi, H., Nishio, T., Unno, M., Tokui, T., Okabe, M., Onogawa, T., Suzuki,
461 T., Asano, N., Tanemoto, M., Seki, M., Shiiba, K., Suzuki, M., Kondo, Y., Nunoki, K.,
462 Shimosegawa, T., Iinuma, K., Ito, S., Matsuno, S., Abe, T., 2001. Identification of thyroid
463 hormone transporters in humans: different molecules are involved in a tissue-specific manner.
464 Endocrinology. 142, 2005-2012.

465

466 Gartung, C., Matern, S., 1997. Molecular regulation of sinusoidal liver bile acid
467 transporters during cholestasis. Yale J. Biol. Med. 70, 355-363.

468

469 Ghanem, C.I., Gómez, P.C., Arana, M.C., Perassolo, M., Ruiz, M.L., Villanueva, S.S.,
470 Ochoa, E.J., Catania, V.A., Bengochea, L.A., Mottino, A.D., 2004. Effect of acetaminophen on
471 expression and activity of rat liver multidrug resistance-associated protein 2 and P-glycoprotein.
472 Biochem. Pharmacol. 68, 791-798.

473

474 Hagenbuch B, Meier PJ., 2004. Organic anion transporting polypeptides of the OATP/
475 SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and

molecular/functional properties. Pflugers Arch. 447, 653-665.

Hsiang, B., Zhu, Y., Wang, Z., Wu, Y., Sasseville, V., Yang, W.P., Kirchgessner, T.G., 1999. A novel human hepatic organic anion transporting polypeptide (OATP2). Identification of a liver-specific human organic anion transporting polypeptide and identification of rat and human hydroxymethylglutaryl-CoA reductase inhibitor transporters. J. Biol. Chem. 274, 37161-37168.

Itagaki, S., Oikawa, S., Ogura, J., Kobayashi, M., Hirano, T., Iseki, K., 2010. Protective effects of rutin on ischemia-reperfusion injury in rat small intestine. Food Chem. 118, 426-429.

Jacob, C., Battaglia, E., Burkholz, T., Peng, D., Bagrel, D., Montenarh, M., 2012. Control of oxidative posttranslational cysteine modifications: from intricate chemistry to widespread biological and medical applications. Chem. Res. Toxicol. 25, 588-604.

Jendrach, M., Mai, S., Pohl, S., Vöth, M., Bereiter-Hahn, J., 2008. Short- and long-term alterations of mitochondrial morphology, dynamics and mtDNA after transient oxidative stress. Mitochondrion. 8, 293-304.

494 Kalliokoski, A., Niemi, M., 2009. Impact of OATP transporters on pharmacokinetics. Br. J.
495 Pharmacol. 158, 693-705.
496
497 Kanai, N., Lu, R., Bao, Y., Wolkoff, A.W., Vore, M., Schuster, V.L., 1996. Estradiol 17
498 beta-D-glucuronide is a high-affinity substrate for oatp organic anion transporter. Am. J. Physiol.
499 270, 326-331.
500
501 Kullak-Ublick, G.A., Ismair, M.G., Stieger, B., Landmann, L., Huber, R., Pizzagalli, F.,
502 Fattinger, K., Meier, P.J., Hagenbuch, B., 2001. Organic anion-transporting polypeptide B
503 (OATP-B) and its functional comparison with three other OATPs of human liver.
504 Gastroenterology. 120, 525-533.
505
506 Kusuhara, H., Sugiyama, Y., 2009. In vitro-in vivo extrapolation of transporter-mediated
507 clearance in the liver and kidney. Drug Metab. Pharmacokinet. 24, 37-52.
508
509 Loguercio, C., Federico, A., 2003. Oxidative stress in viral and alcoholic hepatitis. Free
510 Radic. Biol. Med. 34, 1-10.
511

512 Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with
513 the Folin phenol reagent. J. Biol. Chem. 193, 265-275.
514
515 Macias, R.I., Hierro, C., de Juan, S.C., Jimenez, F., Gonzalez-San Martin, F., Marin, J.J.,
516 2011. Hepatic expression of sodium-dependent vitamin C transporters: ontogeny, subtissular
517 distribution and effect of chronic liver diseases. Br. J. Nutr. 106, 1814-1825.
518
519 Miyazaki, M., Mars, W.M., Runge, D., Kim, T.H., Bowen, W.C., Michalopoulos, G.K.,
520 1998. Phenobarbital suppresses growth and accelerates restoration of differentiation markers of
521 primary culture rat hepatocytes in the chemically defined hepatocyte growth medium containing
522 hepatocyte growth factor and epidermal growth factor. Exp. Cell Res. 241, 445-457.
523
524 Murray, H.W., Cohn, Z.A., 1980. Macrophage oxygen-dependent antimicrobial activity. III.
525 Enhanced oxidative metabolism as an expression of macrophage activation. J. Exp. Med. 152,
526 1569-1609.
527
528 Niemi, M., Pasanen, M.K., Neuvonen, P.J., 2006. SLCO1B1 polymorphism and sex affect
529 the pharmacokinetics of pravastatin but not fluvastatin. Clin. Pharmacol. Ther. 80, 356-366.

Obaidat, A., Roth, M., Hagenbuch, B., 2012. The expression and function of organic anion transporting polypeptides in normal tissues and in cancer. Annu. Rev. Pharmacol. Toxicol. 52, 135-151.

Ogura, J., Fujikawa, A., Maruyama, H., Kobayashi, M., Itagaki, S., Iseki, K., 2011. Alteration of P-gp expression after intestinal ischemia-reperfusion following 16-h fasting in rats. Yakugaku Zasshi. 131, 453-462.

Ogura, J., Kobayashi, M., Itagaki, S., Hirano, T., Iseki, K., 2008. Alteration of Mrp2 and P-gp expression, including expression in remote organs, after intestinal ischemia-reperfusion. Life Sci. 82, 1242-1248.

Ogura, J., Kuwayama, K., Takaya, A., Terada, Y., Tsujimoto, T., Koizumi, T., Maruyama, H., Fujikawa, A., Takahashi, N., Kobayashi, M., Itagaki, S., Hirano, T., Yamaguchi, H., Iseki, K., 2012. Intestinal ischemia-reperfusion increases efflux for uric acid via paracellular route in the intestine, but decreases that via transcellular route mediated by BCRP. J. Pharm. Pharm. Sci. 15, 295-304.

548

549 Payen, L., Courtois, A., Loewert, M., Guillouzo, A., Fardel, O., 2001. Reactive oxygen
550 species-related induction of multidrug resistance-associated protein 2 expression in primary
551 hepatocytes exposed to sulforaphane. Biochem. Biophys. Res. Commun. 282, 257-263.

552

553 Rojkind, M., Domínguez-Rosales, J.A., Nieto, N., Greenwel, P., 2002. Role of hydrogen
554 peroxide and oxidative stress in healing responses. Cell Mol. Life Sci. 59, 1872-1891.

555

556 Stirpe, F., Dellacorte, E., 1965. Regulation of xanthine dehydrogenase in chick liver. effect
557 of starvation and of administration of purines and purine nucleosides. Biochem. J. 94, 309-313.

558

559 Takikawa, H., 2002. Hepatobiliary transport of bile acids and organic anions. J.
560 Hepatobiliary Pancreat. Surg. 9, 443-447.

561

562 Tanaka, Y., Chen, C., Maher, J.M., Klaassen, C.D., 2006. Kupffer cell-mediated
563 downregulation of hepatic transporter expression in rat hepatic ischemia-reperfusion.
564 Transplantation. 82, 258-266.

565

566 Ullery, J.C., Marnett, L.J., 2012. Protein modification by oxidized phospholipids and
567 hydrolytically released lipid electrophiles: Investigating cellular responses. Biochim. Biophys.
568 Acta. 1818, 2424-2435.
569
570 Varela, D., Simon, F., Riveros, A., Jørgensen, F., Stutzin, A., 2004. NAD(P)H
571 oxidase-derived H(2)O(2) signals chloride channel activation in cell volume regulation and cell
572 proliferation. J. Biol. Chem. 279, 13301-13304.
573
574 Wen, J., Xiong, Y., 2010. OATP1B1 388A>G polymorphism and pharmacokinetics of
575 pitavastatin in Chinese healthy volunteers. J. Clin. Pharm. Ther. 35, 99-104.
576
577 Wu, J., Ji, H., Wang, Y.Y., Wang, Y., Li, Y.Q., Li, W.G., Long, Y., Xia, Y.Z., Hong, H., 2009.
578 Glutathione depletion upregulates P-glycoprotein expression at the blood-brain barrier in rats. J.
579 Pharm. Pharmacol. 61, 819-824.
580
581 Wu, L., Nicholson, W., Knobel, S.M., Steffner, R.J., May, J.M., Piston, D.W., Powers,
582 A.C., 2004. Oxidative stress is a mediator of glucose toxicity in insulin-secreting pancreatic islet
583 cell lines. J. Biol. Chem. 279, 12126-12134.

584

585 Ziemann, C., Bürkle, A., Kahl, G.F., Hirsch-Ernst, K.I., 1999. Reactive oxygen species

586 participate in mdr1b mRNA and P-glycoprotein overexpression in primary rat hepatocyte cultures.

587 Carcinogenesis. 20, 407-414.

588

589

590 **Figure legends**

591 Fig. 1. Effects of H_2O_2 on mRNA and protein expression levels of OATPs in HLE cells.

592 Effect of H_2O_2 on mRNA levels of OATPs in HLE cells (left side).

593 Effect of H_2O_2 on protein expression levels of OATPs in HLE cells (right side).

594 HLE cells were exposed to 1 mM H_2O_2 for 6 h or 48 h.

595 Band intensity was determined by densitometry using Scion image program.

596 Each column represents the mean with S.D. of 3-6 measurements.

597 *; significantly different from control ($p<0.05$)

598 **; significantly different from control ($p<0.01$)

599 N.S.; not significant

600

601 Fig. 2. Effects of H_2O_2 on uptake of various compounds by HLE cells.

602 (A) Effects of H_2O_2 on uptake of [^{14}C]CA.

603 (B) Effects of H_2O_2 on uptake of [^{3}H]TCA.

604 (C) Effects of H_2O_2 on uptake of [^{3}H]E3S.

605 HLE cells were exposed to 1 mM H_2O_2 for 6 h or 48 h.

606 HLE cells were incubated with uptake medium containing 1 μM [^{14}C]CA for 10 sec, 50 nM

607 [^{3}H]TCA for 30 sec or 5 nM [^{3}H]E3S for 1 min at 37°C and at pH 7.4.

608 Each column represents the mean with S.D. of 3-6 measurements.

609 *; significantly different from control ($p<0.05$)

610 **; significantly different from control ($p<0.01$)

611 N.S.; not significant

612

613 Fig. 3. Effects of H_2O_2 on mRNA and protein expression levels of Oatps in rat primary

614 hepatocytes.

615 Effect of H_2O_2 on mRNA levels of Oatps in rat primary hepatocytes (left side).

616 Effect of H_2O_2 on protein expression levels of Oatps in rat primary hepatocytes (right side).

617 Rat primary hepatocytes were exposed to 1 mM H_2O_2 for 12 h or 48 h.

618 Band intensity was determined by densitometry using Scion image program.

619 Each column represents the mean with S.D. of 3-6 measurements.

620 *; significantly different from control ($p<0.05$)

621 **; significantly different from control ($p<0.01$)

622 N.S.; not significant

623

624 Fig. 4. Effects of H_2O_2 on uptake of various compounds by rat primary hepatocytes.

625 (A) Effects of H_2O_2 on uptake of [^{14}C]CA.

626 (B) Effects of H_2O_2 on uptake of [^{3}H]TCA.

627 (C) Effects of H_2O_2 on uptake of [^{3}H]E3S.

628 Rat primary hepatocytes were exposed to 1 mM H_2O_2 for 12 h or 48 h.

629 Rat primary hepatocytes were incubated with uptake medium containing 1 μM [^{14}C]CA for 2 min,

630 50 nM [^{3}H]TCA for 3 min or 5 nM [^{3}H]E3S for 3 min at 37°C and at pH 7.4.

631 Each column represents the mean with S.D. of 3-6 measurements.

632 **; significantly different from control ($p<0.01$)

633 N.S.; not significant

634

635 Fig. 5. Lipid peroxidation in the liver of rats fed powdery diet containing 2% inosine.

636 MDA levels in the liver of rats fed powdery diet containing 2% inosine for 1 day, 2 days or 5

637 days.

638 Each column represents the mean with S.D. of 4-5 measurements.

639 **; significantly different from control ($p<0.01$)

640

641 Fig. 6. Effect of inosine-induced oxidative stress on mRNA and protein expression levels of

642 Oatps in the liver.

643 Rats were fed powdery diet containing 2% inosine for 1 day, 2 days or 5 days.

644 Band intensity was determined by densitometry using Scion image program.

645 Each column represents the mean with S.D. of 3-5 independent experiments.

646 *; significantly different from control ($p<0.05$)

647 N.S.; not significant

648

649 Fig. 7 Plasma concentration, biliary excretion and liver accumulation of pravastatin after

650 intravenous administration in rats under the condition of oxidative stress.

651 Rats were fed powdery diet containing 2% inosine for 1 day (A) or 5 days (B).

652 Pravastatin was injected at a dose of 10 mg/kg. Blood samples and bile samples were collected at

653 specified times after injection. Liver samples were harvested at 60 min after injection.

654 Each point and column represent the mean with S.D. of 3-4 measurements.

655 *; significantly different from control ($p<0.05$)

656 **; significantly different from control ($p<0.01$)

657 N.S.; not significant

658

659 Supplementary Fig. 1. Effect of H_2O_2 on viability of HLE cells and rat primary hepatocytes.

660 (A) Effect of H_2O_2 on viability of HLE cells.

661 HLE cells were exposed to 1 mM H_2O_2 for 6 and 48 h.

662 (B) Effect of H_2O_2 on viability of rat primary hepatocytes.

663 Rat primary hepatocytes were exposed to 1 mM H_2O_2 for 12 and 48 h.

664 Each column represents the mean with S.D. of 6-8 measurements.

665

666 Supplementary Fig. 2. Eliminative effects of catalase on H_2O_2-induced alterations in mRNA and

667 protein expression levels of OATPs in HLE cells.

668 Eliminative effect of catalase on H_2O_2-induced alteration in mRNA levels of OATPs (left side).

669 Eliminative effect of catalase on H_2O_2-induced alteration in protein expression levels of OATPs

670 (right side).

671 HLE cells were exposed to 1 mM H_2O_2 in the absence or presence of PEG-catalase (100 U/mL)

672 for 6 h or 48 h. Each solution contained 1% glycerol.

673 Band intensity was determined by densitometry using Scion image program.

674 Each column represents the mean with S.D. of 3-6 measurements.

675 Statistical significance was evaluated using ANOVA followed by Tukey's test.

676 *; significantly different from control ($p<0.05$)

677 **; significantly different from control ($p<0.01$)

678 †; significantly different from H_2O_2 ($p<0.05$)

679 ††; significantly different from H_2O_2 ($p<0.01$)

680 N.S.; not significant.

681

682 Supplementary Fig. 3. Eliminative effects of catalase on H_2O_2-induced alteration in uptake by

683 HLE cells.

684 (A) Eliminative effect of catalase on H_2O_2-induced alteration in uptake of [^{14}C]CA.

685 (B) Eliminative effect of catalase on H_2O_2-induced alteration in uptake of [^{3}H]TCA.

686 (C) Eliminative effect of catalase on H_2O_2-induced alteration in uptake of [^{3}H]E3S.

687 HLE cells were exposed to 1 mM H_2O_2 in the absence or presence of PEG-catalase (100 U/mL)

688 for 6 h or 48 h. Each solution contained 1% glycerol.

689 HLE cells were incubated with uptake medium containing 1 μM [^{14}C]CA for 10 sec, 50 nM

690 [^{3}H]TCA for 30 sec or 5 nM [^{3}H]E3S for 1 min at 37°C and at pH 7.4.

691 Each column represents the mean with S.D. of 3-6 measurements.

692 Statistical significance was evaluated using ANOVA followed by Tukey's test.

693 *; significantly different from control ($p<0.05$)

694 ††; significantly different from H_2O_2 ($p<0.01$)

695 N.S.; not significant.

696

697 Supplementary Fig. 4. Eliminative effects of catalase on H_2O_2-induced alterations in mRNA and

698 protein expression levels of Oatps in rat primary hepatocytes.

699 Eliminative effect of catalase on H_2O_2-induced alteration in mRNA levels of Oatps (left side).

Eliminative effect of catalase on H_2O_2-induced alteration in protein expression levels of Oatps (right side).

Rat primary hepatocytes were exposed to 1 mM H_2O_2 in the absence or presence of PEG-catalase (100 U/mL) for 12 h or 48 h. Each solution contained 1% glycerol.

Band intensity was determined by densitometry using Scion image program.

Each column represents the mean with S.D. of 3-6 measurements.

Statistical significance was evaluated using ANOVA followed by Tukey's test.

*; significantly different from control ($p<0.05$)

**; significantly different from control ($p<0.01$)

†; significantly different from H_2O_2 ($p<0.05$)

††; significantly different from H_2O_2 ($p<0.01$)

N.S.; not significant.

Supplementary Fig. 5. Eliminative effects of catalase on H_2O_2-induced alteration in uptake by rat primary hepatocytes.

(A) Eliminative effect of catalase on H_2O_2-induced alteration in uptake of [^{14}C]CA.

(B) Eliminative effect of catalase on H_2O_2-induced alteration in uptake of [^{3}H]TCA.

(C) Eliminative effect of catalase on H_2O_2-induced alteration in uptake of [^{3}H]E3S.

718 Rat primary hepatocytes were exposed to 1 mM H_2O_2 in the absence or presence of PEG-catalase

719 (100 U/mL) for 12 h or 48 h. Each solution contained 1% glycerol.

720 Rat primary hepatocytes were incubated with uptake medium containing 1 μM [^{14}C]CA for 2 min,

721 50 nM [^{3}H]TCA for 3 min or 5 nM [^{3}H]E3S for 3 min at 37°C and at pH 7.4.

722 Each column represents the mean with S.D. of 3-6 measurements.

723 Statistical significance was evaluated using ANOVA followed by Tukey's test.

724 *; significantly different from control ($p<0.05$)

725 **; significantly different from control ($p<0.01$)

726 †; significantly different from H_2O_2 ($p<0.05$)

727 N.S.; not significant.

728

729 Supplementary Fig. 6. Effects of H_2O_2 on uptake of pravastatin by HLE cells and rat primary

730 hepatocytes.

731 (A) Effect of H_2O_2 on uptake of pravastatin by HLE cells.

732 HLE cells were exposed to 1 mM H_2O_2 for 6 h or 48 h. HLE cells were incubated with uptake

733 medium containing 10 μM pravastatin for 3 min at 37°C and at pH 7.4.

734 (B) Effect of H_2O_2 on uptake of pravastatin by rat primary hepatocytes.

735 Rat primary hepatocytes were exposed to 1 mM H_2O_2 for 12 h or 48 h. Rat primary hepatocytes

736 were incubated with uptake medium containing 10 μM pravastatin for 5 min at 37°C and at pH

737 7.4.

738 Each column represents the mean with S.D. of 3-6 measurements.

739 *; significantly different from control ($p<0.05$)

740 **; significantly different from control ($p<0.01$)

741 N.S.; not significant

742

743 Supplementary Fig. 7. Effect of inosine-induced oxidative stress on Mrp2 mRNA level in the rat

744 liver.

745 Mrp2 mRNA level in the liver of rats fed powdery diet containing 2% inosine for 1 day, 2 days or

746 5 days.

747 Each column represents the mean with S.D. of 3-5 independent experiments.

748 *; significantly different from control ($p<0.05$)

749 N.S.; not significant

750

751 Supplementary Fig. 8. Effects of H_2O_2 on MRP2/Mrp2 mRNA levels in HLE cells and rat

752 primary hepatocytes.

753 (A) Effect of H_2O_2 on MRP2 mRNA level in HLE cells.

754 HLE cells were exposed to 1 mM H_2O_2 for 6 h or 48 h.

755 (B) Effect of H_2O_2 on Mrp2 mRNA level in rat primary hepatocytes.

756 Rat primary hepatocytes were exposed to 1 mM H_2O_2 for 12 h or 48 h.

757 Each column represents the mean with S.D. of 3-6 measurements.

758 *; significantly different from control ($p<0.05$)

759 **; significantly different from control ($p<0.01$)

760 N.S.; not significant

Table 1 Primer sequences for real-time PCR

Gene	Gene bank accession no.	Sequences	Product size (bp)
OATP1A2	NM_134431	Forward 5'-AAG ACC AAC GCA GGA TCC AT-3'	101
		Reverse 5'-GAG TTT CAC CCA TTC CAC GTA CA-3'	
OATP1B1	NM_006446	Forward 5'-GAA TGC CCA AGA GAT GAT GCT T-3'	154
		Reverse 5'-AAC CCA GTG CAA GTG ATT TCA AT-3'	
OATP1B3	NM_019844	Forward 5'-GTC CAG TCA TTG GCT TTG CA-3'	111
		Reverse 5'-CAA CCC AAC GAG AGT CCT TAG G-3'	
OATP2B1	NM_007256	Forward 5'-TCA AGC TGT TCG TTC TGT GC-3'	198
		Reverse 5'-GTG TTC CCC ACC TCG TTG AA-3'	
GAPDH	NM_002046	Forward 5'-AAG GTC ATC CCT GAG CTG AA-3'	95
		Reverse 5'-TTC TAG ACG GCA GGT CAG GT-3'	
Oatp1a1	NM_017111	Forward 5'-ACC TGG AAC AGC AGT ATG GAA AA-3'	163
		Reverse 5'-ACC GAT AGG CAA AAT GCT AGG TAT-3'	
Oatp1a4	U95011	Forward 5'-TGT GAT GAC CGT TGA TAA TTT TCC A-3'	81
		Reverse 5'-TTC TTC ACA TAT AGT TGG TGC TGA A-3'	
Oatp1b2	NM_31650	Forward 5'-CCT GTT CAA GTT CAT AGA GCA GCA-3'	88
		Reverse 5'-TGC CAT AGT AGG TAT GGT TAT ATT TC-3'	
Oatp2b1	NM_080786	Forward 5'-ACG ACT TTG CCC ACC ATA GC-3'	117
		Reverse 5'-CCA CGT AAA GGC GTA GCA TGA-3'	
Gapdh	AF106860	Forward 5'-ATG GGA AGC TGG TCA TCA AC-3'	221
		Reverse 5'-GTG GTT CAC ACC CAT CAC AA-3'	

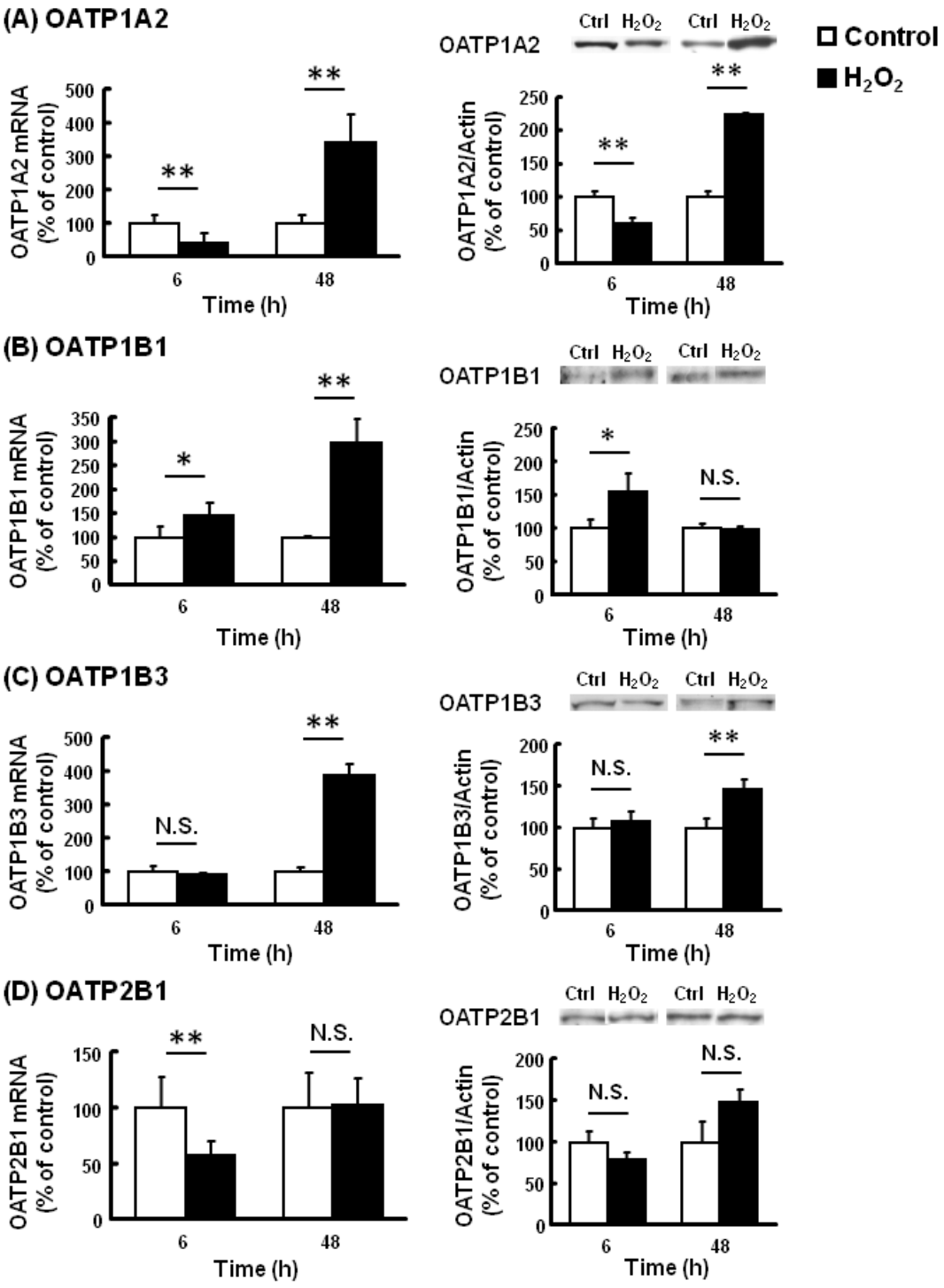

(A) OATP1A2
OATP1A2 mRNA (% of control)
Time (h)
OATP1A2
Ctrl H2O2 Ctrl H2O2
OATP1A2/Actin (% of control)
Control
H2O2
(B) OATP1B1
OATP1B1 mRNA (% of control)
OATP1B1
OATP1B1/Actin (% of control)
N.S.
(C) OATP1B3
OATP1B3 mRNA (% of control)
OATP1B3
OATP1B3/Actin (% of control)
(D) OATP2B1
OATP2B1 mRNA (% of control)
OATP2B1
OATP2B1/Actin (% of control)

Fig. 1

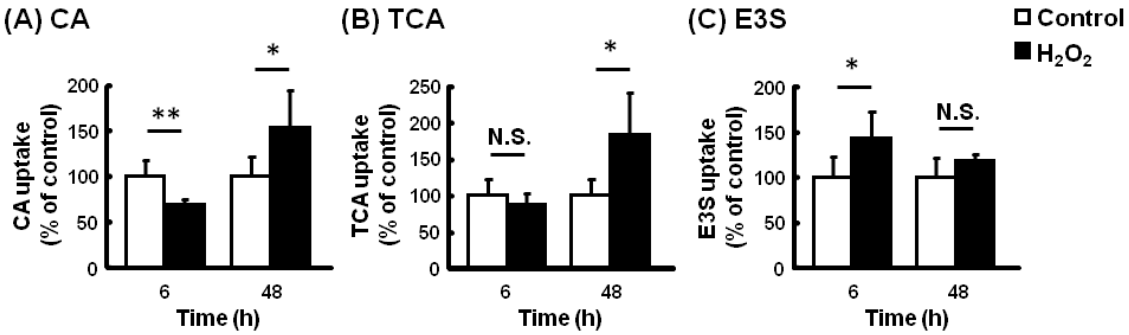

(A) CA
(B) TCA
(C) E3S
Control
H2O2
CA uptake (% of control)
TCA uptake (% of control)
E3S uptake (% of control)
**
*
N.S.
Time (h)
6
48
0
50
100
150
200
250

Fig. 2

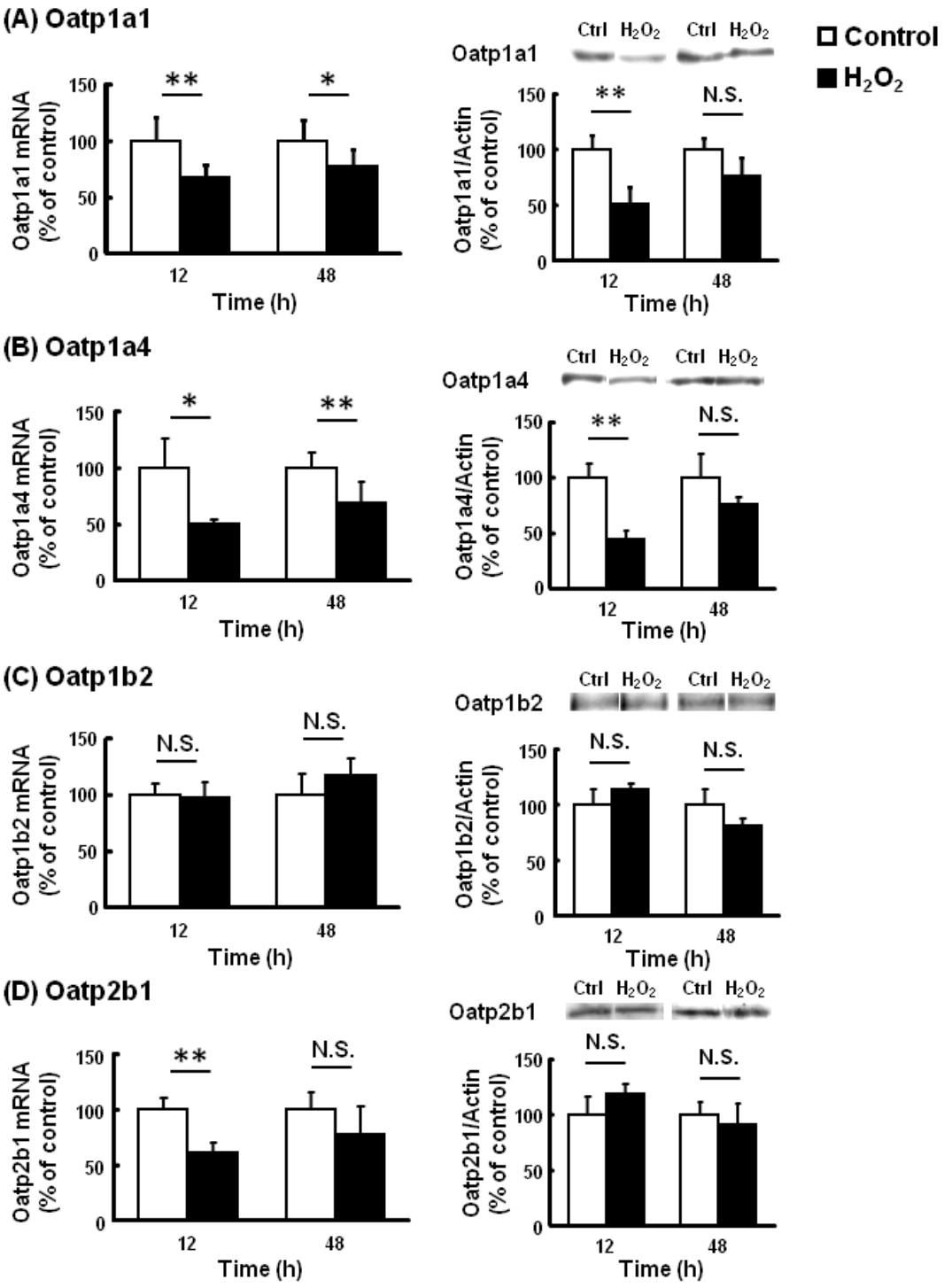

(A) Oatp1a1
Oatp1a1 mRNA (% of control)
Time (h)
Ctrl H2O2 Ctrl H2O2
Oatp1a1
Oatp1a1/Actin (% of control)
N.S.
(B) Oatp1a4
Oatp1a4 mRNA (% of control)
Oatp1a4
Oatp1a4/Actin (% of control)
(C) Oatp1b2
Oatp1b2 mRNA (% of control)
Oatp1b2
Oatp1b2/Actin (% of control)
(D) Oatp2b1
Oatp2b1 mRNA (% of control)
Oatp2b1
Oatp2b1/Actin (% of control)
Control
H2O2

Fig. 3

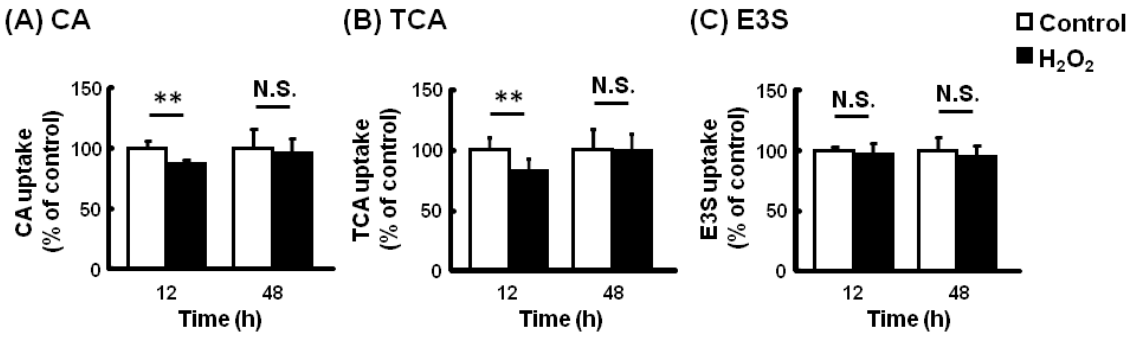

(A) CA
(B) TCA
(C) E3S
Control
H2O2
CA uptake (% of control)
TCA uptake (% of control)
E3S uptake (% of control)
150
100
50
0
**
N.S.
12
48
Time (h)

Fig. 4

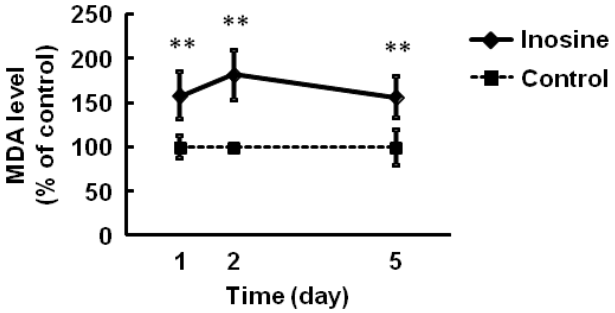

250
200
150
100
50
0
MDA level
(% of control)
**
**
**
1
2
5
Time (day)
Inosine
Control

Fig. 5

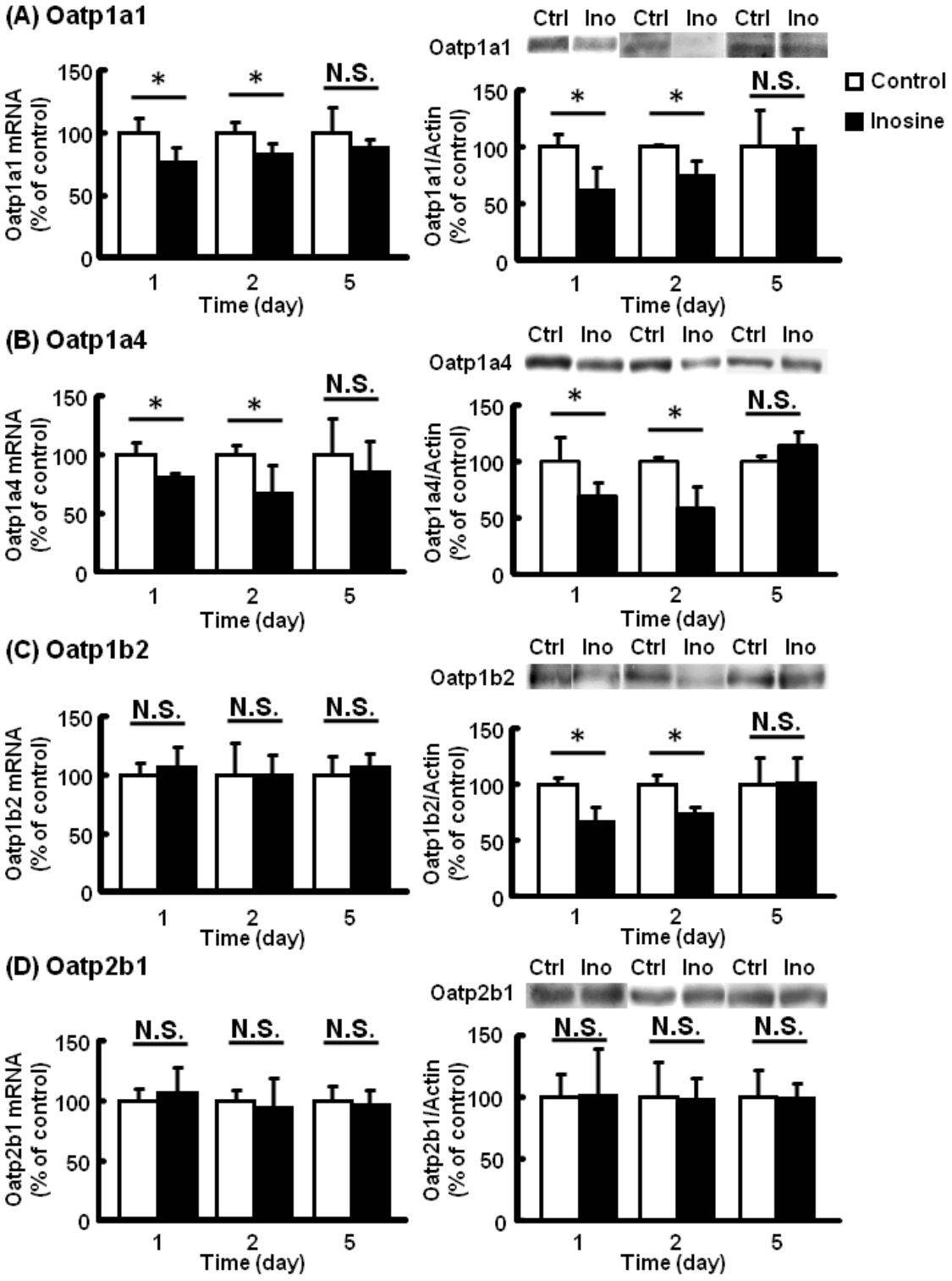

(A) Oatp1a1
Ctrl Ino Ctrl Ino Ctrl Ino
Oatp1a1
Oatp1a1 mRNA (% of control)
Oatp1a1/Actin (% of control)
150
100
50
0
*
*
N.S.
1
2
5
Time (day)
Control
Inosine
(B) Oatp1a4
Oatp1a4
Oatp1a4 mRNA (% of control)
Oatp1a4/Actin (% of control)
(C) Oatp1b2
Oatp1b2
Oatp1b2 mRNA (% of control)
Oatp1b2/Actin (% of control)
(D) Oatp2b1
Oatp2b1
Oatp2b1 mRNA (% of control)
Oatp2b1/Actin (% of control)

Fig. 6

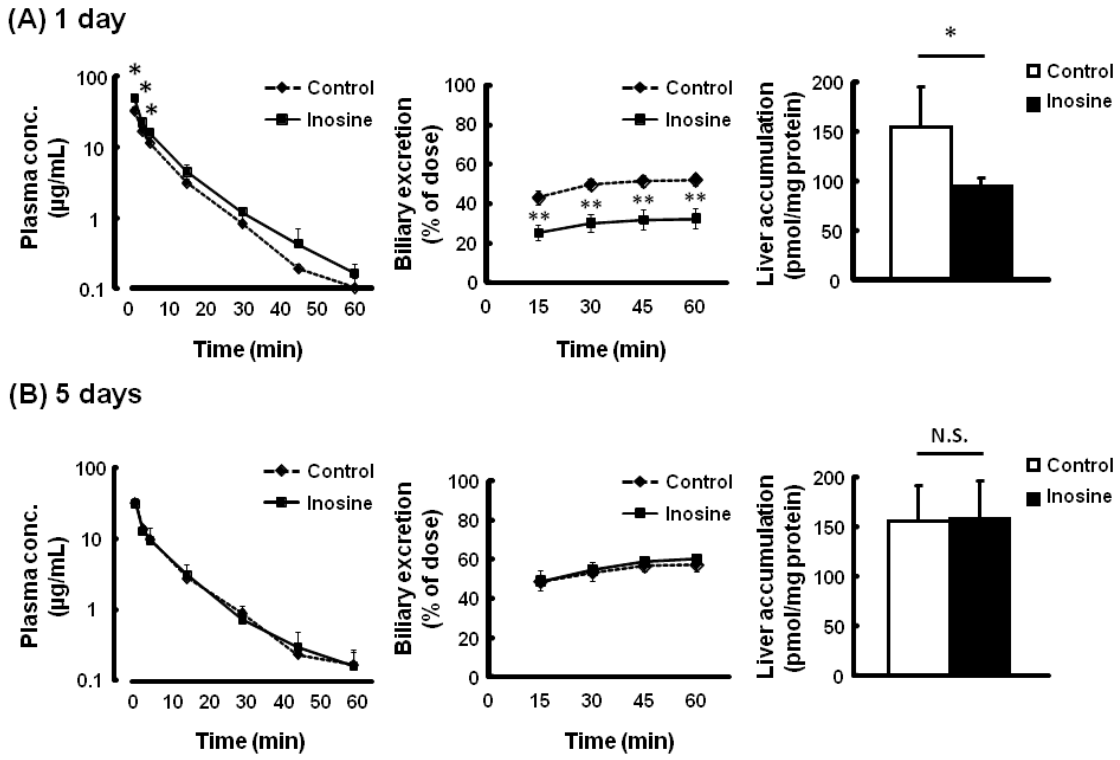
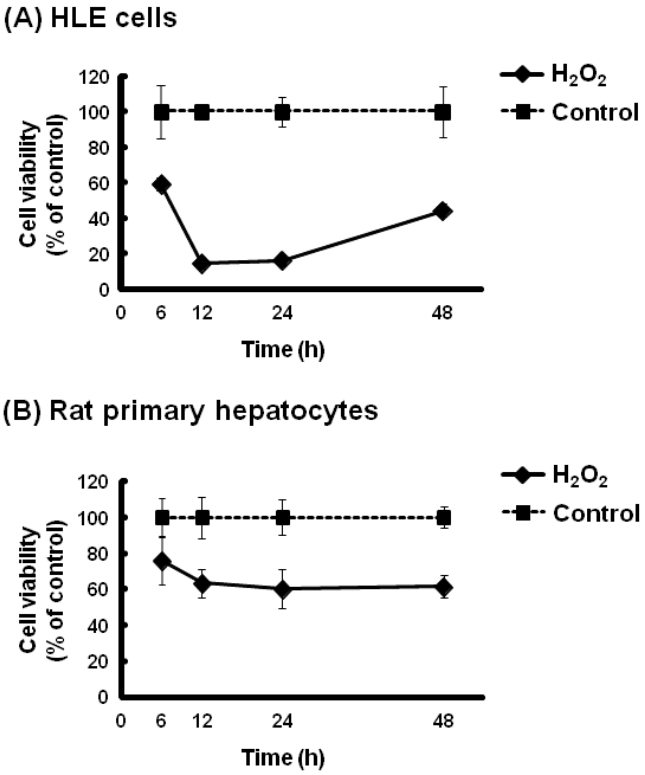

(A) 1 day
Plasma conc. (μg/mL)
Control
Inosine
Time (min)
Biliary excretion (% of dose)
Liver accumulation (pmol/mg protein)
(B) 5 days
N.S.

Fig. 7

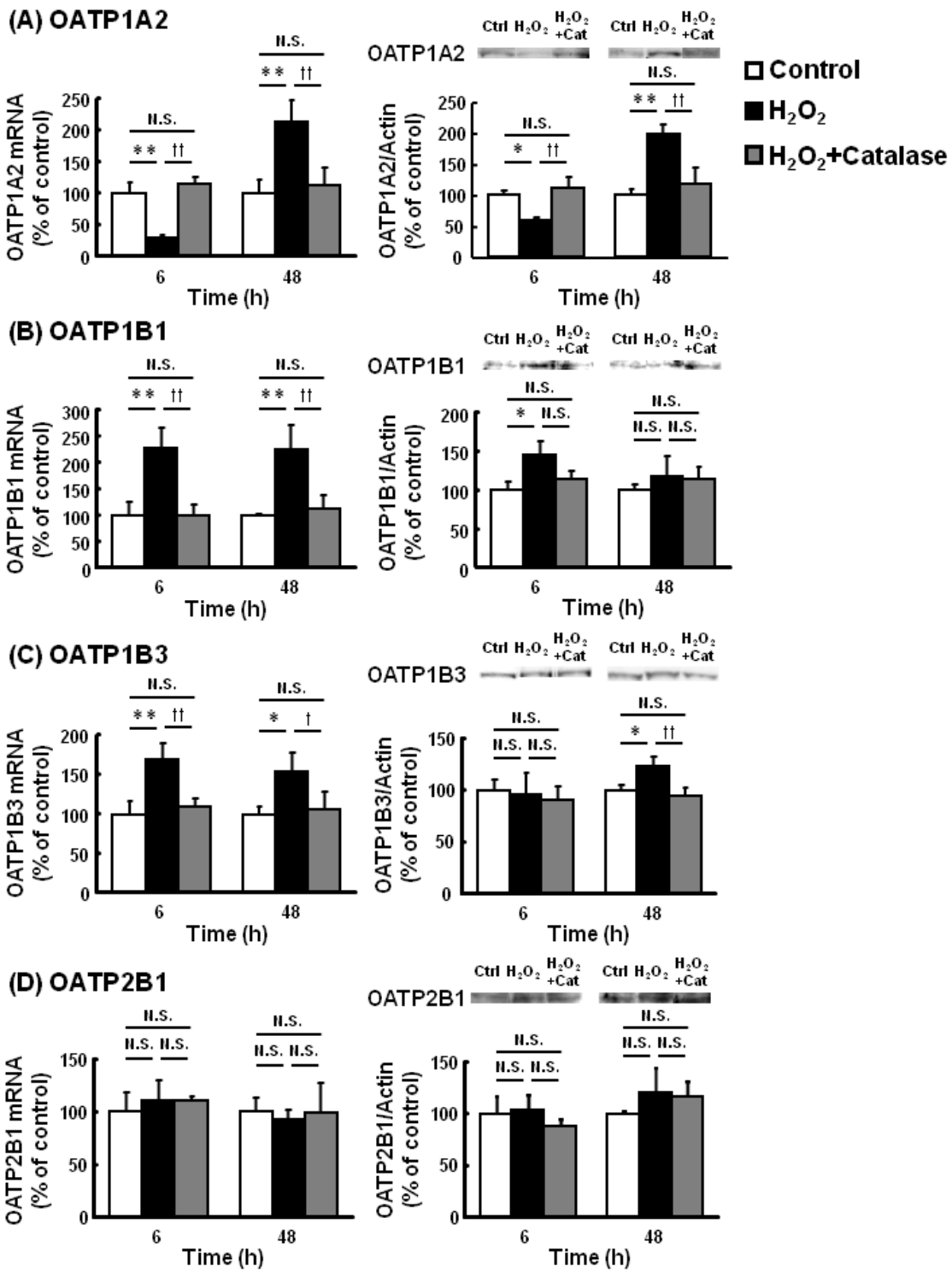
Table 2 Pharmacokinetic parameters of pravastatin after intravenous administration in rats fed powdery diet containing 2% inosine

	1 day		5 days	
	Control	Inosine	Control	Inosine
AUC (mg/L/min)	191.24±19.02	273.16±36.89*	181.27±21.46	171.88±48.37
V_d (L)	0.23±0.08	0.17±0.05	0.25±0.03	0.31±0.08
CL_{tot} (mL/min)	11.94±1.24	8.87±0.88*	13.13±1.48	14.39±2.44

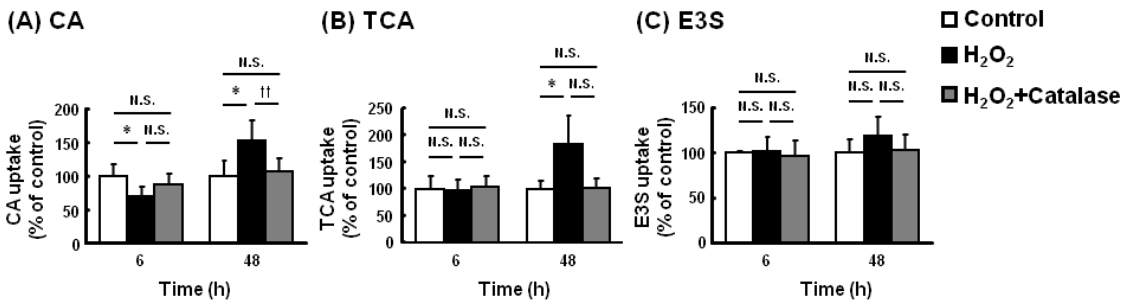
*; significantly different from control ($p<0.05$)



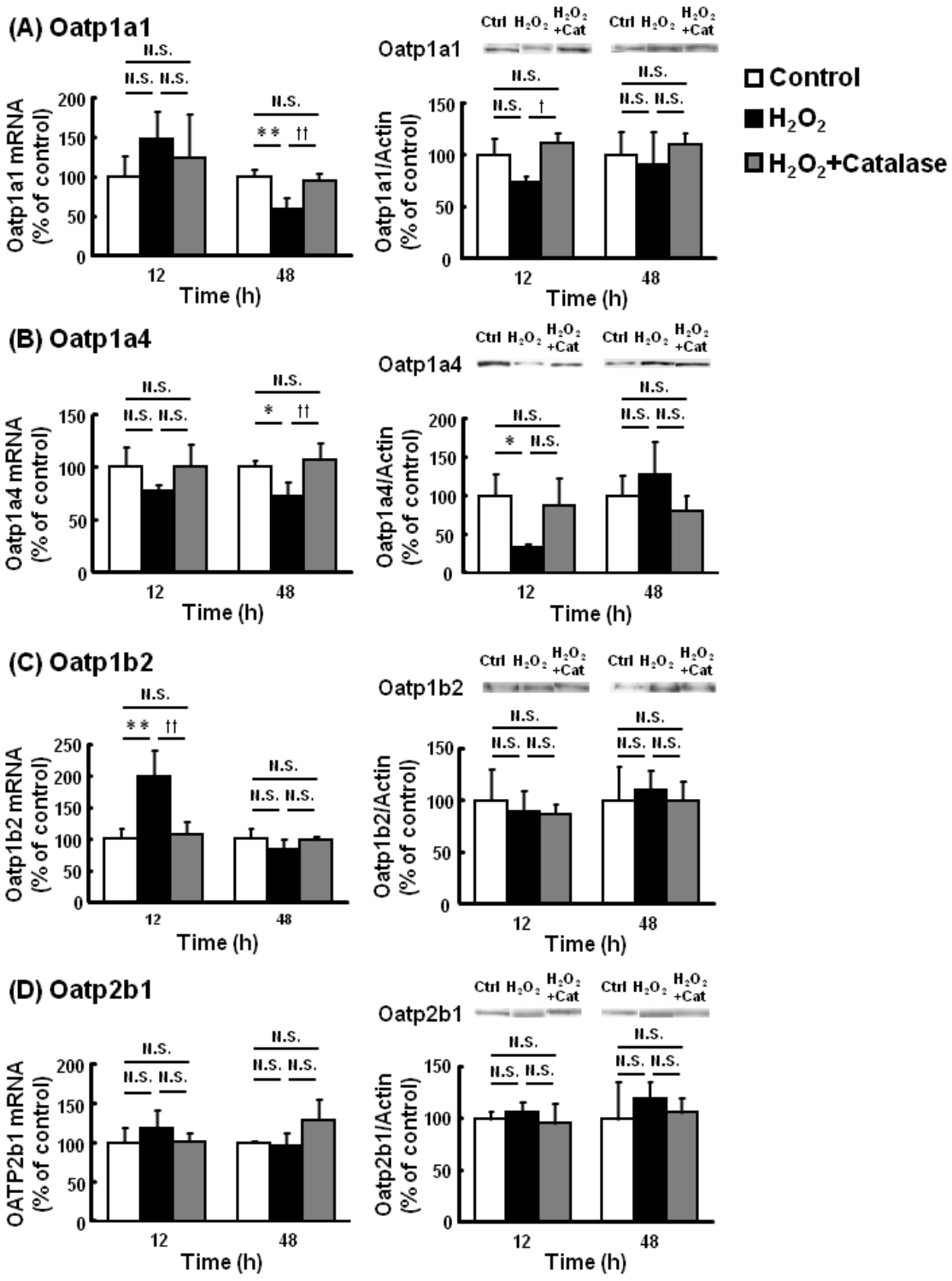
Supplementary fig. 1



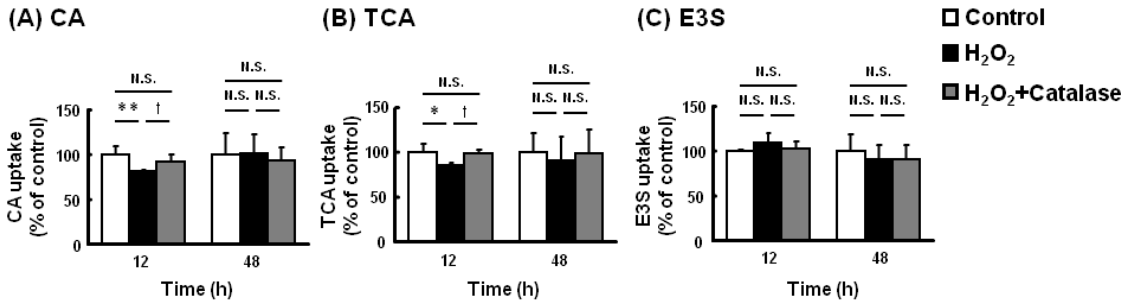
Supplementary fig. 2



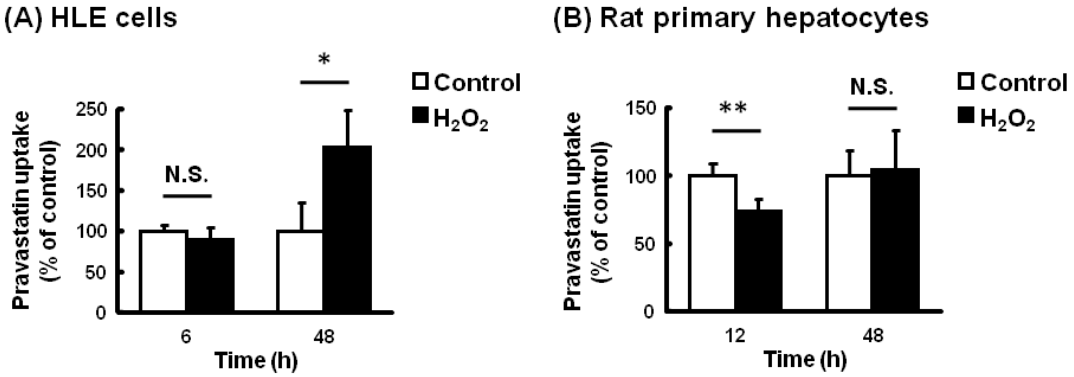
Supplementary fig. 3



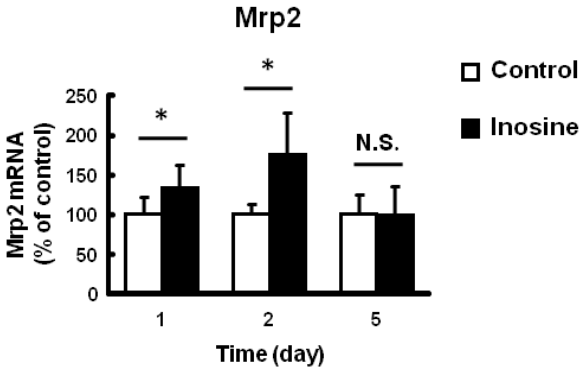
Supplementary fig. 4



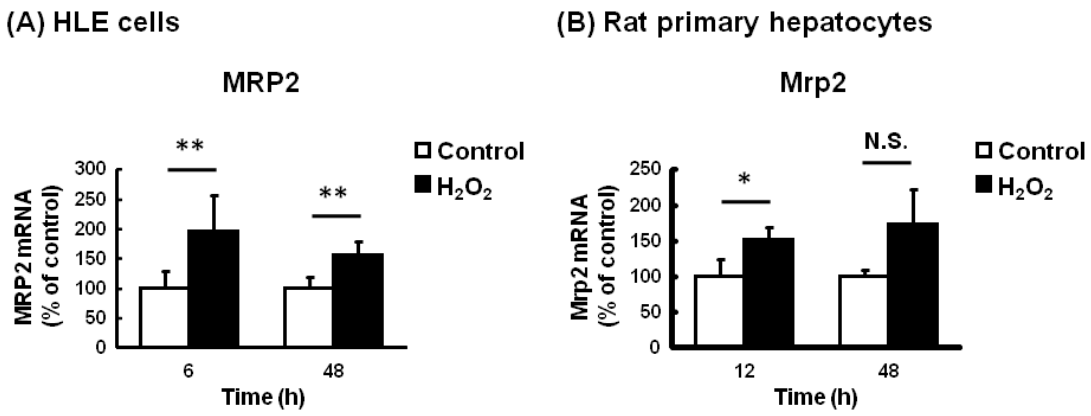
Supplementary fig. 5



Supplementary fig. 6



Supplementary fig. 7



Supplementary fig. 8