Increased translation efficiency and antizyme-dependent stabilization of ornithine decarboxylase in amino acid-supplemented human colon adenocarcinoma cells, Caco-2

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The mechanisms of the response of ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis, to amino acid supplementation were studied in the human colon adenocarcinoma cell line, Caco-2. Supplementation of serum-deprived, subconfluent Caco-2 cells with any one of a series of amino acids (10 mM) resulted in increased ODC activity, reaching a maximum of approx. 12.5-fold after approx. 4 h, over control cells either not supplemented or supplemented with iso-osmolar D-mannitol. Glycine, L-asparagine and L-serine, as well as their D-enantiomers, were the strongest effectors and acted in a concentration-dependent manner; millimolar concentrations of most of these amino acids being sufficient to significantly increase ODC activity. In contrast, supplementation with D-methionine, L-lysine, L-aspartate or L-glutamate had little or no effect on ODC activity, whereas supplemental L-methionine, L-arginine, L-ornithine or L-cysteine was inhibitory. Polyamine assays showed that the putrescine content of cells varied in accordance with the changes in ODC activity. Western-blot and Northern-blot analyses revealed specifically increased levels of ODC protein but not mRNA, respectively, in response to supplementation with an ODC-inducing amino acid. Suppression of the increase in cycloheximide-treated cells confirmed a requirement for protein synthesis. Pulse-labelling of cells with [^{35}S]methionine showed a 3-fold increase in the synthesis of ODC protein after 4 h of supplementation with glycine or L-serine. Supplemental glycine also augmented, reversibly, the half-life of ODC by almost 4-fold and simultaneously decreased the activity of putrescine-induced free antizyme. These results suggest that translational, but not transcriptional, regulation of ODC takes part in ODC induction by amino acids in Caco-2 cells. However, it also appears to occur in concert with decreased enzyme inactivation and/or degradation.

Key words: glycine, polyamine biosynthesis, nutrient, ODC, post-translational regulation.

INTRODUCTION

Therapeutic enteral supplementation with conditionally essential amino acids has proven to be beneficial to intestinal recovery after impairment of epithelial renewal during gut injuries or disease [1]. Mammalian cell growth and tissue regeneration, in particular in the rapidly renewing intestinal epithelium, is highly dependent on the polyamines, putrescine, spermidine and spermine [2]. Moreover, the nutritional state of the organism is clearly an important factor in polyamine biosynthesis [3,4] and transport [5]. Whole-animal studies have reported that ornithine decarboxylase (ODC, EC 4.1.1.17), the first and rate-limiting enzyme in polyamine biosynthesis, is subject to regulation by food [6,7]. Attempts have been made to discriminate the components of food and/or the factors brought into play by eating that may be responsible for ODC activation. Dietary amines [7], hormones [6], proteins [3,8,9], amino acids [10–12] and sugars [8] have been reported to stimulate intestinal, renal or hepatic ODC *in vivo*.

Regarding stimulation by amino acids, strong differences were observed between tissues, e.g. the liver and the small intestine, in the animal [11]. Moreover, intraperitoneal injection of glycine could stimulate hepatic ODC, but not intestinal ODC, despite similar tissue accumulation of the amino acid. Since glycine was among the strongest inducers of ODC in the rat small intestine when administered intragastrically, it was proposed that amino acids must act on the apical surface of enterocytes to trigger ODC induction. Stimulation of the membrane Na^+/H^+ exchanger was found to be required for induction by L-asparagine and L-glutamine [13,14], and studies in cultured cell lines suggested that ODC induction by amino acids, as well as sugars, may be dependent on the coupled influx of Na^+ and organic solute [15–17]. However, intragastric administration of passively diffusing D-alanine or D-serine could also induce rat intestinal ODC [11,12,18], while actively transported amino acids such as L-phenylalanine, L-glutamate and α-amino isobutyric acid (α-AIB) [19] could not induce ODC in porcine jejunal epithelial cells [14]. These results, and reports of no increase in volume of rat intestinal crypt IEC-6 cells after supplementation with Na^+-coupled co-transported L-asparagine, [20,21] indicate that cell swelling may not be a prerequisite for ODC induction.

Marked discrepancies also exist between the reports on the respective role of the multiple regulatory steps that may control ODC activity in response to amino acid supplementation. From

Abbreviations used: α-AIB, α-amino isobutyric acid; DMEM, Dulbecco's modified Eagle's medium; eIF, eukaryotic initiation factor; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; neAA, non-essential amino acids; ODC, ornithine decarboxylase.

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in vivo studies in the rat, it was concluded that the translation efficiency of intestinal ODC mRNA was predominantly increased after intragastric ingestion of L-serine or L-alanine, since neither the ODC mRNA level nor its protein stability were found to vary significantly [12]. On the other hand, each D-stereoisomer of these amino acids was able to substantially augment the ODC mRNA level, however, by an unknown mechanism supposedly associated with cell stress, possibly induced by D-amino acid toxicity. Among numerous L-amino acids able to induce ODC activity in cultured cells, L-asparagine is the one that appears to display the most ubiquitous and potent effect. For that reason and since it is important in certain cancers [22], L-asparagine has been chosen as the test amino acid in most *in vitro* studies. Translational and post-translational regulation of ODC by L-asparagine was evidenced in rat primary cultured hepatocytes [23], as well as in the mouse neuroblastoma DF-40 cell line [24]. However, only the latter study demonstrated substantial post-transcriptional stabilization of ODC mRNA. Although increased amounts of ODC transcripts in response to L-asparagine were reported to be associated with transformation of mammalian cells [25], a similar regulation was also observed in normal, undifferentiated, rat intestinal crypt IEC-6 cells [20,21]. However, it was recently found that, when exposing IEC-6 cells to a salt/glucose solution, supplemented with L-asparagine, ODC activity increased through post-translational mechanism(s) without change at the protein level [26].

As a step towards the clarification of the molecular mechanisms of ODC regulation by amino acids, we re-address this issue in human colon adenocarcinoma Caco-2 cells, which are known to have the ability to differentiate as enterocyte-like cells. The present study demonstrates that, in these cells, ODC activity increases following supplementation with any one of a variety of amino acids, glycine being the most effective amino acid, as observed previously *in vivo* in rat small intestine [11,12]. Amino acid supplementation in Caco-2 cells led specifically to increased amounts of ODC protein, but not mRNA, owing to higher biosynthesis efficiency and increased half-life of ODC. Transient variations in the enzyme stability were concurrent with opposite changes in the activity of antizyme, a polyamine-inducible protein able to bind and inactivate ODC, as well as enhancing its ATP-dependent degradation by the 26 S proteasome [27]. Our data point out that Caco-2 cells constitute a valuable model for future studies on the signals triggered by amino acids to cause induction of ODC synthesis, on the one hand, and antizyme decay, on the other hand.

MATERIALS AND METHODS

Materials

D,L-[1-^{14}C]Ornithine (43.8 mCi/mmol) was purchased from Du Pont New England Nuclear (Boston, MA, U.S.A.). ^{35}S-labelled L-methionine (587 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Orsay, France). Dulbecco's modified Eagle's medium (DMEM), non-essential amino acids (neAA), antibiotics and fetal calf serum (FCS) were purchased from Gibco-BRL (Cergy-Pontoise, France). The amino acids used were of the highest purity grade available from Sigma and, in most cases, they were cell culture tested. The antiserum to mouse ODC was obtained from Eurodiagnostica (Lund, Sweden). The Caco-2 cell line (passages 190–205) [28] and the human ODC cDNA were generously provided by Dr A. Zweibaum (INSERM U 178, Villejuif, France) and Dr O. A. Jänne (Institute of Biomedicine, University of Helsinki, Finland), respectively.

Cell culture and treatment

Cells were seeded at 12×10^3 cells/cm^2 and routinely grown at 37 °C under a O_2/CO_2 (19:1) atmosphere, in DMEM containing 25 mM D-glucose and supplemented with 20 % (v/v) heat-inactivated FCS, 1 % (v/v) neAA, 100 units/ml penicillin and 100 μg/ml streptomycin. The culture medium was changed 48 h after seeding, then every day. For the experiments 3-day-old cells were incubated without serum for 24 h prior to use. Then they were rinsed with PBS and exposed to serum-deprived DMEM either not supplemented (DMEM control) or supplemented with a single amino acid or iso-osmolar D-mannitol (D-mannitol control). Medium osmolarities, measured by using an osmometer (Roebling, Berlin, Germany), averaged 0.32 osmol/litre.

ODC activity assay

After rinsing twice with cold PBS cells were scraped with a cell lifter (Costar, Corning, NY, U.S.A.) and homogenized in buffer containing 125 mM Tris/HCl (pH 7.5)/200 μM pyridoxal-phosphate. The cell suspensions were then centrifuged at 38000 ***g*** for 20 min and the resulting supernatants, supplemented with 0.1 mM EDTA and 5 mM dithiothreitol, were stored at −80 °C until use. ODC activity was determined as reported previously [5] by measuring the release of $^{14}CO_2$ from D,L-[1-^{14}C]ornithine in the presence of 160 μM unlabelled L-ornithine and 200 μM pyridoxal-phosphate. One unit of ODC activity is defined as the amount releasing 1 nmol of CO_2 from L-ornithine/h at 37 °C.

RNA isolation and Northern-blot analysis

Total cellular RNA was isolated according to the procedure of Chomczynski and Sacchi [29], and Northern-blots were performed as described by Sambrook et al. [30]. RNA was cross-linked to the membrane (Hybond N$^+$, Amersham Pharmacia Biotech) by UV irradiation. The blot was then prehybridized for 2 h at 42 °C in 50 % (v/v) formamide/6 × SSC (where 1 × SSC is 0.015 M sodium citrate/0.15 M NaCl)/5 × Denhardt's reagent (where 1 × Denhardt's reagent is 0.02 % Ficoll 400/0.02 % polyvinylpyrrolidone/0.02 % BSA)/0.5 % SDS. Gel-purified *Eco*RI/*Cla*I fragments (1150 bp) of ODC cDNA were labelled by random priming with [α-^{32}P]dCTP using dCTP-devoid, ready-To-Go® DNA labelling beads (Amersham Pharmacia Biotech) and used as ODC probes. Hybridization was carried out overnight at 42 °C. The blots were washed at 42 °C for 15 min twice in 2 × SSC/0.1 % SDS, then once in 0.5 × SSC/0.1 % SDS and once in 0.1 × SSC/0.1 % SDS. Labelled bands were visualized and quantified using a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and the IMAGEQUANT software. Each blot was re-hybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe in order to take into account variations in the amount of RNA in different samples or loading errors.

Analyses of ODC protein

Cell extracts were prepared in 125 mM Tris/HCl (pH 7.5)/200 μM pyridoxal-phosphate buffer supplemented with 1 % (v/v) Triton X-100, before centrifugation at 12000 ***g*** for 10 min [31]. Proteins (80 μg) from the supernatant were separated by SDS/PAGE on a 10 % (w/v) polyacrylamide gel and electrophoretically transferred on to a nitrocellulose membrane in 48 mM Tris/39 mM glycine/20 % (v/v) methanol. First, the membranes were blocked for 1 h at room temperature with 5 % (w/v) non-fat dry milk in PBS/0.1 % Triton X-100 prior to a further 3 h incubation with the anti-ODC antibody at 1:500

dilution. Secondly, the membranes were washed three times with a blocking solution of 0.5% non-fat dry milk in PBS/0.5% Triton X-100 before a 1 h incubation with horseradish peroxidase-conjugated goat anti-(rabbit IgG) at 1:5000 dilution. After three washes, the immunocomplexes were revealed using the ECF Western-blotting kit (Amersham Pharmacia Biotech). Quantification was performed after scanning of the film using the STORM PhosphorImager and IMAGEQUANT software.

ODC and total protein synthesis measurements

Cells treated with 10 mM amino acid for 3.5 h were washed with pre-warmed PBS, then exposed for 10 min to L-methionine-deprived DMEM, containing 10 mM glycine, before being pulse-labelled for 25 min with a supplement of L-[^{35}S]methionine. This was followed by extraction of the cells and immunoprecipitation of the ODC protein, which was analysed by SDS/PAGE and autoradiography as described previously [31].

For measuring total protein synthesis, cells were exposed for 3 h to DMEM, containing 10 mM glycine, then for 1 h to a similar medium depleted of unlabelled L-methionine and supplemented with 5.8 μCi/ml of L-[^{35}S]methionine. After removal of the radioactive medium the cells were processed as described in [32]. Results are given as L-[^{35}S]methionine d.p.m. incorporated/mg of cell protein per h.

Free antizyme assay

In order to determine the activity of free antizyme, 3-day-old subconfluent Caco-2 cells were deprived of serum for 24 h, while being supplemented during the last 12 h with or without 10 mM putrescine. They were subsequently exposed for various times to DMEM, containing 10 mM putrescine, that was either not supplemented (DMEM control) or supplemented with 10 mM of a single amino acid or D-mannitol (D-mannitol control). Treatments were stopped by rinsing the cells twice with PBS and homogenization in 125 mM Tris/HCl (pH 7.5)/200 μM pyridoxal-phosphate before storage at -80 °C until use.

The activity of free antizyme was determined by measuring the loss of ODC activity attributed to antizyme addition [33]. Samples of antizyme-enriched homogenates obtained from amino acid-supplemented or control cells were mixed with a constant amount (approx. 70 units) of partially purified ODC. After 10 min on ice, the mixture was assayed for ODC activity as described above. One unit of antizyme is defined as the amount inhibiting one unit of ODC activity. Inhibition of ODC activity up to 75% has previously been shown to be linear with respect to the amount of antizyme added [34].

Miscellaneous methods

For polyamine analysis cells were harvested in 0.2 M perchloric acid and the acid fraction containing the polyamines was stored at -20 °C before assays by the HPLC method described in [35]. Protein concentrations of cell extracts were measured by the bicinchoninic acid ('BCA') method [36]. When applicable, data were examined statistically by analysis of variance, with $P < 0.05$ considered significant.

RESULTS

Induction of ODC activity by a single amino acid supplementation

To determine the amino acid pattern of ODC induction in Caco-2 cells, serum-starved subconfluent cells were incubated for 4 h in DMEM either not supplemented or supplemented with 10 mM of any one of a series of amino acids or 10 mM D-mannitol, which was used as an osmolarity control. A number of these amino acids were able to increase ODC activity individually (Figure 1). Glycine was by far the most efficient amino acid, but substantial increase also resulted from supplementation with the stereoisomers of asparagine or serine, as well as with L-proline, L-threonine or the sulphonate, taurine. On the other hand,

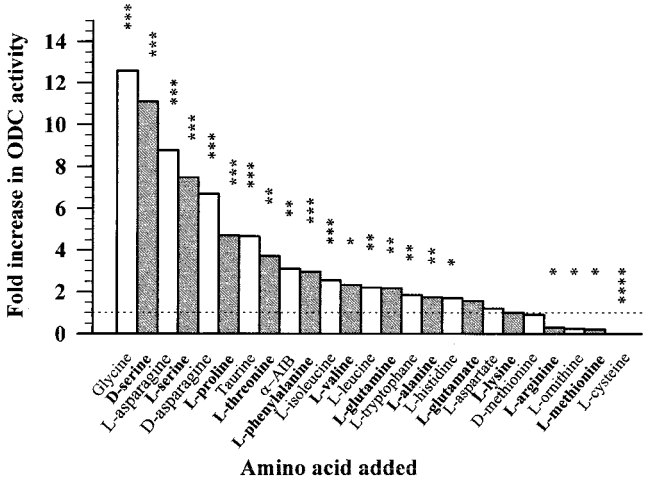

Figure 1 Effect of L-amino acids and amino acid D-isomers on ODC activity in subconfluent Caco-2 cells

Three-day-old cells were exposed for 24 h to an FCS-deprived DMEM medium, then for 4 h to DMEM supplemented with 10 mM of any one of the indicated L- or D-amino acids or D-mannitol (control). Data are expressed as the fold increase in ODC activity in amino acid-supplemented cells relative to control cells, and are means of at least three separate experiments, each done in triplicate. The dashed line (fold increase of 1) refers to the average control activity (approx. 0.5 nmol/h per mg protein). ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$ compared with control.

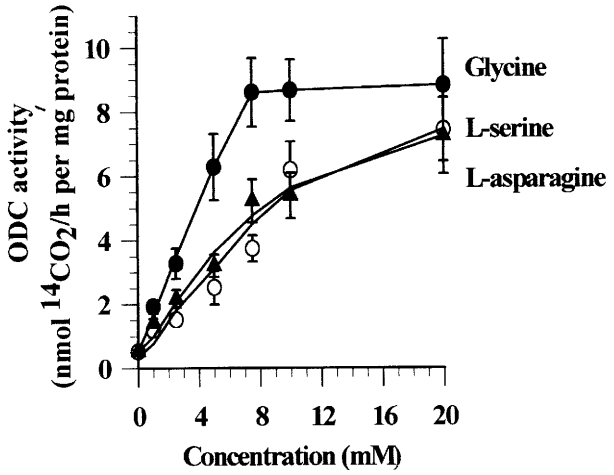

Figure 2 Amino acid concentration dependence of ODC activity in subconfluent Caco-2 cells

Three-day-old cells were exposed for 24 h to an FCS-deprived DMEM medium, then for 4 h to DMEM supplemented with increasing concentrations of glycine (●), L-serine (○) or L-asparagine (▲). Data are means ± S.D. of at least two separate experiments, each done in triplicate. Where not shown, S. D. were smaller than symbols. As compared with D-mannitol-supplemented control cells (not illustrated), the increases in ODC activity were significant ($P < 0.05$) throughout the kinetics except at 1 mM L-asparagine and L-serine ($P = 0.08$ and 0.13, respectively).

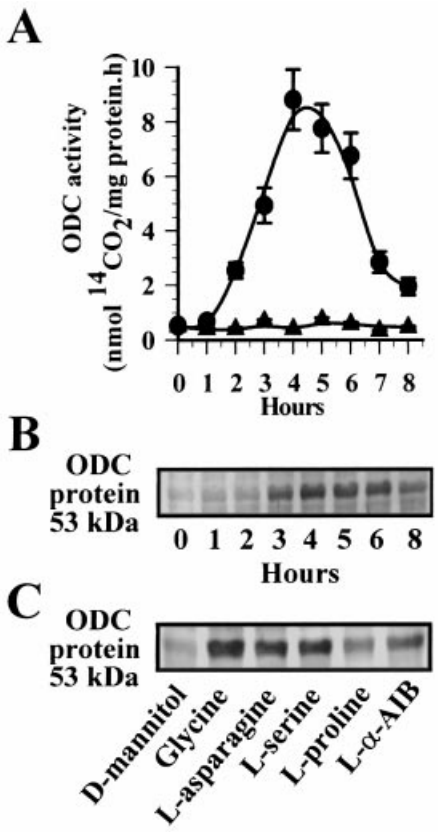

Figure 3 Changes in ODC activity and protein amount following amino acid supplementation in subconfluent Caco-2 cells

Three-day-old cells were exposed for 24 h to an FCS-deprived DMEM medium, then for various times to DMEM supplemented with 10 mM of a single amino acid or D-mannitol (control). (**A**) Time-dependent variations in ODC activity in glycine- (●) or D-mannitol- (▲) supplemented cells. Data are means ± S.D. of at least two separate experiments, each done in triplicate. (**B**) Western-blot analysis of the time course for the changes in ODC protein after glycine supplementation. (**C**) Western-blot analysis of the changes in ODC protein after 4 h of supplementation with 10 mM D-mannitol (control) or one of the indicated amino acids. In (**B**) and (**C**) three experiments were performed that showed similar results.

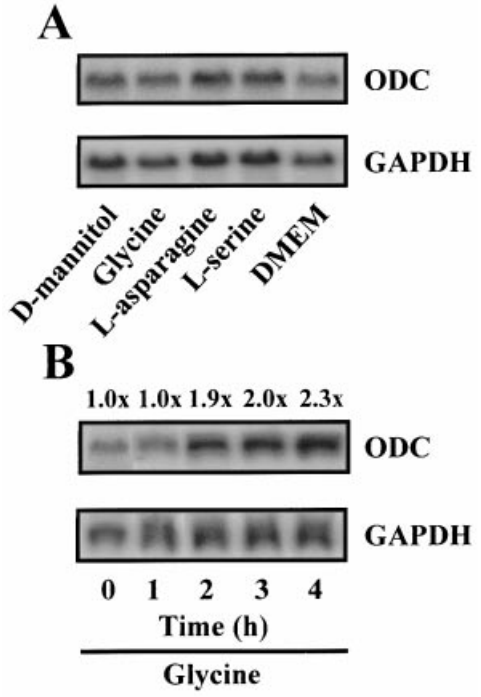

Figure 4 Effect of amino acid supplementation on ODC mRNA amount in subconfluent Caco-2 cells

Three-day-old cells were exposed for 24 h to an FCS-deprived DMEM medium, then: (**A**) for 4 h to fresh medium with 10 mM of either D-mannitol, glycine, L-asparagine or L-serine, or without any addition (DMEM); (**B**) for various times to DMEM supplemented with 10 mM glycine. The data illustrate Northern-blot analysis of ODC mRNA and GAPDH (control for RNA loading) in total RNA preparations from cells. In (**B**) values at the top indicate the fold increase of the GAPDH-normalized ODC mRNA band relative to time 0 of supplementation. Three experiments were performed that showed similar results.

D-methionine, the dibasic amino acid, L-lysine, and the acidic amino acids, L-aspartate and L-glutamate, had little or no effect on ODC activity, while the polyamine precursors, L-methionine, L-arginine and L-ornithine, as well as the free thiol-containing amino acid cysteine, were inhibitory. Globally similar observations were seen in differentiated, 18-day-old Caco-2 cells (results not shown). The increase in ODC activity was dependent on the concentration of supplemental L-asparagine, L-serine or glycine (Figure 2). At 1 mM of any one of these amino acids, ODC activity was increased by more than 1.3-fold relative to the corresponding control. The increase reached its maximum level with 7.5 mM glycine and apparently with higher concentrations of L-serine or L-asparagine.

The observation that glycine is the most potent stimulator of ODC activity in Caco-2 cells is in good agreement with previous *in vivo* observations in rat intestinal ODC [11]. Glycine was therefore chosen as a model inducer in our study of the mechanism of ODC induction by amino acids. Throughout the study, glycine was compared with other ODC-inducing amino acids where appropriate.

Increased amount of ODC protein, but not mRNA, in amino acid-supplemented cells

The time course for ODC induction by 10 mM glycine was transient with a peak at approx. 4–5 h (Figure 3A). Similar time courses were obtained with other ODC-inducing amino acids, unlike non-metabolized α-AIB that resulted in maximal ODC activity up to at least 8 h, the longest time period examined so far (results not shown). Comparatively no significant change occurred when supplementation was carried out with 10 mM D-mannitol instead of the amino acid. In addition, data from Western-blots (Figure 3B) showed that, qualitatively, the variations in ODC activity were accompanied by parallel changes in the amount of ODC protein. Cells supplemented for 4 h with 10 mM L-asparagine or L-serine displayed almost the same increased levels of ODC protein as cells supplemented with glycine (Figure 3C). On the other hand, the increase was lower with supplemental L-proline or α-AIB, while no significant change took place after supplementation with D-mannitol.

To establish whether or not the ODC mRNA level was also increased following amino acid supplementation, Caco-2 cells were exposed for 4 h to FCS-deprived, fresh DMEM without or with 10 mM of either D-mannitol, glycine, L-asparagine or L-serine (Figure 4A). The results showed no significant differences between these various conditions. Similar observations were noted following cell exposure to D-serine or D-asparagine (results

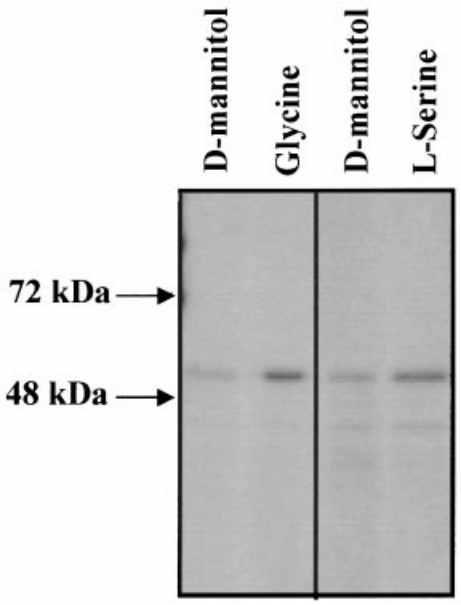

Figure 5 Effect of supplemental glycine or L-serine on the incorporation of [^{35}S]methionine into newly synthesized ODC protein

Three-day-old cells were exposed for 24 h to an FCS-deprived DMEM medium, then for 4 h to DMEM supplemented with either 10 mM glycine, L-serine or D-mannitol (control). Synthesis was determined as described in the Materials and methods section by pulse-labelling cells with [^{35}S]methionine, immunoprecipitating ODC, then analysing cytosolic extracts by SDS/PAGE and autoradiography. Two experiments, each done in duplicate, were performed that showed similar results.

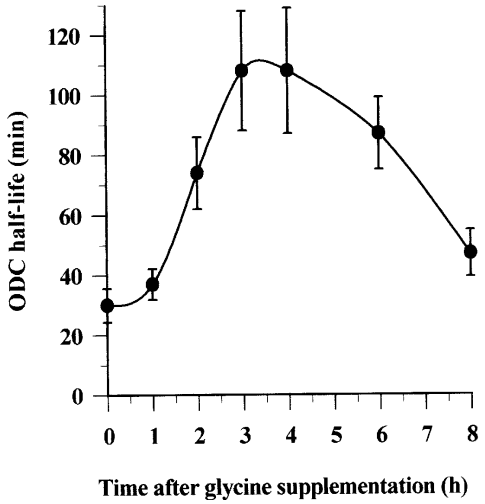

Figure 6 Time course for the effect of glycine supplementation on the stability of ODC activity

Three-day-old cells were exposed for 24 h to an FCS-deprived DMEM medium and incubated for the indicated times in DMEM supplemented with 10 mM glycine. Cells were then treated with cycloheximide (5 μg/ml) for a further 15, 30, 60, 120 or 180 min, harvested and processed as indicated in the Materials and methods section. The data are provided as ODC half-life values which were calculated by linear regression ($r \geqslant 0.93$) from semi-logarithmic plots of percentage ODC activity remaining after exposure to cycloheximide as a function of time. Data are means $\pm$ S.D. of at least two separate experiments, each done in triplicate.

not shown). However, when the ODC mRNA level was measured as a function of the duration of supplementation with glycine (Figure 4B) or D-mannitol (results not shown), it appeared that it increased rapidly up to a maximum of approx. 2-fold relative to its zero-time level.

Increased mRNA translation efficiency, and protein stabilization of ODC, after amino acid supplementation

Since amino acid supplementation, as compared with D-mannitol supplementation, led to increased amounts of ODC protein without specific variations in the ODC mRNA level, we hypothesized that the increase could result from higher efficiency of protein synthesis and/or stability. To examine whether protein synthesis was necessary for ODC induction by amino acid supplementation, we first treated Caco-2 cells with increasing concentrations of cycloheximide concomitant with glycine supplementation. The experiments showed that concentrations of 0.1 and 5 μg/ml of the protein synthesis inhibitor were sufficient to reduce ODC activity by approx. 50 and 97%, respectively (results not shown). Secondly, we compared ODC protein synthesis in cells supplemented with 10 mM D-mannitol or one of the amino acids, glycine or L-serine (Figure 5). The data showed that, after a 3.5 h incubation with 10 mM glycine or L-serine, the 25 min incorporation of radiolabelled L-methionine into ODC protein was 3–4- or 2–3-fold that measured in D-mannitol-supplemented control cells, respectively. This augmentation seemed specific for ODC since the overall protein synthesis, as measured by a 1 h incorporation of radiolabelled L-methionine in the trichloroacetic acid-precipitable cellular fraction, was similar after supplementation with any one of the above-mentioned amino acids or D-mannitol (results not shown).

Since stabilization has been shown to contribute substantially to ODC regulation in many instances, we searched for possible changes in the half-life of ODC activity throughout the time course of its response to glycine supplementation. For that purpose subconfluent Caco-2 cells were treated with 5 μg/ml cycloheximide at various times after addition of the amino acid supplement and the resulting decrease in ODC activity was measured during a further 3 h. In glycine-supplemented cells, the half-life of ODC activity augmented in a time-dependent manner (Figure 6), whereas it remained essentially steady after a 4 h supplementation with D-mannitol (results not shown). The augmentation was transient, reaching a maximal level of approx. 3.6-fold after 3–4 h. However, the ODC half-life after 8 h remained approx. 1.6-fold higher than that at zero-time of glycine supplementation.

Decreased activity of free antizyme after supplementation with an ODC-inducing amino acid

Since ODC degradation can be up-regulated specifically by antizyme [27,33,34], we investigated whether amino acid supplementation could alter the activity of this modulator protein. Assays of free antizyme, as described in the Materials and methods section, showed that a decrease in the antizyme activity was detectable as early as 1 h after exposure of putrescine-treated cells to 10 mM glycine relative to putrescine-treated control cells exposed to 10 mM D-mannitol (Figure 7). The activity of free antizyme decreased further after 2 and 4 h of glycine supplementation, especially in cells that had not been pre-treated with putrescine. In the latter case, the decrease remained substantial, although lower, afterwards, since it appeared to last at least over 9 h of supplementation with the amino acid.

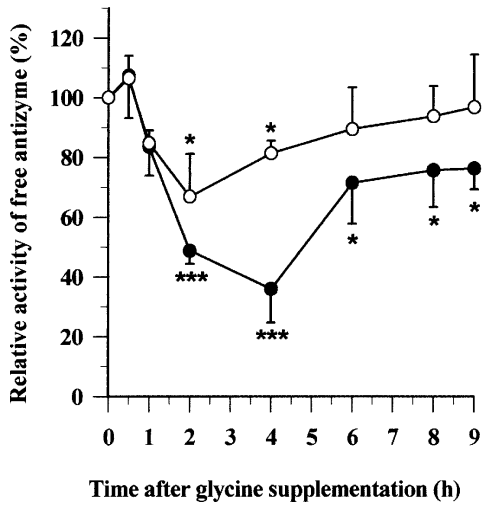

Figure 7 Time course for the effect of glycine supplementation on the activity of free antizyme

Three-day-old cells were exposed for 24 h to a DMEM medium deprived of FCS and supplemented during the last 12 h with (○) or without (●) 10 mM putrescine. They were then incubated for the indicated times in DMEM containing 10 mM putrescine and either 10 mM glycine or D-mannitol (control). The results (percentage of activity of free antizyme in the corresponding control cells) derive from linear regression analysis ($r \geqslant 0.95$) of plots of decreasing activity of an ODC source after addition of increasing amounts of antizyme preparation from glycine- or D-mannitol-supplemented cells. Data are shown as means + SD (○) and means − SD (●) of at least two separate experiments, each done in triplicate. $^{***}P < 0.001$ and $^{*}P < 0.05$ compared with the corresponding D-mannitol control.

Table 1 Effect of a 4 h supplementation with a single amino acid on polyamine levels in subconfluent Caco-2 cells

Three-day-old cells were exposed to FCS-deprived DMEM for 24 h, then to the same medium supplemented with either a single amino acid or D-mannitol (10 mM) for 4 h. Polyamines were measured as described in the Materials and methods section. Results represent means ± S.D. ($n = 3$).

	Polyamines (nmol/mg protein)			
Supplementation	Putrescine	Spermidine	Spermine	Total
D-mannitol (Control)	0.78 ± 0.01	5.92 ± 0.66	9.57 ± 1.45	16.27 ± 1.03
glycine	3.65 ± 0.66†	5.12 ± 1.38	11.05 ± 3.03	19.82 ± 5.05
L-serine	4.12 ± 0.97†	7.23 ± 1.90	11.51 ± 0.20	22.86 ± 3.03*
L-asparagine	2.73 ± 0.55†	7.23 ± 0.84	8.79 ± 2.43	18.75 ± 2.83
D-serine	2.52 ± 0.10‡	7.06 ± 1.31	10.48 ± 1.44	20.06 ± 2.80
D-asparagine	2.14 ± 0.17‡	5.56 ± 1.31	8.93 ± 2.51	16.64 ± 3.95
D-methionine	1.17 ± 0.16*	5.19 ± 0.39	10.52 ± 1.28	16.88 ± 0.95
L-arginine	N. D.	5.73 ± 0.43	10.10 ± 1.03	15.83 ± 1.44
L-methionine	N. D.	6.59 ± 0.12	10.99 ± 0.79	17.64 ± 0.91
L-cysteine	N. D.	4.88 ± 1.07	9.90 ± 2.65	14.78 ± 3.56

$^{*}P < 0.05$, †$P < 0.01$ and ‡$P < 0.001$ compared with control. N. D., not detectable.

Polyamine concentrations after amino acid supplementation

We addressed whether induction of ODC evoked changes in the polyamine concentration of amino acid-supplemented cells. Polyamine assays showed essentially that the putrescine content of Caco-2 cells supplemented for 4 h with one of the ODC-inducing amino acids increased many fold as compared with that in cells exposed to D-mannitol (Table 1). Additionally, it was moderately but significantly increased after supplementation with D-methionine, in spite of the absence of augmented ODC activity (Figure 1). On the other hand, putrescine was undetectable in cells supplemented with L-methionine, L-arginine or L-cysteine, in accordance with the observed decrease in ODC activity. Despite some fluctuations, no significant differences in spermidine and spermine concentrations were measurable. Total polyamine content only varied significantly after L-serine supplementation, although the trend was for an increase after supplementation with most ODC-inducing amino acids.

DISCUSSION

In vivo rat intestinal cells respond to intragastric supplementation with a single amino acid, by increasing notably the activity and the amount, but not the mRNA level, of ODC, a key enzyme in the regulation of polyamine biosynthesis [11,12]. The data in the present study document that (1) similar variations in ODC expression took place in subconfluent cells of the human colon adenocarcinoma cell line, Caco-2, following supplementation with any one of a series of amino acids, (2) increased amounts of ODC protein resulted from both increased protein synthesis and stability, and (3) consistent with the latter finding, the antizyme activity of cells decreased following supplementation with an ODC-inducing amino acid.

Glycine, L-asparagine, L-serine and their D-stereoisomers were by far the most potent ODC-inducing amino acids in Caco-2 cells. More precisely, a maximal induction in ODC activity, averaging 12-fold, was found to occur in a transient manner 4 h after supplementing with 7.5 mM glycine. The time course of induction exhibited a roughly similar pattern to that observed after intragastric administration of glycine *in vivo* [11] or L-asparagine supplementation in cultured cells [16,20,23]. Our results also indicate that a group of L-amino acids, similar to that reported for induction of rat intestinal ODC [11], increased ODC activity in Caco-2 cells. Enzyme stimulation by the non-metabolized amino acid analogue, α-AIB, shows that the amino acids exert their effect directly without a prerequisite for processing in Caco-2 cells, as also observed in other cell lines [16,26].

ODC regulation has repeatedly been shown to involve multiple pathways and to be dependent on cell and tissue type, especially concerning its polyamine requirement for function [2,37]. The existence of discrepancies between cell lines regarding the mechanisms of ODC control by L-amino acids is therefore not surprising. We show in the present study that if the ODC mRNA level actually increased following amino acid supplementation, it also increased after D-mannitol supplementation as well as after mere substitution of 24 h-old FCS-deprived DMEM with fresh similar medium. This indicates that, in contrast with L-asparagine-supplemented rat intestinal undifferentiated IEC-6 cells [20] or mouse neuroblastoma cells [24], subconfluent Caco-2 cells respond to L-amino acid supplementation without increasing specifically their ODC mRNA content. Our data show that, in these cells, ODC induction after amino acid supplementation clearly results from regulation at both the translational and post-translational levels.

The importance of the translational regulation of ODC in mammalian cells has been well demonstrated [37,38]. It has supposedly been reported to depend on secondary structure that may form in the long 5′ untranslated region of ODC mRNA. Accordingly, ODC translation could be up-regulated by the activation of translation initiation factors, such as eukaryotic initiation factor (eIF)-4E, which are able to melt mRNA secondary structure. Several recent studies have shed some light on the mechanisms involved in the regulation of translation of such mRNAs by amino acids [39,40]. In particular, regulation by

L-leucine of ODC synthesis in L6 myoblasts was found to be associated with availability of eIF-4E and phosphorylation of ribosomal protein S6 [41]. However, the involved signalling components, as well as their relationships, remain to be identified. Furthermore, it has long been known that the cell concentration of spermidine, spermine and, to a lesser extent, putrescine down-regulates the efficiency of ODC mRNA translation [2,37]. In the present study, increased intracellular levels of putrescine may contribute to the decrease in ODC expression that took place after 4–5 h of supplementation with glycine or any ODC-inducing amino acid. However, such a decrease was not observable when supplementing cells with non-metabolized α-AIB (results not shown). This suggests that amino acid consumption during the course of incubation may also play a part in the decline of ODC activity beyond 4–5 h of amino acid supplementation. Furthermore, it is possible that, as reported previously [42], the time-dependent decrease in antizyme levels (see Figure 7) may lead to diminished degradation of nascent ODC and, consequently, be responsible for higher apparent protein biosynthesis in glycine-supplemented cells relative to control cells.

Stabilization of ODC was found *in vitro* in cultured cells following L-asparagine supplementation [20,23,24], but not *in vivo* in intestinal cells of rats given a casein, glycine or L-serine load intragastrically [11,12]. We report in the present study that glycine supplementation increased ODC half-life in a transient manner in subconfluent Caco-2 cells. This increase seems to be satisfactorily correlated with that in ODC activity (see Figures 3A and 6). However, the data indicate that, during the first 3 h of supplementation, ODC half-life rapidly reached its maximal value whereas ODC activity was still increasing. This was consistent with the observation of a rapid decrease in the activity of free antizyme, which has been reported both to inactivate ODC and to enhance its energy-dependent degradation by the 26 S proteasome [27], in putrescine-treated cells supplemented with glycine. The decrease in antizyme activity, which was also transient, was particularly dramatic when the onset of antizyme induction by putrescine coincided with the beginning of amino acid supplementation. This suggests that expression of antizyme and/or that of so far unknown regulatory protein(s) involved in antizyme expression may be highly sensitive to changes in the amino acid supply. Antizyme is expressed as two constantly produced labile proteins with half-lives estimated at approx. 24 and 83 min, and thus cells may rapidly adjust its level in response to various environmental changes or stresses [33]. Moreover, owing to its rather low physiological level antizyme would probably act as a catalytic agent in normal cells. Therefore, although evidences were obtained using putrescine-induced cells, it can be thought from the present study that slight decreases in the antizyme level could contribute to ODC induction that follows physiological increases in the amino acid supply of cells normally expressing antizyme.

Antizyme stability was reported to decrease in putrescine-stimulated rat hepatoma HTC cells exposed to hypo-osmotic medium [33]. Millimolar concentrations of actively transported amino acids can induce mammalian cell swelling secondary to their internalization and prior to an osmo-regulatory volume decrease [43]. As already sketched for ODC induction [15–17,23], a parallel might be drawn between the decreases in antizyme level after supplementation with such an amino acid and after hypotonic shock. Moreover, the mechanisms by which hypo-tonicity induces ODC activity appear to be post-transcriptional in most cells [31,44]. However, amino acid supplementation and hypotonicity may not trigger ODC induction through similar mechanisms since (1) certain D-amino acids, namely D-asparagine and D-serine, which are not taken up by Na^+-dependent transport systems in intestinal cells [45], seem to regulate ODC translationally and/or post-translationally, as shown in the present study; (2) conversely, no induction was observed in the present study with L-aspartate and L-glutamate, on the one hand, and L-lysine, on the other hand, although they can be taken up by the Na^+-dependent, acidic amino acid transport system X_{AG-} [46] and the basic amino acid transport system [47], respectively; (3) the substrate preference of the broad-specificity, Na^+-dependent, neutral L-amino acid transporter in Caco-2 cells [48] is not correlated with the herein reported order of potency of amino acids to induce ODC; (4) in contrast with amino acid supplementation, hypo-osmotic conditions have been found to cause a dramatic reduction in general protein synthesis in certain cell types, suggesting disruption of cell function [44]; and (5) no change in the volume of IEC-6 cells after exposure to 10 mM L-asparagine has been repeatedly reported [20,21]. Further studies, e.g. regarding the involved signalling pathway(s), are required in order to delineate the molecular basis for ODC stimulation during the cell response to amino acid supplementation, on the one hand, and to hypo-osmotic conditions, on the other hand.

In addition to L-cysteine, all amino acids that are precursors of polyamine biosynthesis, namely L-methionine, L-arginine and L-ornithine, led to a dramatic decrease in ODC activity when supplemented to subconfluent Caco-2 cells. In all cases, this decrease was followed by a depletion of intracellular putrescine. Inactivation of ODC by supplemental L-cysteine probably resulted from changes in the redox thiol status of the protein and formation of iron reactive species [49]. L-Methionine or L-arginine, as such, were unable to alter ODC activity; however, ODC inactivation after L-methionine catabolism to L-cysteine seemed plausible (results not shown). Moreover, the inhibitory effect of L-arginine might rely on intracellular biosynthesis of nitric oxide which has recently been reported to inactivate ODC *in vitro* [50].

In conclusion, the results of this and other studies [23,24,26] stress the importance of the translational and post-translational regulation of ODC by amino acids in mammalian cells. A remarkable feature of the latter regulation is the dramatic decrease in the activity of antizyme that follows exposure of subconfluent Caco-2 cells to glycine. The cell signals that trigger ODC induction and antizyme decay, as well as the resulting metabolic consequences of these changes, remain to be elucidated.

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