Polyamines upregulate the mRNA expression of cationic amino acid transporter-1 in human retinal pigment epithelial cells

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Polyamines upregulate the mRNA expression of cationic amino acid transporter-1 in human retinal pigment epithelial cells. Am J Physiol Cell Physiol 293: C729–C737, 2007. First published May 9, 2007; doi:10.1152/ajpcell.00018.2007.—We previously showed that ornithine was mainly transported via cationic amino acid transporter (CAT)-1 in human retinal pigment epithelial (RPE) cell line, human telomerase RT (hTERT)-RPE, and that CAT-1 was involved in ornithine cytotoxicity in ornithine-δ-aminotransferase (OAT)-deficient cell produced by a OAT-specific inhibitor, 5-fluoromethylornithine (5-FMO). We showed here that CAT-1 mRNA expression was increased by ornithine in OAT-deficient RPE cells, which was reversed by an inhibitor of ornithine decarboxylase (ODC), α-difluoromethylornithine (DFMO). Polyamines, especially spermine, one of the metabolites of ODC, also enhanced the expression of CAT-1 mRNA. ODC mRNA expression was also increased by ornithine and polyamines, and gene silencing of ODC by siRNA decreased ornithine transport activity and its cytotoxicity. In addition, the mRNA of nuclear protein c-myc was also increased in 5-FMO- and ornithine-treated hTERT-RPE cells, and gene silencing of c-myc prevented the induction of CAT-1 and ODC. Increases in expression of CAT-1, ODC, and c-myc, and the inhibition of these stimulated expression by DFMO were also observed in primary porcine RPE cells. These results suggest that spermine plays an important role in stimulation of mRNA expression of CAT-1, which is a crucial role in ornithine cytotoxicity in OAT-deficient hTERT-RPE cells.

ornithine transport; ornithine decarboxylase; c-myc

Gyrate atrophy of the choroid and retina is a progressive chorioretinal degeneration caused by a deficiency of the mitochondrial matrix enzyme ornithine-δ-aminotransferase (OAT) (48), and patients with the disease exhibit hyperornithinemia and ornithinuria (23, 45). Ornithine is mainly converted to glutamate and proline via Δ1-pyrroline-5-carboxylic acid by OAT in the mitochondria of human retinal pigment epithelial (RPE) cells (36), and under conditions in which OAT is deficient or inactivated, it is likely to be metabolized to polyamines, such as putrescine, spermidine, and spermine, by ornithine decarboxylase (ODC) (38). Ueda and colleagues (34, 47) of our group established the in vitro model of gyrate atrophy using 5-fluoromethylornithine (5-FMO), a specific irreversible inhibitor of OAT.

In mammalian cells, amino acids are transported through biological membranes by various transport systems, with different carrier proteins participating in that amino acid transport, exhibiting distinct transport properties (10, 30, 49). The transport of large neutral amino acids (NAAs), such as leucine, isoleucine, and valine, is mediated by system L, which includes L-type amino acid transporter (LAT)-1 and LAT2 (24, 43). System L is Na+/independent and the major route of branched or aromatic amino acids. On the other hand, cationic amino acids (CAAs), including lysine, arginine, histidine, and ornithine, are transported through cellular membranes by four distinct transport systems known as y+ (LAT)-1 and LAT2 (15, 31) and b0,+ (13). System y+ includes CAA transporter (CAT)-1, -2A/2B, -3, and -4, which are found ubiquitously and transport CAAs specifically. System y+ is Na+/independent and mediates the bidirectional transport of CAAs. System y+L, including y+LAT1 and y+LAT2, is an exchangeable transporter that recognizes CAAs in the absence of sodium, although it requires the cation to interact with NAAs. System L and system y+L require interaction via a disulfide bridge with type II membrane glycoprotein member, namely the heavy-chain 4F2hc, which is necessary to provide trafficking for the transporters to the cell membrane (33). We recently investigated the ornithine transport system in human RPE cells, and we found that CAT-1, NAA transporters LAT1 and LAT2, and CAA/ NAA transporter y+LAT2 are expressed in the human RPE cell line hTERT-RPE cells and that ornithine was mainly transported by CAT-1 in the cells (25). Although we suggested in that study that CAT-1 was involved in the mechanism of ornithine cytotoxicity by the use of the short interfering RNA (siRNA) of CAT-1, the relationship between amino acid transporters and ornithine cytotoxicity remains largely unknown.

The polyamines are ubiquitous low-molecular weight aliphatic cations. Although it is well known that polyamines are essential for growth and maintenance of the functions of normal cells (31, 42), excess levels inhibit protein synthesis and exhibit toxic effects toward cells. Furthermore, both accumulation and depletion of polyamines induce cell death such as apoptosis (1, 32, 40, 44, 52). We previously demonstrated that excessive spermine induces apoptotic RPE cell death and suggested that spermine may be one of the mechanisms of RPE degeneration (26). The nuclear protein c-myc is a transcription factor involved in the cell proliferation, differentiation, and induction of apoptosis (2, 19). Myc/Max heterodimer binds to the DNA at the E-box sequence (CACGTG) and activates transcription of c-myc target genes (3, 6). ODC has...
E-box sequences in promoter region and is regulated by c-myc (7).

In the present study, we found that the polyamines, especially spermine, metabolites of ornithine by ODC, have the ability to stimulate the mRNA expression of CAT-1 and that c-myc might play an important role in the induction of CAT-1 in hTERT-RPE cells. Induction of CAT-1 could play a crucial role in ornithine cytotoxicity in OAT-deficient hTERT-RPE cells.

MATERIALS AND METHODS

Cell culture. The human RPE cell line hTERT-RPE (39), previously established by gene transfer of human telomerase RT-cDNA, was kindly provided by Donald J. Zack (Wilmer Ophthalmological Institute, Johns Hopkins University, Baltimore, MD). This RPE cell line is reported to have several characteristics of other normal RPE cell lines, such as expression of Rpe65 and in vitro differentiation capacity (39). hTERT-RPE cells were cultured in DMEM/Ham F-12 medium (1:1; Sigma-Aldrich, St. Louis, MO) or Ham F12 medium (Invitrogen-Gibco, Grand Island, NY) supplemented with 10% FBS, 100 U/ml of penicillin, and the same antibiotics. Cells at passage 1 were used in the experiments.

Ornithine cytotoxicity. Control or ODC siRNA-transfected hTERT-RPE cells were seeded into well culture dishes at a concentration of 2 × 10^4/well and incubated for 24 h. The cells were treated with 0.5 mM 5-FMO and 10 mM ornithine in F12 medium for 24 and 48 h, respectively. After 24 h of incubation in serum-free medium, the cells were resuspended in DMEM containing 2 mM glutamine, 20% FBS, and the same antibiotics. Cells at passage 2 to 4 were used in the experiments.

Measurement of 1^4Cornithine uptake. The uptake of 1^4Cornithine by RPE cells was determined using L-[U-1^4C]ornithine (250 Ci/nmol, Amersham Biosciences, Tokyo, Japan). hTERT-RPE cells were seeded into Falcon 24-well culture dishes at a concentration of 2 × 10^4/well and incubated for 24 h. The cells were treated with 0.5 mM 5-FMO for 30 min, and 10 mM ornithine was added to the medium. After another 24 h of incubation in serum-free medium, the cells were equilibrated for 30 min in incubation buffer containing 125 mM NaCl, 5.6 mM d-glucose, 4.8 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 1.3 mM CaCl2, and 25 mM HEPES (pH 7.4). Ornithine uptake was initiated by adding 10 mM of the incubation buffer containing 1^4Cornithine (1 mM, 0.05 μCi), and measured for 60 min. Uptake was terminated by washing the cells 3 times with 0.5 ml of ice-cold incubation buffer. The cells were lysed with 0.1 M NaOH containing 0.1% Triton-X and neutralized with 0.15 ml of 0.1 M HCl. Radioactivity of the lysate was measured using a liquid scintillation counter (Tri-Carb Liquid Scintillation Analyzer 2700TR: Packard Instruments, Meriden, CT). The protein content was determined with a Dc Protein Assay kit (Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin as the standard. The uptake values are expressed as pmol/mg of protein or nmol/mg of protein. All experiments were performed at least 3 times, and reproducible results were obtained.

Northern blot analysis. Total RNA (10 μg) was isolated from hTERT-RPE cells with TRIzol (Invitrogen-Life Technologies, Carlsbad, CA), electrophoresed on a 1% agarose-10% formaldehyde gel, transferred to a nylon membrane (Schleicher & Schuell Biscience, Keene, NH), and then hybridized with 32P-labeled 785-base-pair (bp) CAT-1, a 760-bp ODC, a 1,020-bp c-myc, and a 228-bp GAPDH probe. Hybridization was carried out as described previously (35).

Real-time PCR. Total RNA (1 μg) was isolated from hTERT-RPE cells and reverse transcribed into cDNA using a SuperScript first-strand synthesis system for RT-PCR (Invitrogen-Life Technologies). PCR reactions were performed using Taq DNA polymerase (TaKara, Shiga, Japan), anti-Taq high (Toyobo, Osaka, Japan), and the double-strand specific fluorescent dye SYBRgreen I. The specific primers used for amino acid transporters were previously reported (25). The PCR conditions were as follows: hold at 94°C for 30 s, 40 cycles of amplification (94°C for 30 s, 60°C for 30 s, 72°C for 30 s), and a final extension at 72°C for 5 min. mRNA abundance was normalized to GAPDH expression and is presented as relative to the control (non-treated cells). Aliquots (10 μl) were taken from the PCR mixtures and analyzed by electrophoresis on a 2% agarose gel in 1 × Tris-acetate-EDTA buffer. The presence of corresponding PCR products was confirmed by the expected sizes of the amplification products.
of 2.5 × 10⁵/well. After 24 h, the cells were incubated with 0.5 mM 5-FMO and 10 mM ornithine in F12 medium for 24 h. Ornithine cytotoxicity was evaluated morphologically by microphotographs taken with a digital camera (SPOT; Diagnostic Instruments, Sterling Heights, MI) through an inverted confocal microscope (IX70, Olympus, Tokyo, Japan). For quantitative examination, we used 3-(4,5-dimethyl thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, as described previously (34).

Measurement of polyamines. Acid-soluble polyamines (putrescine, spermidine, and spermine) were analyzed using an HPLC system (Tosoh, Tokyo, Japan), as described previously (22). hTERT-RPE cells were incubated with 0.5 mM 5-FMO and 10 mM ornithine in F12 medium for 12, 24, and 48 h. The cells were washed twice with PBS, trypsinized, and collected by centrifugation at 500 g for 1 min. The collected cells were then resuspended with 0.3 ml of 5% (vol/vol) trichloroacetic acid, and the supernatants obtained following centrifugation (20,000 g for 3 min) were used for the measurement of polyamines. Total protein content was determined as described above, and polyamine content is presented as nanomoles per milligram of protein.

Statistics. Statistical significance was determined using ANOVA with Bonferroni correction. Data are expressed as the mean +/− SD of 3 or 4 separate experiments. Levels of P < 0.05 were considered statistically significant.

RESULTS

Increase in [14C]ornithine transport and induction of amino acid transporter mRNA. Recently, we reported that ornithine was mainly transported by CAT-1 in hTERT-RPE cells and that knockdown of CAT-1 by siRNA attenuated ornithine cytotoxicity in OAT-deficient hTERT-RPE cells (25). To clarify the mechanisms of ornithine cytotoxicity via CAT-1 in OAT-deficient hTERT-RPE cells, in the present study, we first determined the effect of ornithine on [14C]ornithine uptake in hTERT-RPE cells pretreated with 5-FMO, an OAT inhibitor.
increases Na+
choline (data not shown). These results suggest that ornithine transport activity (25). These results suggest that ornithine increases ornithine transport activity by induction of CAT-1 mRNA expression in OAT-deficient hTERT-RPE cells. The expression of CAT-1 mRNA was also increased in control- or CAT-1-siRNA-transfected hTERT-RPE cells treated with 5-FMO and ornithine. The increases in CAT-1 mRNA levels were 11.8- and 3.6-fold, respectively, of the nontreated cells. We recently showed that ornithine uptake was significantly reduced in hTERT-RPE cells transfected with CAT-1 siRNA (25). In addition to a significant inhibition of ornithine uptake in nontreated hTERT-RPE cells, CAT-1 siRNA reduced the increase of ornithine-evoked ornithine uptake for 60 min by 50% in OAT-deficient cells (Fig. 2B), demonstrating that the ornithine-induced ornithine uptake was primarily mediated by CAT-1 in OAT-deficient hTERT-RPE cells.

Involvement of ODC metabolites in mRNA expression of amino acid transporters. Ornithine is metabolized to polyamines, such as putrescine, spermidine, and spermine, by ODC. To clarify whether polyamines are involved in the induction of amino acid transporter mRNA expression, we examined the effects of DFMO, an ODC inhibitor, on OAT-deficient hTERT-RPE cells. DFMO (5 mM) completely inhibited the ornithine-induced increase of CAT-1, LAT1, and 4F2hc mRNA in OAT-deficient hTERT-RPE cells (Fig. 3). Further, 5-FMO and ornithine in combination increased the expression of ODC mRNA in the cells in a time-dependent manner, whereas either alone did not have an effect (Fig. 4, A and B). Conversely, DFMO at 5 and 10 mM blocked the induction of ODC mRNA expression by 5-FMO and ornithine (Fig. 4C). These results suggest that the increases in CAT-1, LAT1, 4F2hc, and ODC mRNA expression were regulated by ODC metabolites.

(14)C)ornithine uptake decreased linearly for the first 5 min and nearly reached a plateau after 15 min, while that uptake was enhanced and prolonged by treatment with 5-FMO and ornithine. The increase in (14)C)ornithine uptake in treated cells continued for 60 min until it was about 3.9-fold higher compared with the nontreated cells. However, the uptake of (14)C)ornithine by the cells was not affected significantly by Na+ replacement with choline (data not shown). These results suggest that ornithine increases Na+-independent ornithine transport activity in OAT-deficient hTERT-RPE cells. We recently showed that ornithine uptake was significantly reduced in hTERT-RPE cells transfected with CAT-1 siRNA (25). In addition to a significant inhibition of ornithine uptake in nontreated hTERT-RPE cells, CAT-1 siRNA reduced the increase of ornithine-evoked ornithine uptake for 60 min by 50% in OAT-deficient cells (Fig. 2B), demonstrating that the ornithine-induced ornithine uptake was primarily mediated by CAT-1 in OAT-deficient hTERT-RPE cells.

Fig. 4. Induction of expression of ornithine decarboxylase (ODC) mRNA by ornithine in OAT-deficient hTERT-RPE cells. A: total RNA was isolated from cells treated with or without 0.5 mM 5-FMO and 10 mM ornithine for 24 and 48 h. ODC and GAPDH mRNA levels were analyzed by Northern blot analysis, as described in MATERIALS AND METHODS. B: cells were treated for 24 and 48 h with or without 5-FMO, ornithine, and 5-FMO and ornithine. Total RNA was isolated and analyzed by quantitative real-time PCR using a primer set for ODC. C: effects of α-difluoromethylornithine (DFMO) on the induction of ODC mRNA. Cells were treated for 48 h with 5-FMO and ornithine in the presence (5 and 10 mM) or absence of DFMO, then analyzed by real-time quantitative RT-PCR using a primer set for ODC. GAPDH was used as the control. Data represent the means ± SD of three separate experiments.

Next, we investigated whether the ornithine-induced increase in ornithine transport activity in OAT-deficient hTERT-RPE cells was mediated by changes in amino acid transporter mRNA levels. Quantitative real-time RT-PCR results showed that the mRNA levels of LAT-1, 4F2hc, and CAT-1 were increased after 24 and 48 h of treatment with 5-FMO and ornithine, while LAT2, γ-LAT1, and γ-LAT2 mRNA levels were unchanged (Fig. 2A). The increases in LAT-1, 4F2hc, and CAT-1 mRNA levels at 24 h were 14.5-, 10.3-, and 6.3-fold, respectively, of the nontreated cells. However, treatment with 5-FMO or ornithine alone had little effect on the mRNA expression of these amino acid transporters (data not shown). In addition, we recently found that LAT1/LAT2 had no ornithine transport activity (25). These results suggest that ornithine increases ornithine transport activity by induction of CAT-1 mRNA expression in OAT-deficient hTERT-RPE cells. The expression of CAT-1 mRNA was also increased in control- or CAT-1-siRNA-transfected hTERT-RPE cells treated with 5-FMO and ornithine. The increases in CAT-1 mRNA levels were 11.8- and 3.6-fold, respectively, of the nontreated cells.

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Fig. 5. RNA interference of ODC mRNA and (14)C)ornithine uptake in OAT-deficient hTERT-RPE cells. Cells were transfected with scrambled control siRNA or ODC siRNA. At 48 h after transfection, the cells were treated with or without 0.5 mM 5-FMO and 10 mM ornithine for 24 h, then (14)C)ornithine uptake was measured for 60 min, as described in MATERIALS AND METHODS. Data represent the means ± SD of three independent experiments. Inset: expression of ODC mRNA in control and ODC siRNA-transfected cells. Total RNA was isolated from siRNA-transfected cells and Northern blot analysis was performed. *P < 0.05, **P < 0.01 vs. nontreated cells transfected with control siRNA. ††P < 0.01 vs. 5-FMO and ornithine-treated cells transfected with control siRNA. Data represent the means ± SD of three separate experiments.
Attenuation of ornithine uptake and ornithine cytotoxicity by ODC silencing. We also examined the involvement of ODC in ornithine transport activity in OAT-deficient hTERT-RPE cells. When transfected with control siRNA, [14C]ornithine uptake in the cells treated with 5-FMO and ornithine was four-fold higher than that in nontreated cells (Fig. 5). In contrast, when the cells were transfected with ODC siRNA, mRNA expression was markedly reduced in Northern blot analysis, and [14C]ornithine uptake was decreased by 40% (Fig. 5). We also examined the effects of ODC siRNA on ornithine cytotoxicity of 5-FMO- and ornithine-treated hTERT-RPE cells. As shown in Fig. 6A, the morphological changes in hTERT-RPE cells observed at 24 h after treatment with 5-FMO and ornithine were attenuated by ODC siRNA transfection. MTT assays revealed a significant reduction of cell viability in the cells transfected with scrambled siRNA.

Fig. 6. Inhibition of ornithine cytotoxicity by ODC silencing in OAT-deficient hTERT-RPE cells. A: cells were transfected with 20 nM of scrambled siRNA or ODC siRNA. At 48 h after transfection, the cells were treated with or without 0.5 mM 5-FMO and 10 mM ornithine for 24 h. Morphological changes were assessed using a confocal microscope. Original magnification, ×100. B: cellular viability of ODC siRNA-transfected hTERT-RPE cells. Forty-eight hours after transfection with control siRNA or ODC siRNA, the cells were treated with 0.5 mM 5-FMO, 10 mM ornithine, or 0.5 mM 5-FMO and 10 mM ornithine for 24 h, and MTT colorimetric assay was performed. *P ≤ 0.05 vs. without 5-FMO and ornithine. Data represent the means ± SD of four independent experiments.

Fig. 7. Changes of endogenous putrescine (A), spermidine (B), and spermine (C) induced by ornithine in OAT-deficient hTERT-RPE cells. Cells were preincubated in F12 medium for 24 h, then treated with (●) or without (○) 0.5 mM 5-FMO and 10 mM ornithine for 12, 24, and 48 h. Acid-soluble polyamines were extracted from the cells, and aliquots were analyzed using HPLC. *P < 0.05, **P < 0.01 vs. control. Data represent the mean ± SD of three independent experiments.

However, cell viability was not decreased in the cells transfected with ODC siRNA (Fig. 6B), supporting that ODC is involved in the increase in ornithine transport activity and cytotoxicity caused by ornithine in OAT-deficient hTERT-RPE cells.

Effects of ornithine on levels of endogenous polyamines. Before determining which metabolite(s) of ornithine produced by ODC caused a change in ornithine transport activity, we measured the intracellular levels of polyamines (putrescine,
spermidine, and spermine) in 5-FMO- and ornithine-treated hTERT-RPE cells. Compared with spermidine and spermine, putrescine content was one order lower and variable in hTERT-RPE cells (Fig. 7A). When the cells were treated with both ornithine and 5-FMO, the spermidine level became significantly decreased during the 48-h incubation period (Fig. 7B), whereas spermine was gradually increased in the cells at 24 and 48 h (Fig. 7C).

**Increase in mRNA expression of amino acid transporter and ODC by spermine.** Next, we examined the effects of putrescine, spermidine, and spermine on the mRNA expression of CAT-1 and ODC in hTERT-RPE cells. As shown in Fig. 8A, putrescine at 10 mM caused a weak but detectable increase in CAT-1 mRNA levels, but not at 1 and 5 mM. In contrast, spermidine at 1 mM markedly increased the expression of CAT-1, and ODC mRNA (Fig. 8B). Further, spermine at 0.1, 0.2, and 0.5 mM markedly increased CAT-1, and ODC mRNA levels in a dose-dependent manner (Fig. 8C). Taken together, these results suggest that the 5-FMO- and ornithine-induced mRNA expression of CAT-1 and ODC is mediated by spermine.

**Attenuation of the induction of CAT-1 mRNA by c-myc silencing.** c-myc is one of the target genes of transcription factor c-myc (3). To clarify the involvement of c-myc on the induction of CAT-1 and ODC, we examined the effect of c-myc siRNA on the mRNA expression of CAT-1 and ODC in 5-FMO- and ornithine-treated hTERT-RPE cells. As shown in Fig. 9A, c-myc mRNA expression was also increased under the same condition. Furthermore, c-myc mRNA expression was stimulated by polyamines in OAT-deficient hTERT-RPE cells (Fig. 9B). The induction of CAT-1 and ODC mRNA by ornithine was attenuated by c-myc silencing (Fig. 9C), demonstrating that c-myc was involved in the induction of CAT-1 and ODC mRNA.

**Induction of CAT-1, ODC, and c-myc mRNA in porcine RPE cells.** Next, we determined whether the induction of CAT-1, ODC, and c-myc mRNA expression was also observed in freshly prepared porcine RPE cells. As shown in Fig. 10, quantitative real-time RT-PCR results showed that the mRNA expression of CAT-1, ODC, and c-myc was increased after 24 h of treatment with 5-FMO and ornithine, and that the increases of CAT-1, ODC, and c-myc mRNA levels were 3.8-, 5.8-, and 6.9-fold, respectively, of nontreated cells. Further, DFMO (5 mM) inhibited the ornithine-induced increase of CAT-1, ODC, and c-myc mRNA.

**DISCUSSION**

We recently demonstrated that ornithine is transported by CAT-1 and y-LAT2 in hTERT-RPE cells and suggested that ornithine transport via CAT-1 may play a crucial role in ornithine cytotoxicity in those cells (25). The present results extend those findings and demonstrated that induction of CAT-1 by spermine, as well as the consequent increase in ornithine transport activity can be a cause of ornithine cytotoxicity in hTERT-RPE cells. Those conclusions were based on the following results. 1) Treatment of hTERT-RPE cells by 5-FMO and ornithine for 24 h stimulated ornithine uptake (Fig. 1). 2) Quantitative real-time RT-PCR assay results revealed that the mRNA expression of CAT-1 in hTERT-RPE cells treated with 5-FMO and ornithine was increased by about 6.3-fold and 10-fold at 24 and 48 h, respectively, after treatment, compared with that of nontreated control cells (Fig. 2A). 3) CAT-1 silencing by siRNA reduced ornithine uptake in nontreated hTERT-RPE cells, which was consistent with the results of our recent study (25), as well as ornithine-enhanced ornithine transport activity in OAT-deficient hTERT-RPE cells (Fig. 2B). 4) The induction of CAT-1 mRNA disappeared with blockage by polyamine synthesis of the selective ODC inhibitor, DFMO (Fig. 3). 5) Ornithine stimulated the mRNA expression of ODC in OAT-deficient hTERT-RPE cells, which was also reversed by DFMO (Fig 4). 6) ODC silencing reduced ornithine uptake and prevented ornithine cytotoxicity in OAT-deficient RPE cells (Figs. 5 and 6). 7) Intracellular spermidine was gradually decreased in hTERT-RPE cells treated with 5-FMO and ornithine for 48 h, whereas spermine was increased significantly at 24 and 48 h (Fig. 7). 8) Spermine showed a potent activity to induce CAT-1 mRNA expression (Fig. 8), and it was also observed to induce that in the presence of DFMO (S. Kaneko, unpublished observation).

CAT-1 has a lower affinity to CAAs, such as arginine and lysine (K_m, 70–250 μM), and its specificity is restricted to CAAs, whereas y-LAT2 has a higher affinity to CAAs (K_m, 6–10 μM) (11, 12, 30). Whereas y-LAT2, rather than CAT-1, may contribute to the initial influx of ornithine in RPE cells and CAT-1 contributed to the late phase of ornithine transport (Fig. 1), since CAT-1 has a 10-fold higher V_max than system y-L at higher concentrations (4, 12, 13, 30), 2) CAT-1 mRNA was markedly induced by ornithine in OAT-deficient hTERT-RPE cells (Fig. 2A), and 3) CAT-1 siRNA was able to significantly...
reduce ornithine uptake (Fig. 2B), these results demonstrate a large contribution of CAT-1 to ornithine-induced ornithine transport. Although the LAT1 was markedly induced by ornithine in hTERT-RPE cells, we verified in a previous report that LAT1 had no ornithine transport activity. Therefore, we concluded that its contribution for ornithine transport was little. Furthermore, there is another possibility that acceleration of the metabolism of ornithine following the induction of ODC results in the increase of ornithine uptake.

Although recent studies have shown that the expression of amino acid transporters is regulated by various growth stimuli (5, 14–16, 29) or amino acid starvation (17, 20), and is frequently increased in neoplastic cells (41, 46, 53), this is the first report that spermine induces the mRNA expression of CAT-1, as well as ODC. It is well known that polyamines spermidine, spermine, and putrescine influence gene expression via several distinct mechanisms, that is, through direct interactions with nucleic acids, sequence-specific interactions with DNA, and protein modifications (9, 21, 51). While the polyamines are present in cells in a millimolar range, a large number of them bind to RNA, DNA, ATP, and membranes via ionic bonds (21, 50), and their free forms seem to be kept at very low levels in cells due to their versatile functions. Further, intracellular polyamine content in mammalian cells is tightly controlled by cooperative regulation of their synthesis, catabolism, uptake, and elimination (31). Disturbance of such polyamine homeostasis might occur from an excess amount of extracellular ornithine in OAT-deficient hTERT-RPE cells, since an insignificant increase in spermine content could stimulate mRNA expression of CAT-1 and ODC during the early period following ornithine exposure (Figs. 2, 4, and 7). Under conditions of OAT deficiency, induction of such genes shown in the present study further promoted the accumulation of
intracellular spermine content (Fig. 8C), uncontrollable increase in mRNA expression of CAT-1 and ODC, which led to cell death. The concentration of intracellular spermine measured in the present study (~0.6 mM) is comparable to that for induction of CAT-1 and ODC mRNA. In contrast to spermine, spermidine content was significantly decreased. Although polyamines, for example, 10 mM putrescine, 2 mM spermidine, and 0.5 mM spermine, also had the capacity to increase the ornithine transport in hTERT-RPE cells in the absence of 5-FMO, and the effect of putrescine was significantly increased under the condition of OAT inactivation (data not shown). Intracellular spermidine was estimated to be ~0.2–0.6 mM under the condition, which was lower than 1 mM. In addition, the effect of spermidine on mRNA expression was weak compared with spermine (Fig. 9B); therefore, we supposed that the fluctuation of spermidine content might not be important to stimulate mRNA expression of CAT-1 or ODC in the present condition and concluded that the increase of spermine might play a critical role in induction of CAT-1 and ODC mRNA.

In the present study, we demonstrated that c-myc was involved in induction of CAT-1 and ODC (Fig. 9). It was reported that ODC gene has two-E-box sequences in the promoter region, and ODC is a direct transcriptional target of c-myc (3, 6). The result shown in this study is consistent with those of others who demonstrated that polyamines stimulate transcription of c-myc gene in intestinal epithelial cell and human colon cancer cells (8, 28), and it was also observed to the induction of c-myc by polyamines in hTERT-RPE cells (Fig. 9B). Furthermore, E-box sequences for binding of c-myc are also presented in the first and second intron of the CAT-1 gene, suggesting that polyamines stimulate the expression of CAT-1 and ODC via a transcriptional factor such as c-myc. The expression of CAT-1 is also increased by amino acid starvation (17, 20), which affects amino acid response element for enhanced transcription (17, 27), and the internal ribosomal entry site for regulation of translation (18, 37). Therefore, it is possible that the mechanism of increased mRNA may share features with the effect of polyamine and amino acid control mechanisms. This possibility is now under investigation.

In conclusion, the present findings demonstrated that ornithine uptake is increased in OAT-deficient hTERT-RPE cells, which is dependent on the induction of mRNA expression of CAT-1 and ODC. Furthermore, we found that among ornithine metabolites, spermine is involved in the induction via c-myc in those cells. Additionally, we supposed that an in vitro model shown in this study might be useful to investigate the regulatory mechanism of amino acid transporters.

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