Polyamine metabolism of enterocyte-like Caco-2 cells after exposure to *Phaseolus vulgaris* lectin

J F J G Koninkx, D S Brown, W Kok, H G C J M Hendriks, A Pusztai, S Bardocz

**Abstract**

The effect of *Phaseolus vulgaris* isolecitin E4 on polyamine concentrations and ornithine decarboxylase activity of proliferating and differentiating Caco-2 cells was investigated. Values of putrescine, spermidine, and spermine in control cells were highest during the early phase of proliferative cell growth and lowest in the stationary phase. Phytohaemagglutinin E4 significantly increased cellular polyamine values during the late proliferative phase of cell growth. Ornithine decarboxylase activity was high during intensive proliferation and growth, but was lower when proliferation slowed down or ceased. Exposure of Caco-2 cells in the early proliferative phase of cell growth to increasing concentrations of the potent intestinal growth factor phytohaemagglutinin E4 greatly stimulated enzyme activity. In contrast, the activity of ornithine decarboxylase was not stimulated in Caco-2 cells of the late proliferative phase nor was there any increase in the enzyme activity in differentiating and fully differentiated cells of the stationary phase. Accordingly, when proliferating Caco-2 cells possessed the highest ornithine decarboxylase activity, the polyamine values were also at their highest. During differentiation, as the ornithine decarboxylase activity fell close to zero, polyamine values also decreased. In the early proliferative phase of cell growth ornithine decarboxylase activity coincided with DNA synthesis in cells exposed to *Phaseolus vulgaris* isolecitin E4. These findings with Caco-2 cells were similar to those found in brush border cells of the rat small intestine.

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Keywords: Caco-2 cells, *Phaseolus vulgaris* lectin, polyamines, ornithine decarboxylase.

The intestinal mucosa is renewed by a combination of cellular proliferation and differentiation in the crypts, followed by maturation of the differentiated villus cells. Mucosal growth coincides with increased concentrations of putrescine and the polyamines, spermidine and spermine in the small intestine.1-5 which play an essential part in growth, although their exact functions are not clear. One of the earliest biochemical events associated with cell proliferation is an increase in activity of ornithine decarboxylase (ODC),6-8 the first rate limiting enzyme in polyamine synthesis.

The lectin from kidney bean *Phaseolus vulgaris* (phytohaemagglutinin or PHA), a potent growth factor in vivo,9 induces both hyperplastic and hypertrophic cellular adaptation in the rat tissue.10,11 After PHA binds to and is endocytosed by rat intestinal cells,12 it stimulates the metabolism of the epithelial cells.11 As with other acute or chronic proliferative and physiological stimuli,13-15 the PHA induced growth of the rat small intestine is preceded by a slight increase in activity of ODC and increased polyamine concentrations.11

The structural and functional properties of undifferentiated and differentiated enterocytes can be best studied in vitro using the human colon carcinoma cell line Caco-2.8,16-18 which represents a unique in vitro model.20 Thus, it has been shown that enterocyte differentiation of Caco-2 cells begins when the cells stop proliferating.20 Furthermore, at late confluency, Caco-2 cells display the differentiation characteristics of small intestinal enterocytes both structurally (microvilli) and functionally (brush border associated enzymes).20

Studies of inhibition of ODC with α-difluoromethylornithine have shown that polyamines are essential for the proliferation and differentiation of Caco-2 cells.19 Furthermore, these cells seem to be able to increase their polyamine concentration by both de novo synthesis and increased polyamine uptake.9

We have shown recently that after binding and endocytosis *Phaseolus vulgaris* isolecitin E4 (PHA-E4) induces changes in the cellular metabolism (DNA, RNA, (glyco)protein synthesis) of differentiated Caco-2 cells.17,18 However, the effect of this lectin on the polyamine metabolism of Caco-2 cells is unknown.

This study was designed to investigate whether PHA-E4 affects the activity of ODC and the polyamine concentrations of Caco-2 cells during proliferation and differentiation, and to establish the extent of this effect.

**Methods**

**Caco-2 cell culture**

The Caco-2 cells were grown in Dulbecco’s modified Eagle medium (DMEM, Flow Laboratories, Amstelstad, Amsterdam, the Netherlands), which was supplemented with 1% (v/v) non-essential amino acids (Flow), 50 μg/ml gentamycin (Flow), 10 mmol/l sodium bicarbonate (Flow), 25 mmol/l HEPES (Flow), and 20% (v/v) fetal calf serum (FCS, Imperial Laboratories, Rimietter BV, Utrecht, the Netherlands) and cultured at 37°C in a humidified atmosphere of 5% CO₂ in air. The cells were seeded at 4·10⁴ cells/cm² in tissue culture plates (6 flat bottom
Polyamine concentrations in proliferating and differentiating Caco-2 cells after incubation with PHA-E<sub>4</sub>

Two 3, 4, 5, 6, 7, 9, 12, 4, and 19 day old Caco-2 cells were quickly washed with 1×2 ml of DMEM and incubated with PHA-E<sub>4</sub>. Incubation was performed in quadruplicate in 1 ml of DMEM containing 0, 20, 50, 100, 150, and 200 µg of PHA-E<sub>4</sub>/ml (E-Y Laboratories, San Mateo, CA). After exposure for 48 hours, cells were washed with 3×1 ml ice cold PBS and then scraped off into 2 ml of ice cold twice distilled water. The wells of the tissue culture plates were rinsed once with 1 ml of ice cold twice distilled water, after which both volumes were combined. These cell scrapings were sonicated at 0°C for 30 seconds at an amplitude of 24 µm with a MSE Soniprep 150 (Beun de Ronde BV, Abcoude, the Netherlands). The resulting sonicates were snap frozen and freeze dried and the polyamine content analysed by high performance liquid chromatography.25

ODC activity in proliferating and differentiating Caco-2 cells after incubation with PHA-E<sub>4</sub>

Caco-2 cells were grown in tissue culture plates for 2, 3, 4, 5, 6, 7, 9, 12, 14, 16, and 19 days. Cells were washed and incubated as for the polyamine assay. After exposure for 48 hours, the cells were quickly washed with 1 ml PBS and then scraped off into 1 ml of 0·05 mol/l enzyme buffer (0·1 mol/l KH<sub>2</sub>PO<sub>4</sub>, 0·1 mol/l NaOH, pH 7·2 containing 5 mmol/l dithiothreitol and 1 mmol/l pyridoxal-5-phosphate). The wells of the tissue culture plates were rinsed once with 1 ml of enzyme buffer, and both volumes combined. The cell scrapings were sonicated at 0°C for 30 seconds at an amplitude of 24 µm with a MSE Soniprep 150 (Beun de Ronde BV). After centrifugation, the supernatant was removed for measurement of the ODC activity.26

ODC activity in 2 and 3 day old Caco-2 cells was also determined after exposure of the cells to 200 µg of PHA-E<sub>4</sub>/ml in the presence of 1 mg of fetuin/ml.

To verify whether an increase in ODC activity was accompanied by DNA synthesis,
Lectin-induced changes in polyamine metabolism

Figure 3: Polyamine concentrations of proliferating and differentiating Caco-2 cells after exposure to PHA-E. At various time points after cell seeding the Caco-2 cells were incubated for 48 hours with graded amounts of PHA-E. The results are expressed as nmol putrescine/10^6 Caco-2 cells (n=8; A), nmol spermidine/10^6 Caco-2 cells (n=8; B), and nmol spermine/10^6 Caco-2 cells (n=8; C). The first row of this three dimensional graph represents the pooled SD.

the DNA content of early proliferating (3 day old), late proliferating (6 day old), and differentiated (19 day old) Caco-2 cells was determined after exposure to PHA-E for 48 hours. Incubation was performed in quadruplicate in 1 ml of DMEM containing 20, 50, 100, 150, and 200 μg of PHA-E/ml. DNA contents of control Caco-2 cells (3, 6, and 19 day old) grown for 48 hours in the presence or absence of FCS were also measured.

Statistical analyses
Statistical significance between treated and control cells was assessed by one way analysis of variance (ANOVA) plus comparison of means. Differences were considered significant at the p<0.05 value.

Results
Growth characteristics and specific activity of brush border enzymes during proliferation and differentiation of Caco-2 cells
Figure 1 shows the growth characteristics of the Caco-2 cell line. The cells started to proliferate after a lag phase shorter than two days. The logarithmic phase of the growth was between days 3 and 9, after which the number of cells remained approximately constant at 5×10^5 cells/cm^2. Confluency of the cells was reached on day 6.

The specific activity of the brush border membrane associated enzymes, alkaline phosphatase and sucrose-isomaltase, was low during the lag phase and increased rapidly at the end of the logarithmic phase of cell growth (Fig 2). The highest values of specific enzyme activities were measured during the stationary phase when the cells had stopped proliferating.

Changes in the polyamine values of proliferating and differentiating Caco-2 cells after exposure to PHA-E
The polyamine content of proliferating and differentiating control Caco-2 cells and those exposed to PHA-E showed a gradual decrease (Fig 3A, B, C). The highest values were measured during the early phase of proliferative growth (day 3 and 4), whereas the lowest polyamine values were found in the stationary phase (day 9–19).

Exposure of 2 day old Caco-2 cells to increasing concentrations of PHA-E for 48 hours significantly lowered the putrescine content. Changes in the concentrations of spermidine and spermine did not seem to be significant. With the exception of the significantly decreased concentration of spermine in 3 day old Caco-2 cells, no other changes in polyamine concentrations were found in the early phase of proliferative cell growth (3 and 4 day old Caco-2 cells).

In the late phase of cell growth (5, 6, and 7 day old Caco-2 cells) when enterocyte-like differentiation is beginning to take place, the polyamine concentrations of Caco-2 cells exposed to PHA-E were increased in comparison with control cells (Table I).
TABLE I. Putrescine, spermidine, and spermine concentrations of proliferating and differentiating Caco-2 cells after exposure to 200 μg of PHA-E4/ml

<table>
<thead>
<tr>
<th>Days after cell seeding</th>
<th>Putrescine (nmol/10⁶ cells)</th>
<th>Spermidine (nmol/10⁶ cells)</th>
<th>Spermine (nmol/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>PHA-E4</td>
<td>Control</td>
<td>PHA-E4</td>
</tr>
<tr>
<td>2</td>
<td>1.41</td>
<td>0.05*</td>
<td>4.49</td>
</tr>
<tr>
<td>3</td>
<td>1.57</td>
<td>1.59</td>
<td>3.86</td>
</tr>
<tr>
<td>4</td>
<td>0.98</td>
<td>1.04</td>
<td>2.80</td>
</tr>
<tr>
<td>5</td>
<td>0.56</td>
<td>0.57</td>
<td>1.82</td>
</tr>
<tr>
<td>6</td>
<td>0.41</td>
<td>0.59*</td>
<td>1.71</td>
</tr>
<tr>
<td>7</td>
<td>0.36</td>
<td>0.49*</td>
<td>1.22</td>
</tr>
<tr>
<td>9</td>
<td>0.13</td>
<td>0.13</td>
<td>1.12</td>
</tr>
<tr>
<td>12</td>
<td>0.14</td>
<td>0.15</td>
<td>0.63</td>
</tr>
<tr>
<td>14</td>
<td>0.13</td>
<td>0.12</td>
<td>0.52</td>
</tr>
<tr>
<td>19</td>
<td>0.10</td>
<td>0.14</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Results are expressed as means (the pooled SD is given in Fig 3).

*Significantly different from the corresponding control based on Student’s t test (p<0.05).

ODC activity in proliferating and differentiating Caco-2 cells after exposure to PHA-E4

A sudden decrease in ODC activity was observed one day after the Caco-2 cells had reached confluency (Fig 4). Values of ODC activity seemed to be associated with the proliferative phase of cell growth, whereas it was low in the late proliferative and stationary phase.

Exposure of Caco-2 cells to increasing concentrations of PHA-E4 did not always result in a stimulation of ODC activity. Exposure of 2 and 3 day old Caco-2 cells to PHA-E4 greatly stimulated the enzyme activity (Fig 4). When compared with control cells a 3.1- and 2.4-fold increase in ODC activity was achieved after incubation of 2 and 3 day old Caco-2 cells respectively for 48 hours with 200 μg PHA-E4/ml. ODC activity in 4, 5, and 6 day old Caco-2 cells was only slightly stimulated by 50 μg PHA-E4/ml. Little or no stimulation was found in differentiated cells.

Incubation of 2 and 3 day old Caco-2 cells with 200 μg of PHA-E4/ml in the presence of 1 mg of fetuin/ml (a glycoprotein whose covalently linked oligosaccharide side chains are recognised by PHA-E4) failed to boost ODC activity (data not shown).

In both the early (3 day old) and late (6 day old) phase of proliferative Caco-2 cell growth, the DNA content of PHA-E4 exposed cells seemed to be increased significantly (Table II). However, no change in DNA synthesis was found after incubation for 48 hours of 19 day old differentiated Caco-2 cells (stationary phase of cell growth) with increasing concentrations of PHA-E4.

Discussion

Cell cultures displaying enterocyte-like differentiation are suitable models for studying the mechanisms underlying proliferation and differentiation of intestinal epithelial cells at the cellular level. The human colon carcinoma cell line Caco-2, which mimics the in vivo situation very closely, is a particularly important in vitro model.8 16-18 20 Undifferentiated Caco-2 cells can be regarded as the in vitro counterpart for immature crypt enterocytes, whereas the differentiated cells, exhibiting some of the structural and functional properties of small intestinal enterocytes20 (Fig 2), may represent the in vitro counterpart for mature villus enterocytes. In this study we have used this Caco-2 cell line to gain insight into the role of polyamines during proliferation and differentiation of epithelial cells.

Control Caco-2 cells were shown to have high intracellular concentrations of putrescine, spermidine, and spermine (expressed as nmol/10⁶ cells), which is characteristic for rapidly proliferating cells (Figs 1 and 3). Prior to the end of the logarithmic phase of cell growth (day 9) there was a sudden drop in putrescine content (Fig 3A), which was preceded by an abrupt change in ODC activity (day 7; Fig 4), the first enzyme of de novo polyamine biosynthesis. In contrast with putrescine, both spermidine and spermine concentrations decreased gradually when the cells stopped growing (Figs 3B, C).

Stimulation of the growth of gut mucosa in rats, such as in post-starvation feeding, leads to increased concentrations of polyamines in small intestine cells.28 29 The highly proliferating undifferentiated enterocytes contain higher concentrations of polyamines than differentiated Caco-2 cells, which ceased to proliferate. Our findings also show that polyamines are required for normal cell growth and proliferation and, that cell growth in vitro coincides with high concentrations of polyamines.

TABLE II. DNA content of early proliferating, late proliferating (differentiating), and differentiated Caco-2 cells after exposure to PHA-E4

<table>
<thead>
<tr>
<th>μg PHA-E4/ml</th>
<th>Day 3</th>
<th>Day 6</th>
<th>Day 19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cells</td>
<td>55.3 (3.3)</td>
<td>156.7 (3.0)</td>
<td>263.7 (5.7)</td>
</tr>
<tr>
<td>20</td>
<td>67.1 (3.1)*</td>
<td>181.4 (2.6)</td>
<td>267.1 (4.6)*</td>
</tr>
<tr>
<td>50</td>
<td>73.5 (3.1)</td>
<td>187.1 (2.6)</td>
<td>267.0 (3.9)*</td>
</tr>
<tr>
<td>100</td>
<td>73.2 (4.2)*</td>
<td>185.7 (3.4)</td>
<td>268.5 (3.4)*</td>
</tr>
<tr>
<td>150</td>
<td>68.8 (3.5)*</td>
<td>182.8 (3.3)*</td>
<td>269.2 (4.1)*</td>
</tr>
<tr>
<td>200</td>
<td>72.9 (1.6)*</td>
<td>184.2 (3.1)*</td>
<td>269.1 (4.4)*</td>
</tr>
</tbody>
</table>

At 3, 6, and 19 days after cell seeding, the Caco-2 cells were incubated for 48 hours with graded amounts of PHA-E4. The results are expressed as μg DNA/well (SD) (n=4).

*Significantly different (p<0.05) and t test significantly different from the corresponding Caco-2 cells not exposed to PHA-E4. **Growth of control Caco-2 cells was continued for 48 hours in the presence or absence of FCS. No significant differences could be established in the DNA content of these cells.

Figure 4: ODC activity in proliferating and differentiating Caco-2 cells after exposure to PHA-E4. At various time points after cell seeding the Caco-2 cells were incubated for 48 hours with graded amounts of PHA-E4. ODC activity was measured by decarboxylation of [1-14C]ornithine and expressed as pmol/h/10⁶ cells. The first row of this three dimensional graph represents the pooled SD.
Oral administration of spermidine and spermine to neonatal rats seemed to induce early structural and functional mucosal changes characteristic of postnatal maturation.30 Our results on intracellular polyamine concentrations (Table I) show that the spermidine concentrations of differentiating cells exposed to PHA-E4 (days 5, 6, 7, 12, and 14) are significantly higher than those in differentiating (control) cells. The increased spermidine concentrations may suggest that similar to that in vivo, spermidine might participate in the maturation of the enterocyte-like Caco-2 cells.

Measurements on the accumulation of 14C-spermidine in different tissues of the rat showed that the distribution of polyamines correlated with the metabolic activity and growth of these tissues.29 It has been suggested that polyamines are produced in situ as required mainly by a significant increase in tissue ODC activity.6 7 14 However, the extent of induction of ODC activity in the rat small intestinal cells could not fully account for the accumulation of polyamines during refeeding after fasting or in response to media, growth stimulation and dietary changes.16 The amount of polyamines needed to sustain PHA-induced gut growth in rats are derived from extracellular sources such as the circulation and the body storage pool.31–33

In agreement with a previous investigation,34 our results show that ODC activity is expressed mainly in proliferating Caco-2 cells (Fig 4). When cell proliferation has almost stopped (Fig 1) and differentiation has taken place, as suggested by high levels of sucrose-isomaltase activity (Fig 2), the activity of ODC is very low (Fig 4). Repression of the activity of ODC by the small amounts of polyamine left in the cell culture medium 48 hours after the original medium containing FCS has been replaced by DMEM seems to be unlikely. Therefore, as extracellular sources of polyamines are not available, the polyamine requirements needed to support normal Caco-2 cell growth can only be met by biosynthesis of polyamines by ODC. This was evident both in control cells and in cells in which cell proliferation was induced by PHA. Thus, our data on ODC in proliferating and differentiating cells after exposure to PHA-E4 clearly show that this lectin induces a significant increase in enzyme activity, at least during the early phase of cell proliferation (Fig 4). When ODC activity is high and polyamines are needed urgently for cell proliferation, as is the case in 3 day old Caco-2 cells exposed to PHA-E4, our data clearly show that the DNA content of these cells is significantly higher, suggesting increased DNA synthesis (Table II). The increase in activity found in 2 and 3 day old cells exposed for 48 hours did not occur in 5 and 6 day old cells (Fig 4) suggesting that the intracellular concentrations of polyamines were already high enough to support proliferation. Thus, as suggested by the increased DNA contents of 6 day old Caco-2 cells (Table II), DNA synthesis can proceed even when ODC activity is low.

There was, however, no increase in the ODC activity of 2 and 3 day old undifferentiated Caco-2 cells exposed to PHA-E4 in the presence of fetuin. This shows that the lectin induced effect was related to lectin binding to the glycoconjugates of the cell membrane.

A sudden drop in ODC activity occurred one day after the Caco-2 cells reached confluency and prior to the arrest of cell growth. Because cell proliferation still continued, albeit slowly (Fig 1), sufficient intracellular polyamines must have been present to cover the needs for the proliferation of new cells. As the need for polyamines of non-proliferating Caco-2 cells in the stationary phase of cell growth is likely to be low, ODC activity in these cells can also be very low. Our data show clearly that as exposure of 19 day old cells to increasing concentrations of PHA-E4 has failed to change their DNA content (Table II), low ODC activity (Fig 4) coincides with the absence of DNA synthesis of cells in the stationary phase of growth (Table II). As regulation of ODC activity in the cell correlates well with changes in the level of its messenger RNA,35 36 the sharp decrease in ODC activity in the Caco-2 cells may have been preceded by a corresponding decrease in the transcription of the ODC gene.37 38

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