

Short Communication

Cholesterol-Lowering Effect of Ezetimibe in Uridine Diphosphate Glucuronosyltransferase 1A-Deficient (Gunn) Rats

Received March 5, 2007; accepted June 11, 2007

ABSTRACT:

Ezetimibe (EZE) selectively blocks intestinal cholesterol absorption by interacting with Niemann-Pick C1 Like 1 (NPC1L1). After administration, EZE is extensively metabolized in liver and intestine to its phenolic glucuronide form (EZE-G) by uridine diphosphate glucuronosyltransferases (UGTs), among which UGT1A1 and 1A3 exhibit highest activity. EZE-G is excreted into bile and undergoes extensive enterohepatic recirculation. Considering the pharmacokinetic properties of EZE and an *in vitro* binding study showing the high affinity binding of EZE-G to NPC1L1, glucuronidation by UGTs has been believed to be essential for the pharmacological efficacy of EZE. To study the role of glucuronidation by UGTs for the cholesterol-lowering effect of EZE, *in vitro* and *in vivo* studies were performed using Gunn rats, which hereditarily lack the expression

of UGT1A enzymes. The biliary excreted amount of EZE-G was reduced by 73% up to 3 h after administration of EZE (0.3 mg/kg) in Gunn rats, which is consistent with the reduction of *in vitro* EZE glucuronidation activity found in liver and intestinal microsome from Gunn rats. These results indicate that the formation of EZE-G in Gunn rats is much lower than that in Wistar rats. However, *in vivo* study showed that 0.3 mg/kg EZE, which is the clinically relevant dose, reduced cholesterol absorption in both Wistar and Gunn rats to nearly the same degree and the dose dependence was not significantly different between Wistar and Gunn rats at the range 0.001–0.3 mg/kg. These results indicate that a deficiency of UGT1A activity does not necessarily alter the cholesterol-lowering effect of EZE in rats at therapeutic doses.

Ezetimibe (EZE) (Fig. 1) (van Heek et al., 2000) is the first member of a new class of compounds that selectively block intestinal cholesterol absorption (Kosoglou et al., 2005) by interacting with Niemann-Pick C1 Like 1 (NPC1L1), the recently identified sterol transporter (Altmann et al., 2004; Yamanashi et al., 2007). EZE is extensively metabolized in liver and intestine to its phenolic glucuronide form (EZE-G) by uridine diphosphate glucuronosyltransferases (UGTs), of which UGT1A1 and 1A3 exhibit the highest activity, whereas UGT2B isozymes have a lower activity (van Heek et al., 2000; Patrick et al., 2002; Ghosal et al., 2004). After glucuronidation, EZE-G is excreted into bile and undergoes extensive enterohepatic recirculation. This process has been suggested to contribute to the pharmacological action of EZE (van Heek et al., 2000). In accordance with this hypothesis, it was demonstrated that the cholesterol-lowering activity was reduced in TR⁻ rats, which genetically lack the expression of ATP-binding cassette transmembrane transporter C2 (ABCC2) and showed the elevated plasma EZE-G levels and reduced fecal excretion of EZE and EZE-G (Oswald et al., 2006c). Furthermore, *in vitro* binding assay revealed that EZE-G can bind to NPC1L1 with high affinity (Garcia-Calvo et al., 2005). Considering these data, it is likely that glucuronidation by UGTs is essential for the pharmacological action of EZE. To examine this hypothesis, we decided to use Gunn rats, which hereditarily lack the expression of UGT1A isozymes and, therefore, exhibit a hyperbilirubinemic phenotype (Iyanagi, 1991).

Since EZE glucuronidation is predominantly mediated by UGT1A1 and 1A3 isozyme (Ghosal et al., 2004), the EZE glucuronidation capacity in Gunn rats may be much lower than that of control Wistar rats, and therefore, Gunn rats may be suitable for studying the role of glucuronidation. In the present study, we examined whether the hereditary defect in the expression of UGT1A family enzymes results in the reduced cholesterol-lowering effect of EZE by performing *in vitro* and *in vivo* experiments.

Materials and Methods

Materials. EZE was purchased from Sequoia Research Products Ltd. (Pangbourne, UK). [$1\alpha,2\alpha(n)$ - ^3H]Cholesterol (specific activity, 46.0 Ci/mmol) was obtained from GE Healthcare (Piscataway, NJ). Cholesterol was purchased from Wako Pure Chemicals (Osaka, Japan). L- α -Phosphatidylcholine and uridine diphosphate glucuronic acid (UDPGA) were obtained from Nacalai Tesque (Osaka, Japan). Sodium taurocholate was obtained from Sigma-Aldrich (St. Louis, MO). ^{14}C -labeled UDPGA (180 mCi/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). All other chemicals used in this study were either of analytical or molecular biological grade. ^{14}C -labeled and unlabeled EZE-G was synthesized according to the previously reported method (Zaks and Dodds, 1998). Formation of unlabeled EZE-G was confirmed by mass spectrometry and ^1H nuclear magnetic resonance spectroscopy.

Male Gunn (genotype *jjj*) and Wistar rats were obtained from SLC Inc. (Shizuoka, Japan). All animals used in this study were housed in temperature- and humidity-controlled animal cages with a 12-h dark/light cycle and with free access to water and normal animal chow (MF; Oriental Yeast, Tokyo, Japan).

Preparation of Liver and Intestinal Microsomes and Enzyme Assay. For the preparation of microsomes, male Gunn (260–280 g, $n = 3$) and Wistar rats

Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.

doi:10.1124/dmd.107.015628.

ABBREVIATIONS: EZE, ezetimibe; EZE-G, ezetimibe phenolic glucuronide; NPC1L1, Niemann Pick C1 Like 1; UGT, uridine-diphosphate glucuronosyltransferase; UDPGA, uridine diphosphate glucuronic acid; HPLC, high performance liquid chromatography; ABC, ATP-binding cassette transmembrane transporter; DPM, degradations per minute.

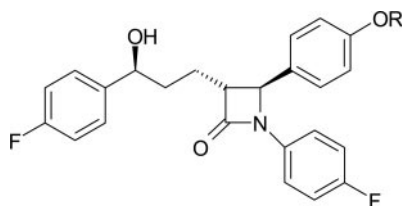


FIG. 1. Chemical structure of EZE ($R = H$) and EZE-G ($R = \text{glucuronide}$).

(245–260 g, $n = 3$) were used. Liver and intestinal microsomes were prepared as described previously (Omura and Sato, 1964; Fasco et al., 1993), and the protein content was determined using the BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as standard.

Enzyme assay was performed as follows (Ghosal et al., 2004). Microsomal incubations were carried out with 100 mM Tris-HCl (pH 7.5), 10 mM magnesium chloride, microsomes (0.1 mg protein/ml for liver and 0.2 mg protein/ml for intestine), 5 mM saccharolactone, 25 $\mu\text{g/ml}$ alamethicin, 2 mM UDPGA, and 0.5–50 μM EZE. Before addition of UDPGA, microsomes were incubated for 15 min on ice to maximize the UGT activity. After 5 min of preincubation at 37°C, UDPGA was added and the incubation was continued for 2.5–10 min. The enzyme reaction was terminated by addition of 10 μl of 70% perchloric acid and the analytical procedure described below was carried out.

Analysis of EZE and EZE-G in Microsome Incubations. The concentrations of EZE and EZE-G were determined by HPLC as described previously (van Heek et al., 2000; Oswald et al., 2006b) with some modification. The analysis was performed on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with a 5- μm YMC-Pack C18 column (250 \times 4.6 mm; YMC, Kyoto, Japan). The mobile phase was 0.1% phosphoric acid/acetonitrile = 60:40 and the flow rate was set at 1.0 ml/min. The analytical column was maintained at 40°C and eluents were continuously monitored at 232 nm.

Specimens were prepared as follows. After termination of the reaction by adding 10 μl of 70% perchloric acid, 20 μl of 3.5 M potassium chloride and 10 μl of internal standard solution (0.05 mg/ml 4-hydroxychalcone dissolved in methanol) were added. Then, specimens were vortexed, centrifuged at 15,000g for 15 min, and 40 μl of supernatant was directly injected onto the HPLC.

Biliary Excretion Study Using Wistar and Gunn Rats. Male Wistar (220–253 g, $n = 3$) and male Gunn rats (235–248 g, $n = 3$) were used for biliary excretion study. EZE was solubilized with ethanol (20 mg/ml) and was added directly to the blank rat plasma to give final concentrations of 0.3 mg/ml. After overnight (18-h) fasting, rats were anesthetized with pentobarbital (50 mg/kg i.p.) and bile duct was cannulated. After cannulation, rats received an intravenous administration of 1 ml/kg EZE-containing plasma from the jugular vein. After EZE dosing, bile specimens were collected for 3 h. Bile specimens were weighted and 25- μl aliquots were subjected to the EZE assay.

Analysis of EZE and Total EZE-G in Bile Specimens. The amount of EZE and EZE-G in bile specimens was determined as follows. Bile specimens (25 μl) were collected in a 10-ml glass tube and diluted with 1 ml of 0.3 M acetate buffer (pH 5.4). Then, β -glucuronidase solution (50 μl , 22,000 units/ml; Wako Pure Chemicals) was added in each tube and the mixture was incubated for 2 h at 60°C. For determination of unconjugated EZE, 50 μl of water was added instead of β -glucuronidase. After incubation, 5 M HCl (1 ml), internal standard solution (25 μl) and ethyl acetate (8 ml) were added and tubes were shaken for 15 min. Then, tubes were centrifuged (3000 rpm, 10 min) and organic layer was transferred to the other tube. Then the organic layer was washed with 5% (w/v) sodium hydrogen carbonate (1 ml) and evaporated under dry nitrogen stream. The residue was reconstituted in 100 μl of acetonitrile and 50 μl was injected onto the HPLC system. The HPLC condition is the same as that in the in vitro enzyme assay.

Preparation of Cholesterol Emulsion. Cholesterol emulsion was prepared as described previously (Tso et al., 1980). In brief, stock lipid solutions were mixed to give a final concentration of 13.3 mM triolein, 2.6 mM cholesterol, 3 mM L- α -phosphatidylcholine, and 3 $\mu\text{Ci/ml}$ [^3H]cholesterol. Solvents were evaporated and 19 mM sodium taurocholate (dissolved in phosphate-buffered saline) was added to give the required lipid concentration. Then the mixture

was sonicated three times for 5 min each, using an ultrasonic homogenizer (UP 200H; Hielscher Ultrasonics, Teltow, Germany) to produce a stable emulsion.

Acute Cholesterol Absorption Study Using Wistar and Gunn Rats. Male Wistar (180–233 g) and male Gunn rats (203–265 g) were used for in vivo study. An acute cholesterol absorption study was conducted as described previously (van Heek et al., 2000) with minor modification. EZE ethanol solution (20 mg/ml) was added directly to the blank rat plasma to give final concentrations of 0.003, 0.01, 0.03, 0.1, and 0.3 mg/ml. Fasted rats (18 h) were anesthetized with pentobarbital (50 mg/kg i.p.; Dainippon Pharmaceutical, Osaka, Japan) and an intraduodenal cannula was inserted as described previously (Tso et al., 1980). After cannulation, rats received intravenously 1 ml/kg blank plasma or EZE-containing plasma from jugular vein. Immediately after drug dosing, 5 ml/kg cholesterol emulsion was delivered directly into the intestine via the duodenal cannula. Three hours after cholesterol loading, rats were sacrificed; then, plasma was isolated and directly analyzed for [^3H]cholesterol in duplicate. Livers were also taken, weighed, and minced. Aliquots of 50–200 mg of tissue were solubilized using Solvable reagent (PerkinElmer Life and Analytical Sciences) and subjected to [^3H]cholesterol assay. Intestinal mucosa was scraped from upper intestine (approximately 40 cm from the pylorus), homogenized with 5 volumes of ice-cold phosphate-buffered saline, and directly analyzed for [^3H]cholesterol in duplicate. Protein concentrations of homogenate were determined using the BCA protein assay kit (Pierce).

For plasma and liver, data are expressed as percentage of administered radioactivity per total plasma volume or total liver. Total plasma volume was assumed to be 4% of the body weight as described elsewhere (Hawk and Leary, 1995). For the intestinal mucosa, data are shown as ^3H DPM per mg of protein.

Determination of Plasma Protein Binding of EZE-G. For the determination of plasma protein binding of EZE-G in Wistar and Gunn rats, [^{14}C]EZE-G was synthesized according to the method described by Zaks and Dodds (1998) by incubating EZE and [^{14}C]UDPGA with rat liver microsome. Specific activity of [^{14}C]EZE-G was 180 mCi/mmol. The purity of [^{14}C]EZE-G was confirmed by thin-layer chromatography with radioactivity detection.

Plasma protein binding was determined by ultrafiltration. ^{14}C -labeled and unlabeled EZE-G was added to plasma specimens from Wistar and Gunn rats to produce the final concentrations of 5, 20, 50, and 200 ng/ml. After incubation for 60 min at 37°C, specimens underwent ultrafiltration using a Centrifree device (Millipore, Billerica, MA) according to the manufacturer's protocol and the filtrates were directly analyzed for ^{14}C radioactivity.

Results and Discussion

Plasma EZE concentration is known to be much lower than that of EZE-G in both humans and rats (Patrick et al., 2002; Oswald et al., 2006c), and one of the reasons to account for this difference may be the rapid glucuronidation of EZE in liver and intestine by UGTs. In addition, detailed studies have shown that most (>80%) of the intraduodenally or intravenously administered EZE was recovered in bile within 2–3 h and the main form (>90%) was EZE-G (van Heek et al., 2000). In addition, it is believed that the pharmacological action of EZE-G is much more potent than that of EZE (van Heek et al., 2000). In the present study, we conducted an in vivo experiment in Gunn rats, which hereditarily lack the expression of UGT1A family members. Because human UGT1A1 and 1A3 are the major enzymes mediating the EZE phenolic glucuronidation (Ghosal et al., 2004), we hypothesized that Gunn rats may exhibit reduced EZE glucuronidation activity, resulting in a reduced cholesterol-lowering effect of EZE.

First, we conducted in vitro metabolism experiments using liver and intestinal microsomes from both Wistar and Gunn rats to confirm the EZE glucuronidation capacity of Gunn rats. The results of metabolism experiments are shown in Fig. 2. Due to the limit of detection, we could not determine the glucuronidation rate of EZE at concentrations lower than 0.5 μM . It was found that the glucuronidation rate of EZE by liver and intestinal microsomes from Gunn rats was reduced by 60–80% compared with that from Wistar rats at EZE concentrations

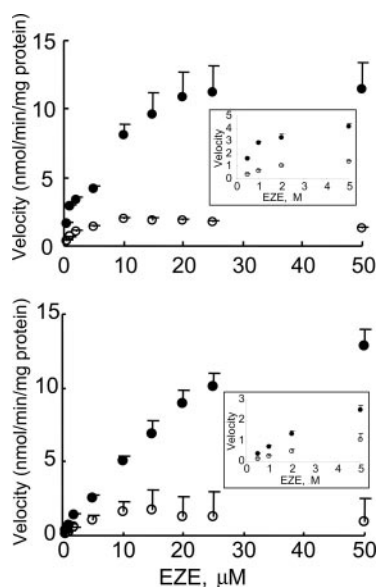


FIG. 2. Ezetimibe glucuronidation activity of liver (top) and intestinal (bottom) microsomes prepared from Wistar and Gunn rats. Intestinal and liver microsomes were prepared and incubated as described under *Materials and Methods*. The incubation time was as follows: Wistar rat liver microsomes, 2.5 min for 0.5, 1, 2, and 5 μM , 5 min for 10 μM , and 10 min for 15, 20, 25 and 50 μM ; Gunn rat liver microsomes, 5 min for 0.5, 1, 2, 5, and 10 μM , and 10 min for 15, 20, 25, and 50 μM ; Wistar rat intestinal microsome, 2.5 min for 0.5, 1, 2, and 5 μM , 5 min for 10 μM , and 10 min for 15, 20, 25, and 50 μM ; Gunn rat intestinal microsomes, 5 min for 0.5, 1, 2, 5, and 10 μM , and 10 min for 15, 20, 25, and 50 μM . Closed and open circles indicate data from Wistar and Gunn rats, respectively. The insets indicate the enlargement of the results at lower concentrations. Data are shown as mean \pm S.D. ($n = 3$).

of 0.5–50 μM (Fig. 2). The remaining glucuronidation activity observed in microsomes from Gunn rats may reflect the activity of the UGT2B isozymes (Ghosal et al., 2004). However, we cannot exactly compare the metabolite formation rate between Wistar and Gunn rats, due to substrate depletion from the incubation medium. Indeed, although 85 and 81% of EZE molecules in the medium remained unmetabolized after incubation of 0.5 μM EZE with microsomes from the small intestine of Wistar and Gunn rats, respectively, the fraction unmetabolized after incubation with liver microsomes from Wistar and Gunn rats was only 39 and 69%, respectively, at the end of experiments.

Furthermore, since we could not determine the glucuronidation rate of EZE in the lower concentration range due to the detection limit, we cannot conclude from the results of the present *in vitro* metabolism studies that the glucuronidation of EZE is reduced in Gunn rats even at clinically relevant concentrations (0.025–0.25 μM). To examine the extent of glucuronide formation, an *in vivo* experiment was conducted to compare the disposition of EZE between Wistar and Gunn rats. After *i.v.* administration of EZE at a dose of 0.3 mg/kg, it was found that the biliary excretion of EZE-G was reduced by approximately 73% in Gunn rats compared with that in Wistar rats (Fig. 3). In our experiments, EZE was not detectable in bile in both Wistar and Gunn rats. Since it has been shown that the expression level of ABCB2 and ABCB1/P-glycoprotein, which may be involved in the biliary excretion of EZE-G (Oswald et al., 2006a), was not altered in Gunn rats (Higuchi et al., 2004), our present *in vivo* results suggest that the hepatic clearance for the formation of EZE-G was significantly reduced by the lower UGT1A activity in Gunn rats even at the clinically relevant EZE dosage.

After confirmation of reduction of EZE glucuronidation in Gunn rats, we conducted an *in vivo* acute cholesterol absorption study using

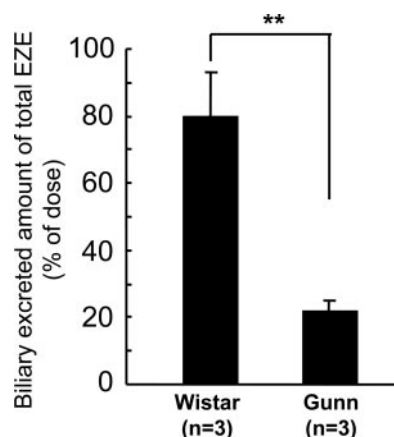


FIG. 3. Biliary excreted amount of EZE and EZE-G in Wistar and Gunn rats. Bile specimens were collected and assayed for total and unconjugated EZE as described under *Materials and Methods*. Cumulative amount of EZE and EZE-G excreted into the bile up to 3 h after *i.v.* administration of EZE (0.3 mg/kg) in Wistar and Gunn rats is shown as percentage of EZE dose. Unmetabolized EZE was not detectable in the bile from both strains of rats. Data are shown as mean \pm S.D. ($n = 3$). **, $p < 0.05$ by Student's *t* test.

Wistar and Gunn rats given different intravenous doses of EZE. The results are summarized in Fig. 4. The cholesterol amount in plasma and intestinal mucosa was reduced by EZE in a dose-dependent manner in both strains of rats. Although the amount of cholesterol in the liver was not significantly reduced by 0.01 mg/kg EZE, 0.3 mg/kg EZE significantly reduced the liver content of cholesterol in both rat strains (Fig. 4). Therefore, contrary to the previous belief, these *in vivo* data indicate that intravenous EZE is effective in both Wistar and Gunn rats at almost the same potency.

Although the exact mechanism behind this result is not clear, one of the possible explanations is that the pharmacological activity of EZE itself is almost the same as that of EZE-G. The higher intestinal mucosal and/or luminal concentrations of EZE in Gunn rats compared with Wistar rats due to the lack in the UGT1A activity may compensate for the reduced concentration of EZE-G in Gunn rats. Recent findings by Hawes et al. (2007) support this hypothesis. They showed that the binding affinities of EZE and EZE-G for NPC1L1 were almost the same (970 nM and 352 nM for EZE and EZE-G, respectively) (Hawes et al., 2007), which is consistent with the hypothesis that EZE itself is also pharmacologically active.

Another factor to be considered in accounting for the difference in the pharmacological activity of EZE and EZE-G between Wistar and Gunn rats is the alterations in the plasma protein binding. For this purpose, we measured the plasma protein binding of ^{14}C -labeled EZE-G. The unbound fraction of EZE-G was almost identical between Wistar and Gunn rats. Indeed, the plasma protein binding of EZE-G was determined to be $62.3 \pm 3.9\%$, $63.6 \pm 1.1\%$, $60.5 \pm 1.3\%$, and $86.4 \pm 0.4\%$ in Wistar rats, and $59.3 \pm 2.8\%$, $60.6 \pm 1.5\%$, $62.0 \pm 2.2\%$, and $86.1 \pm 0.7\%$ in Gunn rats at medium concentrations of 5, 20, 50, and 200 ng/ml, respectively. However, due to the limitations of our assay method (>40 ng/ml), plasma protein binding of EZE was not determined even at 200 ng/ml EZE. This result may be accounted for by considering the high plasma protein binding of EZE. Indeed, the human plasma protein binding of EZE was determined to be 93.9 to 94.5% under *in vivo* experimental conditions, and 99.5 to 99.8% under *in vitro* experimental conditions (Kosoglou et al., 2005). Therefore, we cannot exclude the possibility that the unbound fraction of EZE is different in Wistar and Gunn rats.

Finally, the dose used in the present study should be discussed in relation to the clinical use of EZE. The therapeutic dose of EZE in

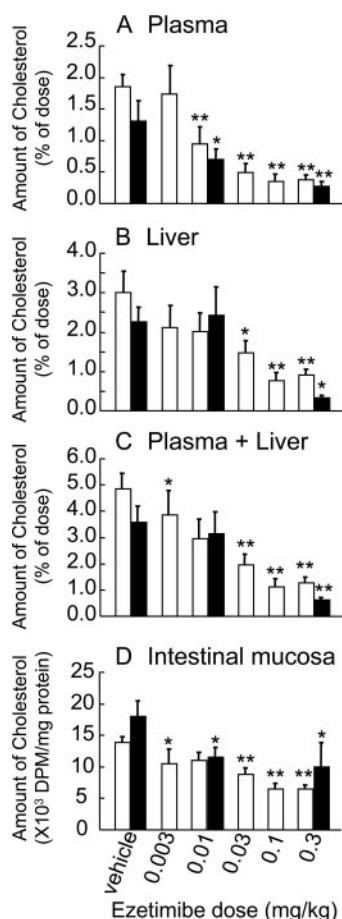


FIG. 4. Cholesterol amount in plasma (A), liver (B), plasma + liver (C), and intestinal mucosa (D) of Wistar and Gunn rats after intravenous administration of several doses of ezetimibe. Open and closed bars indicate the results from Wistar rats and Gunn rats, respectively. The cholesterol amount in intestinal mucosa is shown as DPM/mg protein. Total plasma cholesterol absorption was calculated as described under *Materials and Methods*. Data are shown as mean \pm S.E.M. ($n = 4\text{--}14$). All comparison was performed versus control group (without administration of EZE) of corresponding rat strain. *, $p < 0.05$; **, $p < 0.01$, by nonrepeated analysis of variance followed by Bonferroni test.

human is 10~20 mg/day (~0.2~0.4 mg/kg/day) (Kosoglou et al., 2005). In rats, approximately 90% of EZE is excreted in bile as EZE-G after oral and intravenous administration (van Heek et al., 2000), suggesting that almost all the orally administered EZE is absorbed from small intestine. Therefore, the maximum dose of EZE used in the present study (0.3 mg/kg intravenously) may be the clinically relevant dose, and this dose of EZE effectively blocked the cholesterol absorption in both Gunn and Wistar rats. Together with the results shown in Figs. 2 to 4, it is suggested that the deficiency in UGT1A enzymes and/or genetic polymorphisms of UGT1A gene may not affect the pharmacological efficacy of EZE when this drug is administered at therapeutic dosing regimen.

In conclusion, the cholesterol-lowering effect of EZE is not altered in Gunn rats when EZE is administered at clinically relevant doses.

These results may be accounted for by assuming that EZE itself is also able to inhibit NPC1L1-mediated cholesterol absorption. However, the detailed mechanism of action of EZE and EZE-G needs to be determined in future studies.

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