Characterization of the Transport Properties of Organic Anion Transporting Polypeptide 1 (oatp1) and Na⁺/Taurocholate Cotransporting Polypeptide (Ntcp): Comparative Studies on the Inhibitory Effect of their Possible Substrates in Hepatocytes and cDNA-Transfected COS-7 Cells

HIROKAZU KOUZUKI, HIROSHI SUZUKI, BRUNO STIEGER, PETER J. MEIER, and YUICHI SUGIYAMA

Abstract
In the present study, we compared the inhibitory effects of organic anions (including bile acids) on the uptake of taurocholate (TC) and estradiol 17β-D-glucuronide (E₂17βG), typical substrates for sodium taurocholate cotransporting polypeptide (Ntcp) and organic anion transporting polypeptide (oatp1), respectively, using primary cultured rat hepatocytes and Ntcp- or oatp1-transfected COS-7 cells. The Na⁺-dependent uptake of TC was inhibited by nine bile acids and five nonbile acid organic anions in a concentration-dependent manner, and their inhibitory effects were similar in both primary cultured rat hepatocytes and Ntcp- or oatp1-transfected COS-7 cells. The Na⁺-dependent uptake of E₂17βG was significantly inhibited by 15 organic anions in a concentration-dependent manner. In addition, the Na⁺-independent uptake of E₂17βG mediated by oatp1. These results are consistent with the hypothesis that the hepatic uptake of TC and E₂17βG is predominantly mediated by Ntcp and oatp1, respectively.

Recently, the molecular mechanism underlying the hepatocellular uptake of organic anions has been clarified. Using Xenopus laevis oocytes injected with mRNA from rat liver, sodium taurocholate cotransporting polypeptide (Ntcp) and organic anion transporting polypeptide (oatp1) have been cloned as transporters responsible for the Na⁺-dependent uptake of bile acids and Na⁺-independent uptake of organic anions, respectively (Meier, 1995). Oatp2 and oatp3 have been identified as homologs of oatp1 (Noe et al., 1997; Abe et al., 1998). Moreover, the hepatic expression of oat2 and 3, homologs of oat1 initially cloned from rat kidney, have been demonstrated (Kusuhara et al., 1999). The transport properties of these cDNA products have been investigated by examining the substrate uptake into Xenopus laevis oocytes injected with cRNA and/or into mammalian cells transfected with cDNA (Meier, 1995; Hagenbuch and Meier, 1996; Noe et al., 1997; Sekine et al., 1997, 1998; Kusuhara et al., 1999). It has been demonstrated that Ntcp accepts taurocholate (TC), cholate (CA), taurochenodeoxycholate (TCDC), taursodeoxycholate, glychocholate (GCA), and estrone-3-sulfate as substrates (Meier, 1995; Hagenbuch and Meier, 1996; Kouzuki et al., 1998; Schroeder et al., 1998). In addition, oatp1 has been demonstrated to transport bromosulfophthalein (BS), bile acids (such as TC and CA), anionic steroid conjugates (such as estrone-3-sulfate and estradiol 17β-D-glucuronide; E₂17βG), neutral ste-

Received for publication July 19, 1999.

ABBREVIATIONS: Ntcp, sodium taurocholate cotransporting polypeptide; oatp, organic anion transporting polypeptide; TC, taurocholate; E₂17βG, estradiol 17β-D-glucuronide; BS, bromosulfophthalein; CA, cholate; TCDC, taurochenodeoxycholate; CDCA, chenodeoxycholate; E3040, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole; DBSP, dibromosulfophthalein; GCA, glycocholate; LCA, ursodeoxycholate; UDCA, ursodeoxycholate; GCDCA, glycochenodeoxycholate; BZP, benzylpenicillin; ONO-1301, 7,8-dihydro-5-[E]-[α-(3-pyridyl)benzylidene]aminooxy[ethyl]-1-naphthyoxylacetic acid; BS-SG, glutathione-conjugate of bromosulfophthalein; DMEM, Dulbecco’s modified Eagle’s medium.

0022-3565/00/2922-0505$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Copyright © 2000 by The American Society for Pharmacology and Experimental Therapeutics
JPET 292:505–511, 2000
Printed in U.S.A.

022-3565/002922-0505$03.00/0
roids (such as ouabain, aldosterone, and cortisol), and even some amphibiotic organic cations such as N-(4,4-azo-n-pentyl)-21-deoxyajmalinone, a permanently charged photolabile derivative of the antiarrhythmic drug N-propylajmaline (Meier, 1995; Hagenbuch and Meier, 1996), whereas oatp2 preferentially accepts digoxin as a substrate rather than oatp1 substrates (Noé et al., 1997). Substrates for oat2 include p-aminohippurate, salicylate, acetylsalicylate, prostaglandin E₃, and dicarboxylate (Sekine et al., 1998) and those for oat3 include p-aminohippurate, ochratoxin A, estrone sulfate, and cimetidine, a cationic compound (Kusuhara et al., 1999).

Moreover, Hagenbuch et al. (1996) and our group (Kouzuki et al., 1998, 1999; Akhteruzzaman et al., 1999) have determined the contribution of Ntcp and oatp1 to the hepatic uptake of their ligands. By examining the effect of simultaneous injection of specific antisense sequences against Ntcp and oatp1, Hagenbuch et al. (1996) found that >95% of TC transport is mediated by Ntcp. Our group has suggested that the uptake of E₂₁₇βG by hepatocytes is predominantly mediated by oatp1, based on the agreement in the Vₘₐₓ value for the uptake of E₂₁₇βG and the expression level of oatp1 protein between hepatocytes and transfected COS-7 cells (Kouzuki et al., 1999). Moreover, we have compared the ligand uptake into hepatocytes and into COS-7 cells transfected with Ntcp and oatp1 cDNAs and normalized the ligand uptake with standard substrates (TC and E₂₁₇βG for Ntcp and oatp1, respectively) to determine the contribution of these cloned transporters (Kouzuki et al., 1998, 1999).

The details of the hepatic uptake of organic anions, however, still remain to be clarified. For example, although hepatic uptake of cyclic peptide (BQ-123; an endothelin antagonist) is mediated, at least in part, by an Na⁺-dependent transporter that is competitively inhibited by TC and, moreover, the Na⁺-dependent uptake of TC is inhibited by BQ-123 (Nakamura et al., 1996), no Ntcp-mediated transport of BQ-123 is detected if COS-7 cells are used for Ntcp cDNA transfection (Akhteruzzaman et al., 1999). This complexity of Na⁺-dependent transport has also been reported for Na⁺-dependent uptake of bumetanide (Blitzer et al., 1982; Petzinger et al., 1989, 1996). As far as oatp1 is concerned, the same paradox has been reported for BQ-123 (Nakamura et al., 1996), glucuronide-conjugates of 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole (E3040; Takenaka et al., 1997), and a 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor (pravastatin; Yamazaki et al., 1993). In the present study, we have characterized the nature of the transport mediated by Ntcp and oatp1, particularly focusing on the inhibitory effect of a series of bile acids and non bile acid organic anions on the uptake of their typical substrates (TC and E₂₁₇βG, respectively) by hepatocytes and cDNA-transfected COS-7 cells.

**Experimental Procedures**

**Materials.** COS-7 cells were purchased from American Type Culture Collection (Rockville, MD). [³H]TC (128.4 GBq/mmol) and [³H]E₂₁₇βG (1813 GBq/mmol) were purchased from New England Nuclear (Boston, MA). TC, CA, GCA, chenodeoxycholate (CDCA), TCDDA, cholate (LCA), deoxycholate (DCA), ursodeoxycholate (UDCA), glycochenodeoxycholate (GDCA), indomethacin, and probenecid were purchased from Wako Pure Chemical Industries (Osaka, Japan). E₂₁₇βG, benzylpenicillin (BZP) and estrone-3-sulfate were purchased from Sigma Chemical (St. Louis, MO). Dibromosulfophthalein (DBSP) was obtained from the Societe d’Etudes et de Recherches Biologiques (Paris, France). 7,8-Dihydrod-5-(E)-[α-(3-pyridyl)]benzylideneminoxyethyl]-1-naphthoxyacetic acid (ONO-1301) was kindly donated by ONO Pharmaceutical Co., Ltd. (Osaka, Japan). BQ-123 and a linear peptide (BQ-485) were donated by Banyu Pharmaceutical Co., Ltd. (Ibaraki, Japan). The glucuronide and sulfate conjugates of E3040, prepared according to the method described previously (Hibi et al., 1994), were donated by Eisai Co., Ltd. (Tokyo, Japan). Pravastatin was a gift from Sankyo Co., Ltd. (Tokyo, Japan). The glutathione conjugate of BSP (BSP-SG) was synthesized by the method described by Saxena and Henderson (1995) using BSP (Aldrich, Milwaukee, WI) and glutathione (Wako, Osaka, Japan). All other chemicals were commercially available and of reagent grade.

**Transient Expression of Ntcp and oatp1 cDNA in COS-7 Cells.** The pCAGGS plasmids containing Ntcp and oatp1 cDNAs were used in the present study. The structure of the plasmid construct has been described previously (Kouzuki et al., 1998, 1999).

The procedure for the transfection of plasmids into COS-7 cells has been described previously (Kouzuki et al., 1998, 1999). Briefly, COS-7 cells were cultured in 150-mm dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum. At 30% confluence, cells were exposed to serum-free DMEM containing plasmid (1 µg/ml) and Lipofectamine (1 µg/ml; BRL, Gaithersburg, MD). At 8 h after transfection, the plasmid-Lipofectamine solution was removed and the medium, consisting of DMEM supplemented with 5% fetal bovine serum, was cultured overnight. Then, the transfected cells were treated with trypsin and approximately 1.6 × 10⁶ cells were seeded onto 22-mm dishes and cultured overnight. An uptake study was performed at 48 h after transfection.

**Primary Cultured Rat Hepatocytes.** The procedure for the preparation of primary cultured rat hepatocytes has been described previously (Kouzuki et al., 1998, 1999). Briefly, rat hepatocytes were isolated from male Sprague-Dawley rats (200–250 g, Japan Laboratory Animal Inc., Tokyo, Japan) after perfusing the liver with collagenase. Cell viability was routinely checked by the trypan blue [0.4% (w/v)] exclusion test. After preparation, freshly isolated cells were suspended in Williams’ medium E. Approximately 5 × 10⁶ cells were placed on collagen-coated 22-mm dishes and cultured for 4 h.

**Inhibition Study.** Uptake of [³H]TC and [³H]E₂₁₇βG (1 µM) was examined by the method described previously (Kouzuki et al., 1998, 1999). Briefly, the experiments were initiated by adding the radiolabeled ligands to the medium in the absence or presence of inhibitors, after washing the cultured cells three times and preincubating with Krebs-Henseleit buffer or choline buffer at 37 for 5 min. The Krebs-Henseleit buffer consisted of 142 mM NaCl, 23.8 mM Na₂CO₃, 4.83 mM KCl, 0.96 mM KH₂PO₄, 1.20 mM MgSO₄, 12.5 mM HEPES, 5 mM glucose and 1.53 mM CaCl₂ adjusted to pH 7.3. The composition of the choline buffer was the same as that of the Krebs-Henseleit buffer except that NaCl and NaHCO₃ were replaced with isotonic choline chloride and choline bicarbonate, respectively. At designated times, the reaction was terminated by adding ice-cold Krebs-Henseleit buffer. Just before the designated times, 50 µl of medium was transferred to scintillation vials. Then, cells were washed three times with 2 ml of ice-cold Krebs-Henseleit buffer and solubilized in 500 µl of N NaOH. After adding 500 µl of distilled water, 800-µl aliquots were transferred to scintillation vials. The radioactivity associated with the cells and medium was determined in a liquid scintillation counter (LS 6000SE; Beckman Instruments, Inc., Fullerton, CA) after adding 8 ml of scintillation fluid (Hionic flow; Packard Instrument Co., Downers Grove, IL) to the scintillation vials. The remaining 100-µl aliquots of cell lysate were used to determine protein concentrations by the method of Lowry et al. (1951) with BSA as a standard. Ligand uptake is given as the cell-to-medium concentration ratio, determined as the amount of ligand associated with the cells divided by the medium concentration.
Because the initial velocity of the uptake of TC and E2,17βG was linear up to 2 and 1 min, respectively, the uptake of these ligands at respective times was used to determine the kinetic parameters. $K_i$ values of a series of anionic compounds for the uptake of TC (1 μM) and E2,17βG (1 μM) were calculated assuming a competitive inhibition. Na$^+$-dependent uptake was calculated by subtracting the Na$^+$-independent uptake (measured in choline buffer) from the total uptake (measured in Krebs-Henseleit buffer).

**Results**

**Inhibitory Effect of Organic Anions on the Uptake of TC.** The inhibitory effects of bile acids on the Na$^+$-dependent uptake of TC by primary cultured rat hepatocytes and Ntcp-transfected COS-7 cells were investigated at several concentrations (1–300 μM). The uptake of TC (1 μM) was inhibited by bile acids in a concentration-dependent manner in these two cell types (Fig. 1). Among them, TCDCA and GCDCA were potent inhibitors compared with GCA, CA, and LCA (Fig. 1).

In the same manner, other nonbile acid organic anions inhibited the Na$^+$-dependent uptake of TC by these two cell types in a concentration-dependent manner (Fig. 2). Of these, ONO-1301 was a potent inhibitor compared with BQ-123, DBSP, and indomethacin (Fig. 2). For the uptake of TC, a significant correlation was observed in the inhibitory effect of bile acids and nonbile acid organic anions (100 and 300 μM) between Ntcp-transfected COS-7 cells and primary cultured hepatocytes (Figs. 3 and 4).

**Inhibitory Effect of Organic Anions on the Uptake of E2,17βG.** The inhibitory effects of organic anions on the Na$^+$-independent uptake of E2,17βG by primary cultured rat hepatocytes and oatp1-transfected COS-7 cells were investigated at several concentrations (1–300 μM). The uptake of E2,17βG (1 μM) was inhibited by bile acids (TC and CA) and by nonbile acid organic anions in a concentration-dependent manner in these two cell types (Fig. 5). Of these, ONO-1301, estrone-3-sulfate and BQ-485 were potent inhibitors compared with BQ-123, BSP-SG, E3040 glucuronide, probenecid, and BZP (Fig. 5). For the uptake of E2,17βG, a significant correlation was observed in the inhibitory effect of organic anions (10, 100, and 300 μM) between oatp1-transfected COS-7 cells and primary cultured hepatocytes (Fig. 6).

**Discussion**

In the present study, we compared the inhibitory effects on the uptake of TC and E2,17βG in primary cultured rat hepatocytes and Ntcp- or oatp1-transfected COS-7 cells. The Na$^+$-dependent and/or Ntcp-mediated uptake of TC was inhibited by bile acids in a concentration-dependent manner and their inhibitory effects were similar in both primary cultured rat hepatocytes and Ntcp-transfected COS-7 cells (Figs. 1 and 3). The rank order of the $K_i$ values was TCDCA < GCDCA < CDCA DCA UDCA GCA < CA < LCA in rat hepatocytes and Ntcp-transfected COS-7 cells (Fig. 1). These results should be compared with the previously reported kinetic parameters. With sinusoidal membrane vesicles, Zimmerli et al. (1989) found that the Na$^+$-dependent uptake of TC was competitively inhibited by CA ($K_i = 140$ μM), TCDCA ($K_i = 9$ μM), and CDCA ($K_i = 53$ μM). In addition, it has been shown that the Ntcp-mediated uptake of TC is inhibited to approximately 10 to 20% by TCDCDA (100 μM), to 20 to 30% by CDCA (100 μM), to 30% by UDCA (100 μM), to 20 to 30% by CA (100–200 μM), and to 60% by GCA (100 μM), whereas DCA (100 μM) does not significantly inhibit the uptake in oocytes injected with cRNA and mammalian cells transfected with cDNA (Hagenbuch et al., 1991; Boyer et al., 1994; Platte et al., 1996). The results of the present study are in good agreement with the previous reports, although we have no good reason to account for the discrepancy in the inhibitory nature of DCA between the previous study (Platte et al., 1996) and the present one (Figs. 1 and 3).

We also examined the inhibitory effects of nonbile acid organic anions on TC transport. The Na$^+$-dependent and/or Ntcp-mediated uptake of TC was inhibited by organic anions in a concentration-dependent manner (Fig. 2), and their inhibitory effects were similar in both primary cultured rat hepatocytes and Ntcp-transfected COS-7 cells (Fig. 4). The rank order of $K_i$ values was ONO-1301 < BQ-485 DBSP indomethacin < BQ-123 in the Ntcp-transfected COS-7 cells (Fig. 2). The inhibitory effect of ONO-1301 is consistent with our previous findings; we have reported that the Na$^+$-dependent uptake of TC into isolated rat hepatocytes was inhibited by ONO-1301 with an IC$_{50}$ of ~10 μM (Imawaka and Sugiyama, 1998). In addition, we could demonstrate that the transfection of Ntcp CDNA into COS-7 cells stimulated the Na$^+$-dependent uptake of ONO-1301 (H. Imawaka and Y.S.,

![Fig. 1. Comparison of the inhibition of $^3$H/TC uptake by bile acids in primary cultured rat hepatocytes (left) and Ntcp-transfected COS-7 cells (right). Uptake of $^3$H/TC (1 μM) was measured for 2 min in the presence or absence of Na$^+$. Inhibitors used were 10 to 300 μM GCA (A, ▲), 1 to 300 μM TC (A, □), 10 to 300 μM GCDCA (A, ●), and to 300 μM UDCA (B, ○), 10 to 300 μM DCA (B, ▲), 10 to 300 μM CDCA (B, △), 10 to 300 μM LCA (C, ▼), 10 to 300 μM CA (C, ▽), and 10 to 300 μM TCDCA (C, ○). Each symbol and vertical bar represent the mean ± S.E. of six determinations in two different preparations.](https://www.aspetjournals.org/journals/10.1124/jpet.2000.207.8.507)
unpublished observations). The inhibitory effect of BQ-123 and indomethacin needs to be discussed. Nakamura et al. (1996) found that BQ-123 is taken up by isolated hepatocytes, at least in part, in a Na\(^+\)-dependent manner (\(K_m = 5.9\ \mu\text{M}\)), which was competitively inhibited by BQ-485 (\(K_i = 1.6\ \mu\text{M}\)) and TC (\(K_i = 9.1\ \mu\text{M}\)). The calculated \(K_i\) value (9.1 \(\mu\text{M}\)) of TC for the Na\(^+\)-dependent uptake of BQ-123 was comparable with the \(K_m\) value for the uptake of TC itself (13.1 \(\mu\text{M}\)), which is consistent with the hypothesis that the Na\(^+\)-dependent uptake of BQ-123 may be mediated by Ntcp (Nakamura et al., 1996). However, we found that the contribution of Ntcp to the Na\(^+\)-dependent uptake of BQ-123 was negligible (Akhteruzzaman et al., 1999). Moreover, although we found that indomethacin is partly taken up by rat hepatocytes in a Na\(^+\)-dependent manner, it was revealed that this drug transport was not significantly mediated by Ntcp expressed on COS-7 cells (Kouzuki et al., 2000). Thus, it appears that both BQ-123 and indomethacin reduced the Na\(^+\)-dependent up-
take of TC by hepatocytes and that by transfected COS-7 cells, to a comparable degree without being substrates for this transporter. These ligands may act as “antagonists” for Ntcp. Moreover, the presence of multiplicity for Na\(^+\)-dependent transport of organic anions should be assumed. Although one of the candidates is microsomal epoxide hydrolase (von Dippe et al., 1996), the presence of additional transporter(s) should be hypothesized.

Furthermore, we also examined the inhibitory effects of organic anions (including bile acids) on the uptake of E\(_{17\beta}\)G. The Na\(^+\)-independent and/or oatp1-mediated uptake of E\(_{17\beta}\)G was inhibited by organic anions in a concentration-dependent manner and their inhibitory effects were similar in both primary cultured rat hepatocytes and oatp1-transfected COS-7 cells (Figs. 5 and 6). The rank order of \(K_i\) values was ONO-1301 < estrone-3-sulfate BQ-485 CDCA DBSP E3040 sulfate < indomethacin TC CA pravastatin < E3040 glucuronide probenecid BQ-123 BZP BSP-SG in the oatp1-transfected COS-7 cells. The inhibition of oatp1-mediated E\(_{17\beta}\)G transport by TC, CA, and estrone-3-sulfate is consistent with the previous results that the hepatic uptake of these organic anions shares a common mechanism (Anwer and Hegner, 1978; Brouwer et al., 1987), which were confirmed by the studies with cloned oatp1 product (Jacquemin et al., 1994; Kanai et al., 1996; Bossuyt et al., 1996). The inhibitory effect of TC, CA, estrone-3-sulfate, and E3040 sulfate is also consistent with our previous kinetic analysis, which indicated that the uptake of these substrates is mediated partly by oatp1; we found that the contribution of oatp1 to the Na\(^+\)-independent uptake of TC and CA into rat hepatocytes was more than 50 to 60%, whereas the corresponding values for the estrone-3-sulfate and E3040 sulfate were 20 to
30% (Kouzuki et al., 1999). Moreover, the potency of the inhibitory effect of TC, estrone-3-sulfate, and E3040 sulfate agreed with their \( K_m \) values; the \( K_m \) values for TC and estrone-3-sulfate were determined as 50 and 4.5 \( \mu \)M in Xenopus laevis oocytes injected with oatp1 cRNA (Kullak-Ublick et al., 1994; Bossuyt et al., 1996), whereas the \( K_m \) value of E3040 sulfate was 25 \( \mu \)M in isolated rat hepatocytes (Tak enaka et al., 1997). Much less potency of inhibition of BZP may be ascribed to its low affinity to the uptake transporter (\( K_m = 475 \mu \)M; Tsuji et al., 1986). The inhibition potency of small peptides (BQ-123 and BQ-485) is also consistent with our previous results in isolated rat hepatocytes; the \( K_m \) value for the \( \text{Na}^+ \)-independent uptake of BQ-123 (12 \( \mu \)M) into rat hepatocytes was higher than the \( K_m \) value of BQ-485 (2.5 \( \mu \)M) on the uptake of BQ-123 (Nakamura et al., 1996). However, the inhibitory effect of BQ-123, E3040 glucuronide, and pravastatin should be discussed in more detail, in relation to the previous findings. Nakamura et al. (1996), Takenaka et al. (1997), and Yamazaki et al. (1993) found that DBSP competitively inhibits the \( \text{Na}^+ \)-independent uptake of BQ-123 (\( K_m = 12 \mu \)M), E3040 glucuronide (\( K_m = 59 \mu \)M), and pravastatin (\( K_m = 29 \mu \)M) with \( K_M \) values of 3.5, 8.4, and 6.3 \( \mu \)M, respectively. If we consider the facts that: 1) DBSP is taken up by isolated rat hepatocytes via an \( \text{Na}^+ \)-independent transport system; and 2) the \( K_m \) value for the uptake of DBSP (2.1 \( \mu \)M; Blom et al., 1981) is comparable with the previously described \( K_c \) values, it is plausible that the uptake of BQ-123, E3040 glucuronide, and pravastatin is mediated by oatp1. However, we found that the contribution of oatp1 to the \( \text{Na}^+ \)-independent uptake of these three organic anions was minimal as far as COS-7 cells were used (Kouzuki et al., 1999). Moreover, we found that transporter(s) other than oatp1 may be responsible for the \( \text{Na}^+ \)-independent hepatic uptake of indomethacin (Kouzuki et al., 2000). Collectively, BQ-123, E3040 glucuronide, pravastatin, and indomethacin inhibit the \( \text{Na}^+ \)-independent uptake of \( \text{E}_2\text{G} \) by hepatocytes and that by transfected COS-7 cells to a comparable degree without being substrates for this transporter. These ligands act as “antagonists” for oatp1.

Collectively, for the uptake of both TC and \( \text{E}_2\text{G} \), the pattern of inhibition observed in hepatocytes and transfected COS-7 cells was similar (Figs. 3, 4, and 6). These results can be accounted for by assuming that the hepatic uptakes of TC and \( \text{E}_2\text{G} \) are almost predominantly mediated by Ntcp and oatp1, respectively, or by other transporter(s) with similar transport kinetics.

**References**


Send reprint requests to: Yuichi Sugiyama, Ph.D., Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. E-mail: sugiyama@seizai.f.u-tokyo.ac.jp.