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The inhibitory effects of fluoroquinolones on L-carnitine transport in placental cell line BeWo

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Abstract

L-Carnitine plays an important role in lipid metabolism by facilitating the transport of long-chain fatty acids across the mitochondrial inner membrane followed by fatty acid beta-oxidation. It is known that members of the OCTN family play an important role in L-carnitine transport in the placenta. Investigation of drug–drug or drug–nutrient interaction in the placenta is important for establishment of safety drug medication during pregnancy. The aim of this study was to determine the effects of fluoroquinolones, inhibitors of OCTN2, on L-carnitine transport in the placenta which is known to have a high expression level of OCTN2.

We investigated the inhibitory effect of five fluoroquinolones, ciprofloxacin (CPFX), gatifloxacin (GFLX), ofloxacin (OFLX), levofloxacin (LVFX) and grepafloxacin (GPFX), on L-carnitine transport mediated by OCTN2 in placental cell line BeWo cells. We found that all of the fluoroquinolones inhibited L-carnitine transport, GPFX being the strongest inhibitor. We also found that the inhibitory effects of LVFX and GPFX depended on their existence ratio of zwitterionic forms as, we reported previously.

Furthermore, we elucidated the LVFX transport mechanism in BeWo cells. LVFX was transported actively by transporters. However, we found that LVFX transport was Na^+-independent and L-carnitine had no inhibitory effect on LVFX transport, suggesting that LVFX acts as inhibitor of OCTN2, not as a substrate for OCTN2.

Keywords: L-Carnitine; BeWo; Levofloxacin; Fluoroquinolone

1. Introduction

Fluoroquinolones are a class of antibiotic agents that act by inhibiting DNA gyrase with a broad spectrum of activity against both Gram-positive and Gram-negative bacteria. As reproductive and developmental toxicity, these drugs induced maternal toxicity and embryotoxicity in experimental animals (Watanabe et al., 1992; Morinaga et al., 1996). Moreover, different factors combine to raise teratogenic and fetotoxic concerns regarding their use during pregnancy. But there is no evidence of their toxicity for the fetus, and more information is needed.

Recently, a novel organic cation transporter (OCTN) family has been described. It has been reported that OCTN2 transports L-carnitine Na^+-dependently and that it contributes to L-carnitine homeostasis by transporting L-carnitine in various tissues (Tamai et al., 1997; Lahjouji et al., 2002). It has also been shown that OCTN2 transports L-carnitine in placental cell line BeWo cells (Rytting and Audus, 2005).

It has been proposed that OCTN2 has both anion and cation binding sites for zwitterionic compounds (Ohashi et al., 2001), and there are some reports of carnitine deficiency being induced by several drugs (valproic acid, cephaloridine, emetine and pivalic acid) (Igarashi et al., 1990; Tune and Hsu, 1994; Kuntzer et al., 1990; Holme et al., 1989). Furthermore, we have reported that zwitterionic forms of levofloxacin (LVFX) and grepafloxacin (GPFX) inhibit L-carnitine transport in Caco-2 cells (Hirano et al., 2006).

Fluoroquinolones have toxicity for the fetus. We previously reported that transport of enoxacin, a new quinolone antibiotic, is stimulated by diffusion potential in rat brush-border membrane

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vesicles (Hirano et al., 1994, 1995). Moreover, quinolones are substrates for the ATP-binding cassette transporter superfamily (Yamaguchi et al., 2000; Sasabe et al., 2004; Gonzalez-Alvarez et al., 2005). Therefore, although the transporters may play an important role for permeation of fluoroquinolones from the mother to fetus, the mechanism by which fluoroquinolones are transported has not been investigated in detail. Moreover, Polachek et al. (2005) have reported that only a small fraction of fluoroquinolones transferred from the maternal to fetal compartment in the isolated perfused human placenta. These facts conflict with the toxicity of fluoroquinolones for the fetus during pregnancy. We therefore hypothesized that the toxicity of fluoroquinolones during pregnancy does not depend on the amount of fluoroquinolones transferred to the fetus, but their inhibitory effects on transport of endogenous compound through the placenta affects fetal growth.

In this study, we investigated the effects of fluoroquinolones on L-carnitine transport in BeWo cells, a model for the placenta. We also investigated LVFX transport in BeWo cells in order to obtain further information on toxicity of fluoroquinolones during pregnancy.

2. Materials and methods

2.1. Chemicals

L-[^{3}H]-carnitine (80.0 Ci/mmol) was purchased from Amersham Biosciences Corp. LVFX was kindly donated by Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan). GPFX was kindly supplied by Otsuka Pharmaceutical Co. Ltd. (Tokyo, Japan). Gatifloxacin (GFLX) was kindly supplied by Kyorin Pharmaceutical Co. Ltd. (Tokyo, Japan). Ofloxacin (OFLX) and Ciprofloxacin (CPFX) were purchased from Sigma–Aldrich, Japan (Tokyo, Japan) and MP Biomedicals, Inc. (Solon, USA), respectively. All other reagents were of the highest grade available and used without further purification.

2.2. Cell culture

BeWo cells were obtained from Riken Cell Bank (Saitama, Japan). They were cultivated in nutrient mixture F-12 with Ham Kaighn's modification (Sigma–Aldrich, Japan) supplemented with 15% fetal bovine serum and 1% penicillin–streptomycin at 37 °C under 95% air/5% CO_2. The cells were grown for 4–5 days, and after reaching confluency they were washed with PBS and harvested by exposure to a trypsin–EDTA solution and then passed into new flasks. For the uptake study, BeWo cells were seeded at a density of 2×10^6 cells/mL on 24-well plastic plates (Corning Coster Corp., Cambridge, MA). The cell monolayers were fed a fresh growth medium every 2 days and were then used at 4–5 days for the uptake experiments.

2.3. Uptake experiments

Uptake experiments were carried out as described previously (Itoh et al., 2005). The cells were washed two times with Hanks' balanced salt solution (HBSS) containing 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.3 mM KH_2PO_4, 0.4 mM NaH_2PO_4, 4.2 mM $NaHCO_3$, 25 mM D-glucose, and 10 mM HEPES and then equilibrated in HBSS at 37 °C for approximately 10 min. L-[^{3}H]-carnitine (0.25 μCi/mL) or 50 μM of LVFX premixed with or without inhibitors in warm HBSS was added to the cells in a shaking hot box (37 °C). When required, Na^+ was isosmotically substituted by *N*-methyl-D(−)-glucamine ($NMDG^+$). The dosing solutions were then aspirated, and the cells were washed with ice-cold HBSS two times. In L-carnitine uptake experiments, 0.5 mL of lysing solution (1% SDS in 0.2 N NaOH) was added and shaking of the plate in the hot box was continued. Samples of the lysate were then collected for scintillation spectrometry (1600TR, Packard Instruments, Meriden, CT). In LVFX uptake experiments, 0.5 mL of 1N NaOH was added. After shaking the plate in the hot box, 0.5 mL of 1N HCl was added and samples of the lysate were collected for centrifugation. After centrifugation of the mixture (12,000 rpm for 10 min), the concentration of LVFX in the supernatant was measured.

2.4. Analytical procedures

LVFX was determined using an HPLC system equipped with a Hitachi LC liquid chromatograph pump and UV detector. The column was a Mightysil RP-18GP column (4.6 mm × 250 mm (5 μm), Kanto Chemical). A mobile phase containing 50 mM KH_2PO_4:methanol (3:1, v/v, 2% CH_3COOH) was used. Column temperature and flow rate were 50 °C and 0.9 mL/min, respectively. The wavelength for detection of LVFX was 290 nm. Protein was measured by the method of Lowry et al. with bovine serum albumin as a standard.

Student's *t*-test was used to determine the significance of differences between two group means. For kinetic studies, the Michaelis–Menten constant was fitted by a nonlinear least-squares regression analysis using Origin® (version 6.1J).

3. Results

3.1. Inhibitory effects of fluoroquinolones on L-carnitine transport in BeWo cells

In the first part of this study, we investigated the inhibitory effects of fluoroquinolones (CPFX, GFLX, OFLX, LVFX and GPFX) on L-carnitine uptake in BeWo cells (Fig. 1). It was found that L-carnitine uptake was inhibited in the presence of any kind of the fluoroquinolones. Although the concentration of GPFX was lower than the concentration of other fluoroquinolones, GPFX had the strongest inhibitory effect on L-carnitine uptake among the fluoroquinolones we used in this study.

We previously reported that zwitterionic forms of fluoroquinolones are important for the inhibitory effect of fluoroquinolones on L-carnitine recognition by OCTN2 (Hirano et al., 2006). We therefore investigated the effects of LVFX and GPFX on L-carnitine uptake under pH 5.5 and pH 7.4 buffer conditions in which the existence ratios of zwitterionic forms of LVFX and GPFX are changed (Fig. 2). L-Carnitine uptake in the presence of 10 mM LVFX was decreased to about 50% and

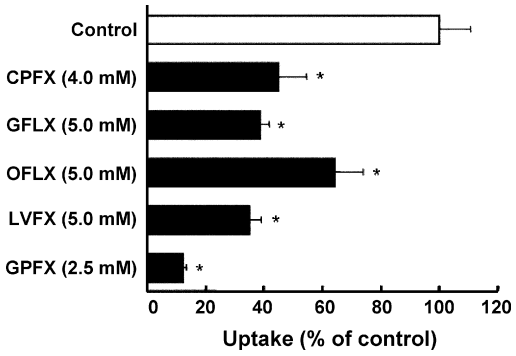

Fig. 1. Inhibitory effects of fluoroquinolones on L-carnitine uptake. L-[^{3}H]-carnitine (5 nM) uptake was measured for 30 min in the presence of each fluoroquinolone. *Significantly different from control at $p < 0.05$.

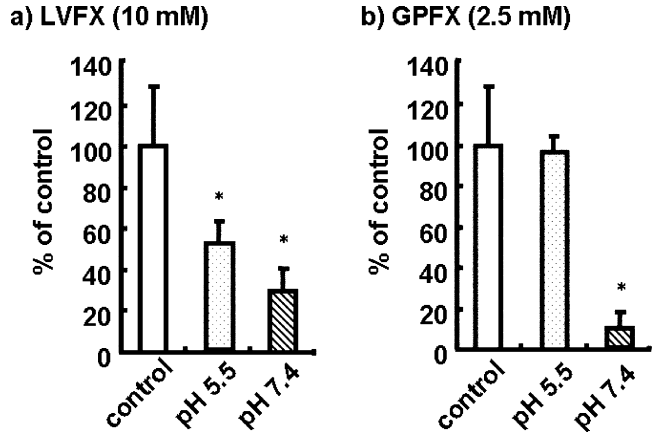

Fig. 2. Inhibitory effects of LVFX (a) and GPFX (b) on L-carnitine uptake at pH 5.5 and pH 7.4. L-[^{3}H]-carnitine (5 nM) uptake at pH 5.5 or 7.4 was measured for 30 min in the presence of 10 mM of LVFX or 2.5 mM of GPFX. *Significantly different from no addition at $p < 0.05$.

30% of the control level under pH 5.5 and pH 7.4 buffer conditions, respectively. On the other hand, while 2.5 mM GPFX inhibited L-carnitine uptake significantly under a pH 7.4 buffer condition, it had no effect on L-carnitine uptake under a pH 5.5 buffer condition.

We also investigated the kinetic nature of the inhibitory effects of LVFX and GPFX on L-carnitine uptake.

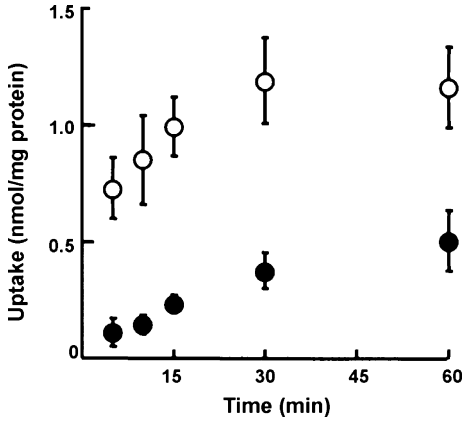

Fig. 4. Time course of LVFX uptake into BeWo cells. LVFX (50 μM) was measured in a buffer maintained at 37 °C (◯) or 4 °C (●).

Eadie–Hofstee plot analysis showed that both LVFX and GPFX inhibit L-carnitine in a non-competitive manner (Fig. 3). Nonlinear least-squares analysis showed K_i values of 3.0 mM for LVFX and 0.3 mM for GPFX.

3.2. LVFX uptake into BeWo cells

We investigated the transport of LVFX, which inhibited L-carnitine under both pH 5.5 and pH 7.4 buffer conditions, into BeWo cells. It was shown that LVFX uptake was linear for 15 min. On the other hand, LVFX uptake was decreased significantly when transport buffer was maintained at 4 °C (Fig. 4).

To study the driving force for LVFX uptake, the effect of replacement of Na^+ ions in the transport buffer was investigated. It was shown that LVFX uptake was Na^+-independent. However, LVFX uptake was significantly decreased under an ATP-depleted condition (Fig. 5).

We also investigated the inhibitory effects of other fluoroquinolones and L-carnitine on LVFX uptake (Fig. 6). Although L-carnitine had no inhibitory effect on LVFX uptake, other fluoroquinolones (GPFX and CPFX) significantly decreased LVFX uptake into BeWo cells. Furthermore, several compounds (including tetraethylammonium, cimetidine, valproic acid) that

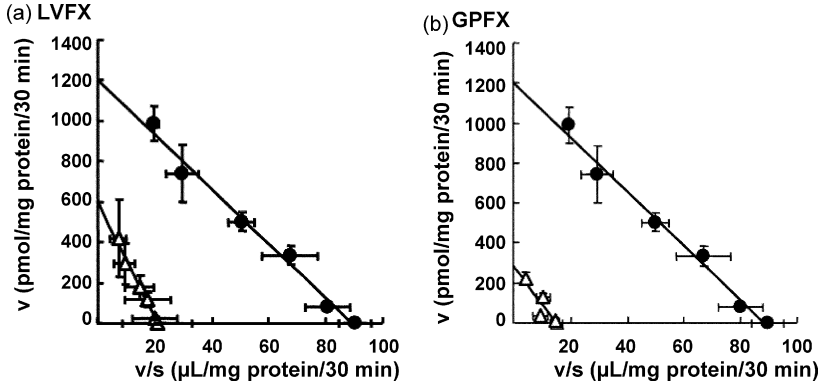

Fig. 3. Eadie–Hofstee plot analysis of the effects of LVFX (a) and GPFX (b) on L-carnitine uptake. L-[^{3}H]-carnitine (5 nM) uptake at pH 7.4 was measured for 30 min in the absence (●) or presence (△) of LVFX at 10 mM (a) or GPFX at 2.5 mM (b).

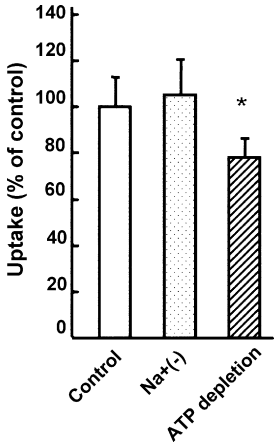

Fig. 5. Effect of replacement of Na^+ and depletion of ATP on LVFX uptake. LVFX (50 μM) uptake was measured for 15 min. Na^+ was replaced with NMDG. Cells were cultured in a buffer containing NaN_3 (5 mM) and NaF (5 mM) for 10 min before uptake experiments to deplete ATP. *Significantly different from control at $p < 0.05$.

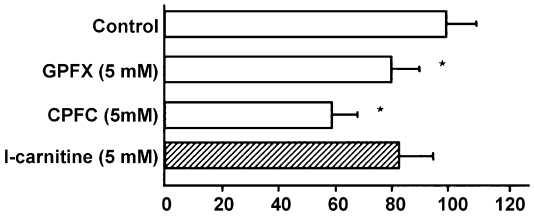

Fig. 6. Inhibitory effect of fluoroquinolones on LVFX uptake. LVFX (50 μM) uptake was measured for 15 min in the presence of each fluoroquinolone. *Significantly different from control at $p < 0.05$.

have been reported to be inhibitors of OCTN2 had no effect on LVFX uptake (data not shown).

To clarify LVFX uptake into BeWo cells in detail, the concentration dependence of LVFX was studied. We analyzed transporter-dependent LVFX uptake by subtracting the uptake under a 4 °C buffer condition from the uptake under a 37 °C buffer condition (Fig. 7). The transporter-dependent LVFX

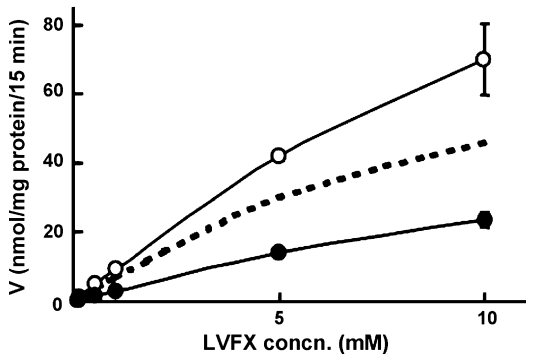

Fig. 7. Concentration dependence of LVFX uptake by BeWo cells. LVFX uptake by BeWo cells at various concentrations of LVFX from 50 μM to 10 mM was measured for 15 min. The LVFX uptake was measured in a buffer maintained at 37 °C (◯) or 4 °C (●). The dashed line shows the saturable component, determined by subtracting LVFX uptake at 4 °C from LVFX uptake at 37 °C at each concentration of LVFX uptake.

uptake into BeWo cells was saturable. Kinetic analysis showed that the Km value for LVFX uptake was 20 mM.

4. Discussion

L-Carnitine plays an important role in lipid metabolism by facilitating the transport of long-chain fatty acids across the mitochondrial inner membrane followed by fatty acid beta-oxidation (Bremer, 1983; Pons and De Vivo, 1995). It is known that carnitine deficiency causes cardiomyopathy, muscle weakness, hypoketoic hypoglycemia, Reye's syndrome and sudden infant death (Treem et al., 1988; Scholte et al., 1990; Ganphaichitr and Leelahagul, 1993).

It has been reported that fatty acid oxidation plays an important role in generation of energy in the placenta (Shekhawat et al., 2003). Since the developing fetus may not be able to synthesize an adequate amount of carnitine, carnitine should be transported actively from the mother to fetus. It has been suggested that fatal teratogenicity induced by some drugs might be associated with low carnitine level (Hug et al., 1991; Wu et al., 2004). It has been reported that fluoroquinolones have adverse effect on musculoskeletal system, consisting mainly myaglia, arthralgia and rhabdomyolysis (Petitjeans et al., 2003). Moreover, L-carnitine deficiencies are among the most common causes of inherited fatty acid oxidation disorders characterized by hypoglycemia-induced rhabdomyolysis (Brownell et al., 1979). We have reported that LVFX and GPFX inhibit L-carnitine transport by OCTN2 using Caco-2 cells. OCTN2 is expressed in apical membrane of syncytiotrophoblast cells and mediates L-carnitine transport from mother to fetus (Lahjouji et al., 2004). These facts suggest that the inhibitory effect of fluoroquinolones on L-carnitine transport in the placenta is one of the causes of their toxicity for the fetus during pregnancy. We therefore elucidated the inhibitory effect of fluoroquinolones on L-carnitine uptake in BeWo cells.

L-Carnitine uptake was decreased by all fluoroquinolones we examined in this study (Fig. 1). It is known that L-carnitine is transported by OCTN2 in BeWo cells. OCTN2 has two substrate recognition sites, a cation recognition site and an anion recognition site. Since fluoroquinolones exist as zwitterions, it is thought that they act as inhibitors of L-carnitine transfer from the mother to the fetus by OCTN2.

We investigated whether inhibitory effects of LVFX and GPFX in BeWo cells were dependent on the existence ratio of zwitterionic forms, the ratios of zwitterionic forms of LVFX and GPFX were about 65–75% in a neutral condition but decreases to 39% for LVFX and 2% for GPFX under an acidic condition (Hirano et al., 2006). It was found that both LVFX and GPFX inhibited L-carnitine uptake more strongly at pH 7.4 than at pH 5.5. Furthermore, GPFX had no effect on L-carnitine uptake at pH 5.5 (Fig. 2). Comparing these results with our previous study (Hirano et al., 2006), it is also suggested that LVFX and GPFX inhibit L-carnitine uptake by OCTN2 in BeWo cells. Furthermore, since pH of placental blood is 7.4, both LVFX and GPFX might act as inhibitor for L-carnitine transport in placenta.

It has been suggested that LVFX transport is mediated by some transporters (Yamaguchi et al., 2001; Matsuo et al., 1998).

However, the mechanism by which LVFX is transported in the placenta has not been elucidated. We therefore investigated the mechanism of LVFX uptake into BeWo cells. LVFX uptake into BeWo cells was Na^+-independent, but an ATP depletion condition decreased LVFX uptake to 80% of the control level (Fig. 5). Moreover, although other fluoroquinolones inhibited LVFX uptake, an inhibitory effect of L-carnitine on LVFX was not observed (Fig. 6). Furthermore, the inhibitory manners of both LVFX and GPFX were non-competitive (Fig. 3). These facts suggested that a small fraction of LVFX was transported actively into BeWo cells but that OCTN2 is not involved in LVFX uptake in BeWo cells. Furthermore, the apparent Km value of LVFX uptake was calculated to be 20 mM, suggesting that affinity of LVFX on this transporter is too low to transport in clinical practice (Fig. 7). It has been reported that Km values of LVFX in Caco-2 cells and LLC-PK1 cells were 9.3 mM and 0.6 mM, respectively (Yamaguchi et al., 2001; Matsuo et al., 1998), suggesting that the LVFX transport mechanism is different from that in other organs. It has also been reported that LVFX might be a substrate of ATP-dependent efflux transporter. In this study, although we showed that an ATP depletion condition decreased LVFX uptake in BeWo cells, the effect of ATP depletion was not strong. Since it has been reported that efflux transporters are expressed in the placenta (Young et al., 2003), involvement of efflux transporters in LVFX transport in BeWo cells is also possible. Further investigation is needed to clarify LVFX transport in the placenta. However, in this study, we found small contribution of transporter for LVFX uptake in BeWo cells, suggesting that the amount of LVFX transferred from the mother to the fetus is also small as reported by Polachek et al. (2005). Taking these facts into consideration, the toxicity of LVFX during pregnancy might not depend on the amount of fluoroquinolones transferred to the fetus, but the inhibitory effect on transport of L-carnitine or other endogenous compounds through the placenta affects fetal growth.

It has been reported that the range of blood concentration of various fluoroquinolone is 0.5–5.0 mg/L in clinical practice (Turnidge, 1999). We know that blood concentration is lower than that of we elucidated in this study. However, it has been reported that OCTN2 highly expressed in placenta as well as in kidney and skeletal muscle (Tamai et al., 1998). Furthermore, Grube et al. (2005) have reported that OCTN2 has an important role for L-carnitine transport in human placenta. These facts suggest that the inhibitory effect of fluoroquinolones on OCTN2 has possibility of decreasing the amount of L-carnitine transfer from mother to fetus. But further investigation for inhibitory effect of fluoroquinolones in lower concentration and chronic administration in pregnant animals are needed.

In summary, L-carnitine uptake was inhibited by fluoroquinolones (CPFX, GFLX, OFLX, LVFX and GPFX). Since the inhibitory effects of LVFX and GPFX on L-carnitine uptake were dependent on the existence ratio of zwitterionic forms, it is suggested that these fluoroquinolones inhibited L-carnitine uptake by OCTN2. However, the manner of these inhibitory effects was non-competitive and LVFX transport was not inhibited by L-carnitine, suggesting that LVFX acts as only an inhibitor of OCTN2.

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