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Channelling of arginine in NO and polyamine pathways in colonocytes and consequences

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1. ABSTRACT

Colon epithelium is renewed within few days through asymmetric mitosis of pluripotent cells followed by differentiation and exfoliation. Absorptive colonocytes do not transport amino acids from lumen to bloodstream but import amino acids from plasma. Among amino acids, colonocytes can use L-arginine as a precursor for nitric oxide (NO) synthesis and also for polyamine synthesis through the stepwise conversion of L-arginine into L-ornithine and urea, conversion of L-ornithine into putrescine and then synthesis of spermidine and spermine. Colonic epithelial cells can transport polyamines produced exogenously by the microbiota. Polyamines are strictly necessary for undifferentiated colonic epithelial cell proliferation, but NO exerts anti-proliferative effect on these cells raising the view that the channelling of arginine in the nitric oxide and polyamine pathways is involved in the control of cellular proliferation. Furthermore, NO has been shown to be a potent inhibitor of ornithine decarboxylase activity in colonic epithelial cells. Unbalance of the chanelling of arginine in the NO and polyamine pathways in colonic cancerous epithelial cells as a determinant promoting their proliferation is discussed.

2. INTRODUCTION

The epithelium of the large intestine represents a selective barrier between the microbiota and luminal compounds and the underlying lamina propria. The colonic epithelium is renewed within 3 to 5 days in humans (1) through asymmetrical mitosis of pluripotent stem cells which are located at the very base of the colonic crypts (2-4). As the daughter cells move up to the top of the colonic crypt, they differentiate into cell lineages which are responsible for the physiological functions of the epithelial cells namely absorptive colonocytes, mucus-producing goblet cells and enteroendocrine cells (5). At the luminal surface, fully mature cells are shed into the lumen by a process referred as detachment-induced apoptosis (or anoikis) which is likely to represent one form of elimination of these cells (6-8). Although the coordination and control of these events are dreadfully complex in nature and not fully understood, studies on the luminal and endogenous signals as well as signalling and metabolic pathways involved in the renewal of the large intestine epithelium have allowed progress in our understanding of these events (9-12).

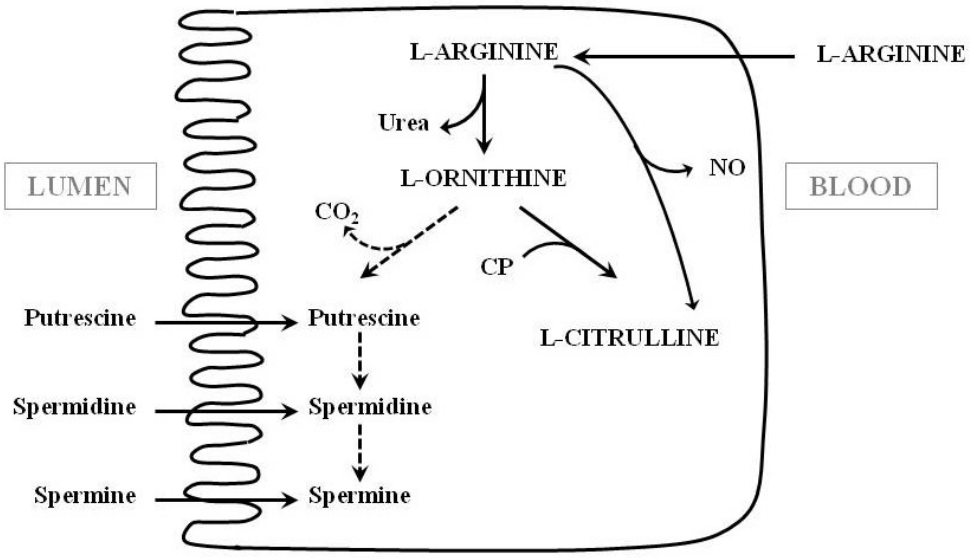

Figure 1. Schematic view of the metabolism of L-arginine in healthy absorptive rat colonocytes. L-ARG: L-arginine; L-ORN: L-ornithine; L-CITR: L-citrulline; CP: carbamoylphosphate. Dotted lines indicate quantitatively minor metabolic pathways.

Amino acids through oxidation and synthesis of various metabolites as well as through activation of signalling pathways within colonic epithelial cells are involved in the process of the rapid renewal of the epithelium (13,14). Among them, L-arginine has been studied for its metabolic fate in colonic epithelial cells originating from both healthy and cancerous mucosa.

The aim of the present article is to present an overview of what is known on the metabolism of L-arginine in healthy and cancerous colonic epithelial cells and to indicate how this metabolism is involved in the control of cell proliferation, differentiation and death. In addition, the potential role of exogenous polyamines produced by the intestinal microbiota in such a control is presented.

3. METABOLISM AND PHYSIOLOGICAL EFFECTS OF POLYAMINES AND NITRIC OXIDE ON NORMAL AND CANCEROUS COLONIC EPITHELIAL CELLS

3.1. Metabolism of L-arginine in the polyamine and nitric oxide pathways in normal and cancerous colonic epithelial cells

Amino acids from the luminal origin are not believed to be absorbed to any significant extent through the colonic epithelium except for a short period after birth (15). Then, in contrast with the situation found in small intestine enterocytes, which import amino acids from both luminal and blood origins (16), colonic epithelial cells import amino acids only from the arterial blood for metabolic and physiologic purposes. In healthy absorptive rat colonocytes, L-arginine is converted to L-ornithine and urea through the catalytic activity of arginase (17) (see Figure 1). Although urea represents a metabolic end product of colonocyte metabolism, L-ornithine can be further converted to L-citrulline through the activity of ornithine carbamoyltransferase (18). L-ornithine is little used for polyamine (putrescine, spermidine, spermine; see Figure 2) production in normal colonocytes due to a very low ornithine decarboxylase (ODC) catalytic activity (17). This ODC activity was found to be equally low in normal human colonocytes isolated from the upper and lower crypt regions (19). Putrescine, spermidine and spermine can be measured in normal human colonocytes with putrescine being at much lower concentration in colonocytes than spermidine and spermine (19). The comparison between ODC catalytic activity and intracellular polyamine concentrations in normal colonocytes suggests that polyamines are likely to originate from extracellular source.

L-arginine can also be converted to nitric oxide and L-citrulline through the catalytic activities of nitric oxide synthase (NOS, see Figure 1) which are present in colon absorptive epithelial cells (20).

In human proliferative colonic adenocarcinoma HT-29 epithelial cells, a model of colonic epithelial cells which is often used by cell physiologists, L-arginine can be converted to L-ornithine and urea (Figure 3), but in sharp contrast with the situation observed in normal colonocytes, a large part of L-ornithine is converted to putrescine due to a very high activity of ODC (21). In these latter cells, a part of newly synthesized putrescine is converted to spermidine. The high ODC activity in cancerous colonic epithelial cells is in accordance with the fact that this activity is higher in human colonocytes isolated from cancerous areas compared with colonocytes isolated from adjacent colonic tissue (22). Also, the intracellular concentrations of putrescine, spermidine and spermine are several fold higher in colonocytes recovered from cancerous areas than in colonocytes recovered from normal mucosa (22). The metabolic capacity to synthesize putrescine from L-arginine

Putrescine $H_2N\,(CH_2)_4\,NH_2$

Spermidine $H_2N\,(CH_2)_3\,NH\,(CH_2)_4\,NH_2$

Spermine $H_2N\,(CH_2)_3\,NH\,(CH_2)_4\,NH\,(CH_2)_3\,NH_2$

Agmatine $H_2N\,C(=NH)\,NH\,(CH_2)_4\,NH_2$

Figure 2. Chemical structure of putrescine, spermidine, spermine and agmatine.

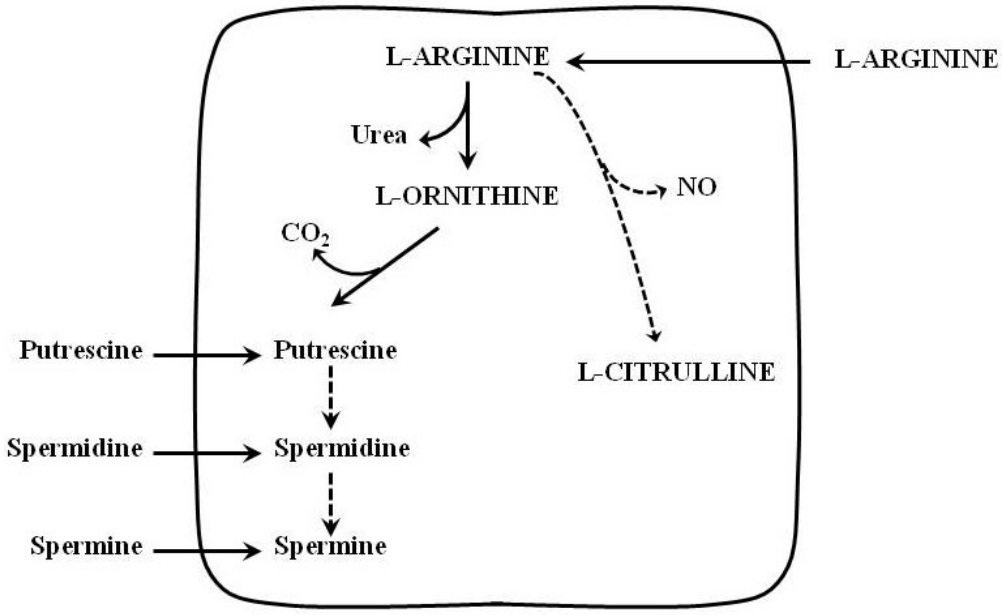

Figure 3. Schematic view of the metabolism of L-arginine in proliferative cancerous colonic epithelial cells (HT-29 cell line). L-ARG: L-arginine; L-ORN: L-ornithine; L-CITR: L-citrulline. Dotted lines indicate quantitatively minor metabolic pathways.

collapses when HT-29 $Glc^{-/+}$ cells move to a less proliferative and more differentiated cell phenotype (21).

In sharp contrast, the capacity of HT-29 $Glc^{-/+}$ cells to produce NO from L-arginine remains low whatever cellular state i.e. proliferative or differentiated (21). In contrast, using the colonic adenocarcinoma Caco-2 cells which spontaneously differentiate at late confluency in small intestine enterocyte-like cells, it was found that increased differentiation is associated with decreased expression of the inducible form of NO synthase (iNOS) (23). In HT-29 cells, the basal NO production is low but can be increased by culturing cells in the presence of pro-inflammatory cytokines (24). This result was confirmed in a study using DLD-1 human colon adenocarcinoma cells which showed that pro-inflammatory cytokines increase the capacity of these cells to produce NO (25-27). Interleukin-22 was found to potentiate the expression of iNOS in the human DLD-1 and Caco-2 colonic adenocarcinoma cells (28). Interestingly, HT-29 and Caco-2 cells were characterized by an increased expression of iNOS after infection with enteroinvasive bacteria (29).

3.2. Exogenous sources of polyamines for colonic epithelial cells: the synthesis of polyamines by the microbiota

Polyamines are present in many, but not all, living cells including prokaryotic and eukaryotic micro-organisms (30). Although polyamines were first discovered in animal tissues, micro-organisms have contributed significantly to the elucidation of the biosynthetic pathways and biological effects through the selection of a wide range

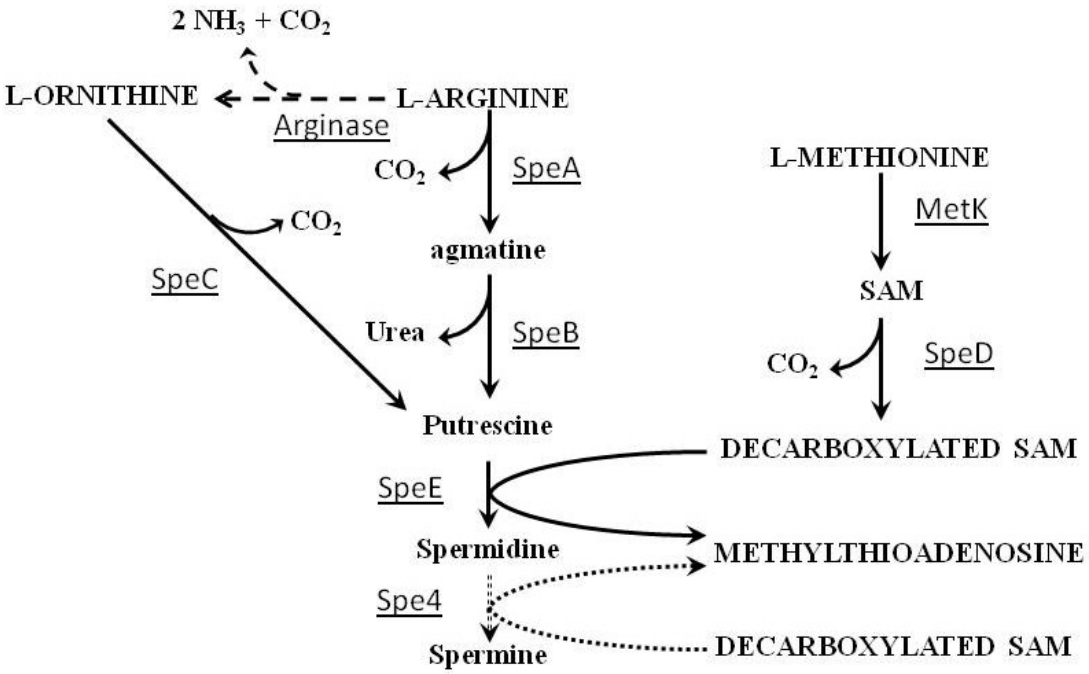

Figure 4. Biosynthetic pathways for polyamine biosynthesis in bacteria. SpeA: arginine decarboxylase; SpeB: agmatine ureohydrolase; SpeC: ornithine decarboxylase; Spe4: spermine synthase; MetK: methionine adenosyltransferase. In *Escherichia coli*, the step from L-arginine to L-ornithine and the step from spermidine to spermine are not present. Spe4 was identified and purified in *Saccharomyces cerevisiae*.

of mutants that either are defective in individual enzymes of the pathways or overproduce these enzymes. Then, comparative studies carried out in animals and micro-organisms found similarities for most of the biosynthetic steps (31).

Putrescine and spermidine are the most common polyamines among the microbial cells while spermine is less widely distributed. Their biosynthetic pathways are well established (Figure 4). In *E. coli* and many other bacteria, putrescine is synthesized by two coexisting routes: (i) the decarboxylation of ornithine to putrescine by ornithine decarboxylase encoded by the *speC* gene, (ii) the decarboxylation of arginine to agmatine by arginine decarboxylase (*speA* gene), agmatine can be subsequently converted to putrescine and urea by agmatine ureohydrolase (*speB*). It should be noted that many bacteria do possess arginase activity which catalyzes the conversion of arginine to ornithine thus enabling putrescine synthesis through arginine by the two distinct pathways described above (31). In *E. coli*, spermidine biosynthesis involves methionine which is converted to S-adenosyl methionine by methionine adenosyltransferase (*metK*). S-adenosyl methionine is then decarboxylated by S-adenosyl methionine decarboxylase (*speD*). This latter compound is implicated as co-substrate for the conversion of putrescine to spermidine through the action of spermidine synthase (*speE*) (32). Spermidine can be converted to spermine by spermine synthase. A highly specific spermine synthase (encoded by *spe4*) was characterized in fungi such as *Saccharomyces cerevisiae*. Although spermine is present in many bacteria, *de novo* synthesis is quite controversial (30, 32, 33). It is not established that bacteria commonly contain a specific spermine synthase. Nevertheless Pegg and Mickael (30) argue that spermine would be synthetized through a non-specific aminopropyltransferase activity using a minor substrate. Aminopropyltransferases (ie spermidine synthase and spermine synthase) are key enzymes for polyamine synthesis. All spermidine synthases derive from a common ancestor that arose prior to the separation between prokaryotes and eukaryotes. Then, the different activities were generated by unusually frequent events of diversification of existing functions (33). Similarly, the general scheme described above (Figure 4) may have slight modifications depending on the type of micro-organism.

Gram-negative bacteria such as *Escherichia coli* contain high concentrations of polyamines. Generally the intracellular contents of spermidine in bacteria are comprised between 1 and 3mM while those of putrescine are comprised between 0.1 and 0.2 mM. However, in *E coli*, putrescine is the predominant polyamine (10-30 mM) followed by spermidine (1-3 mM) while spermine is not synthesized (31, 32). The concentrations of polyamines are markedly affected by culture conditions and many biochemical factors were shown to affect polyamines biosynthesis. The pH of the medium, the amount of aeration, the presence and concentrations of amino acid precursors, the age of the culture and the growth rate are key factors that affect polyamine synthesis. For instance, in *E. coli*, cells harvested in stationary phase have higher

polyamine concentrations than those harvested in mid-logarithmic phase. Cells that accumulate ornithine or arginine contain more polyamines because of genetic or metabolic blocks (31).

Polyamines are very important for bacterial growth (31, 32, 34). Even if deficient mutants that do not contain any polyamine are still able to grow, their growth rate is about only 30% of the rate observed when polyamines are added. Polyamines are fully protonated under physiological conditions and they can interact with a lot of cellular polymers or structures such as nucleic acids and especially RNA, proteins, phospholipids and membranes. Polyamines obviously play an important role in protein synthesis, DNA replication, cell wall stabilization in Gram positive bacteria and outer membrane functions in Gram negative bacteria. Experimental works in microbiology along the ten past years highlight that polyamines play a key role in the establishment of a series of physiological processes such as the response to physiological stress, the biofilm formation and the microbial pathogenesis. Nevertheless high concentrations of spermine are bactericidal. The toxicity of spermine is greater at pH 8.0 than at pH 7.0, probably as a consequence of enhanced import at higher pH.

Drastic changes in polyamine concentrations accompany developmental transitions or stress exposures. The intracellular concentrations are finely regulated by biosynthesis, degradation, uptake and excretion (34). However the regulatory mechanisms are not fully understood. In *E. coli*, the relative degree of putrescine biosynthesis by the arginine decarboxylase (ADC) pathway or the ornithine decarboxylase (ODC) pathway is governed by the respective intracellular concentrations of arginine and ornithine. ODC and ADC are present in two different forms. One is called the biodegradative form and is induced by growth under semi-anaerobic conditions at low pH in rich media containing an excess of amino-acids. In these cultural conditions, biodegradative ODC and ADC forms are not present in all *E. coli* cells tested; but they account respectively for 7 % and 3 % of the proteins contained in crude extracts. The second form is called the biosynthetic form. It is a constitutive form and is present in all *E. coli* cells when they are grown at neutral pH with aeration. Biosynthetic ODC and ADC account respectively for only 0.023 % and 0.07 % of the proteins of the crude extracts (31). ODC and ADC activities are reversibly inhibited by either putrescine or spermidine. Their activity is also reduced reversibly by antizymes whereas ODC activity is enhanced by GTP binding. The transcription of the *speC* gene (ODC) is repressed by cAMP. Spermidine is the main regulator of S-adenosyl methionine decarboxylase through the reduction of its activity and expression (31,32). Most of the biosynthetic enzymes are feedback regulated by the polyamines themselves.

E. coli have several transport systems that allow polyamine uptake. There are two systems that function at neutral pH, one is spermidine preferential and the other is putrescine specific. Additionally, there are two antiporters, one that exchange putrescine for ornithine and one that exchange lysine for cadaverine. There are also uniporters for putrescine and cadaverine. The preferred order of uptake is: putrescine, spermidine, spermine. It is worth to note that the polyamine uptake system is regulated by feedback inhibition by polyamines themselves (32, 34). Both antiporters mentioned above function as uptake proteins for cadaverine and putrescine at neutral pH. At acidic pH they excrete putrescine and cadaverine thus helping neutralizing the external pH. To date, there is only one excretion system functioning at neutral pH that has been evidenced. It excretes spermidine when this polyamine is over-accumulated (35).

During logarithmic growth, there is no obvious metabolism of either putrescine or spermidine. However when cells reach the stationary phase, large amounts of glutathionylspermidine are found. If cultures are stored in the cold, intracellular monoacetylputrescine and monoacetylspermidine are formed. The addition of high concentrations of spermidine or spermine results in the formation of monoacetylspermidine and monoacetylspermine (31).

If we consider colonic bacteria, it appears that many members such as bacteroides, clostridia, bifidobacteria, enterobacteria are known to produce polyamines and for some in large quantities. Haenisch *et al.* (36) have studied the ability of ten representative bacterial species to accumulate agmatine and to release it (by efflux or bacterial lysis) in culture media. Most of the bacteria produced large amounts of agmatine. The culture media of *Bacteroides vulgatus*, *Lactobacillus reuteri*, *Lactobacillus acidophilus* and *Veillonella dispar* had high contents of agmatine. Agmatine release of *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, *Bifidobacterium bifidum* and *E. coli* Nissle was lower but still important. However, little is known about the ability of colonic bacteria to produce polyamines in the physiological conditions of the colonic luminal content. Allison and Macfarlane (37) studied *in vitro* the influence of pH, nutrient availability and growth rate on the production of amines and polyamines by *Bacteroides fragilis* and *Clostridium perfringens* under batch or continuous cultures. The authors observed that the putrescine production of *C. perfringens* was greater at neutral pH than at acidic pH; that it increased with growth rate and lastly that it was repressed by carbohydrates. In this study, putrescine production of *Bacteroides fragilis* was not more than trace quantities. Thus the optimal conditions defined in this study for putrescine production by *Clostridium perfringens* are commonly found in the left colon. *In vivo*, the importance of microbially formed polyamines in the colon is supported by the observation that urinary excretion of cadaverine (exclusively from prokaryotic origin) decreased during antibiotic elimination of bacteria of the digestive tract and increased after restoration of the microbiota (38). More directly, the comparison of polyamine fecal contents of germ-free and conventional rats fed the same diet (39) showed that the high spermidine luminal content of conventional rats is from microbial origin. It is worth to notice that surprisingly putrescine has been found in large amounts in the luminal contents of germ-free rats (4.19 – 3.89 – 2.09 μmol/g dry

weight respectively in the cecal, colonic and fecal contents). According to these results, putrescine can thus be considered as a major endogenous polyamine within the gut lumen. Putrescine concentrations fell to undetectable values in the cecum and colon and to 0.21 µmol/g dry weight in the fecal contents of conventional rats. Noack *et al.* (40) carried out a study with gnotobiotic rats mono-associated with *Bacteroides thetaiotaomicron* or di-associated with *Bacteroides thetaiotaomicron* and *Fusobacterium varium* fed the same diet. They observed that the cecal contents of monoassociated rats contain large amounts of spermidine (1.51 µmol/dry total cecal content) which represents the major polyamine detected whereas the cecal contents of diassociated rats contain even more spermidine (2.53 µmol/dry total cecal content) and also putrescine (3.01 µmol/dry total cecal content) which became in this case the predominant polyamine. Spermine is also detected in such experimental conditions (0.6 µmol/dry total cecal content).

Then, the question that could be raised is how do nutritional factors play a role in the microbial polyamine production processes in the large intestine luminal contents? Studies performed on animal models, companion animals, livestock or human volunteers show that nutritional factors do influence polyamine production. Dietary fibers (indigestible) when added to diets, stimulate bacterial growth and thus increase polyamine concentrations of cecal or colonic contents. When gnotobiotic rats di-associated with *Bacteroides thetaiotaomicron* and *Fusobacterium varium* are fed with a pectin-enriched diet, cecal concentrations of putrescine, spermidine and spermine were respectively increased by 40, 37 and 100% when compared with a pectin-free diet (40). Dietary pectin, guar gum and fructans also modulate microbial polyamine synthesis in the ceca of rats. Guar gum and pectin led to the appearance of cadaverine (8.2 µmol/g dry weight for guar gum) and to elevated putrescine concentrations (for guar gum and pectin respectively 1.35 and 2.27 µmol/g dry weight versus 0.2 µmol/g dry weight for fiber-free diets) in cecal contents (41). The ingestion of oligofructose also leads to an increase of putrescine concentration in the cecal contents of rats (42). On the contrary, the reduction of protein contents at the expense of amino acids supplementation, leads to putrescine and cadaverine decrease in the cecal digesta of early-weaned pigs (43). Noack *et al.* (39) showed in conventional rats fed a polyamine-deficient or a polyamine rich diet that microbial polyamine synthesis is inversely related to alimentary polyamine intake. The few studies enrolling healthy human volunteers show that the consumption of fructo-oligosaccharides or lactic acid bacteria (i.e. *Bifidobacterium lactis*) is concomitant with the increase of polyamine concentrations in fecal samples (44-46). Lyophilized preparations of the biotherapeutic yeast *Saccharomyces boulardii* contain polyamines, mainly spermidine (3.8 nmol/mg lyophilized powder) and spermine (2.9 nmol/mg lyophilized powder) that are released in the intraluminal fluids. According to the authors this latter process could be, at least in part, at the origin of a number of the trophic effects of *S. boulardii* preparations (47).

One can then draw the conclusion that microbial polyamine production in the colon must be taken into account as one important exogenous source for colonic epithelial cell supply. Nevertheless, the overlook of the specialized literature shows that parameters such as physiological and biochemical conditions, composition of the microbiota and nutritional factors are implicated in the overall process of polyamine microbial metabolism. Further studies are then needed to properly identify the factors involved as well as the interactions between these factors.

3.3. Uptake, metabolism and release of polyamines by normal and cancerous colonic epithelial cells

Surprisingly, little information is available on the capacity of normal colon epithelial cells to import polyamines from the extracellular medium. Since polyamines are present at very low concentrations in the blood plasma (less than 1 micromolar in humans, 48,49); it can be presumed that the main source of polyamines for colonic epithelial cells is the unbound polyamines present in the large intestine lumen. In rats, micromolar concentrations of putrescine, spermidine and spermine have been measured in the colonic lumen (50.). Since polyamines present in the small intestine lumen appears to be efficiently absorbed (51-53), it can be proposed that polyamines in the large intestine lumen are originating from the metabolic activity of the microbiota and from the intracellular contents of mature colonocytes shed into the lumen. Experiments in the rat model suggest that luminal polyamines can be taken up by colonocytes (54). The presence of acetyl-spermidine and acetyl-spermine in human colonocytes suggests that these cells are able to convert spermidine in putrescine and spermine in spermidine (19). The metabolism of polyamines has been much more extensively studied in cancerous colonic epithelial cells than in normal colonocytes. HT-29 $Glc^{-/+}$ cells have the capacity to accumulate exogenous putrescine, spermidine and spermine with undetectable catabolism of these compounds (21). After accumulation of radioactive putrescine and spermidine, HT-29 $Glc^{-/+}$ cells display capacity for the release of radioactive compounds in the extracellular medium although it was not determined in this study if polyamines were released from cells in their acetylated form. Experimental works with HT-29 cells have shown that spermidine excretion represents a way to regulate polyamine intracellular concentrations (55). The coordinated capacities of cells originating from human colonic adenocarcinoma to synthesize, import and export polyamines represent important parameters for determining intracellular polyamine concentrations (56). Using differentiated Caco-2 cells, Barlier *et al.* (57) have shown putrescine transport and excretion in the apical-to-basolateral direction. Using LoVo human colon adenocarcinoma cells, it was shown that these cells can accumulate putrescine from both the apical and basolateral membranes (58). In Caco-2 cells undergoing culture medium replacement in order to stimulate cell proliferation, polyamine endogenous synthesis and polyamine uptake were increased (59) suggesting that both processes are involved in the supply of polyamines to proliferating cells.

3.4. Synthesis of polyamines and cancerous colonic epithelial cell growth

The capacity of colonic adenocarcinoma cells to synthesize polyamines is strictly necessary for cell proliferation since inhibition of the conversion of L-ornithine to polyamines inhibits drastically cell proliferation (60-63). Addition of putrescine or spermidine in the culture medium in addition to ODC inhibitor reverses almost totally the anti-proliferative effect of ODC inhibition (60-63) indicating that exogenous putrescine and/or spermidine through entry into adenocarcinoma cells can substitute for endogenous production. Endogenous production of polyamines in Caco-2 cells appears to be more closely associated with cell proliferation than with cell differentiation (64). Using isolated nuclei from the human colon carcinoma cell line COLO 320, it has been shown that spermidine can increase the transcription of growth-associated genes (65). Furthermore, inhibition of endogenous polyamine synthesis is associated with altered cell cycle phase distributions (62) in HT-29 cells suggesting that adequate intracellular polyamine content is required for cell cycle progression. This result can not be however extrapolated to all cancerous cells originating from colon adenocarcinoma since in Caco-2 cells in which polyamine endogenous synthesis is inhibited , there is a proportional slowdown in all cell cycle phases (63).

3.5. Exogenous nitric oxide exerts anti-proliferative effect on cancerous colonic epithelial cell growth

Various nitric oxide donors have been tested for their effects on the proliferation of colonic epithelial cell originating from human colonic adenocarcinoma. The NO-donor sodium nitroprusside (SNP) at a concentration as low as 1 micromolar was able to almost abolish the growth of HT-29 Glc-/+ colonic cancerous cells (66). This effect was largely reverted by the NO trap haemoglobin demonstrating that the release of NO from SNP is mainly responsible for the growth-inhibitory effect of this compound. Although the effect of SNP was not associated with short term effect on cell metabolism (i.e. protein synthesis and cellular oxidation of L-glutamine), SNP was found to increase HT-29 cell necrosis after long term treatment (66). In this cell model, it has been reported that the sensitivity of these cancerous cells to NO donors is dependent on the position of cells in the cell cycle (67). In DLD-1 human colon adenocarcinoma cells engineered to produce relatively high level of nitric oxide continuously, it was found that these cells grew more slowly than the wild-type parental cells reinforcing the view that NO acts as a modulator of colon cancer growth *in vitro* (68). In these DLD-1 cells, the induction of the expression of the inducible isoform of NO synthase was found efficient for the inhibition of cell proliferation and for the induction of apoptosis (69). The endogenous weak production of nitric oxide from L-arginine was however apparently not involved in the control of HT-29 $Glc^{-/+}$ proliferation at late confluency since inhibition of this production has no effect on the cellular growth (70). Using Caco-2BBe monolayers, Salzman *et al.* reported that NO donors used at high millimolar concentrations deplete intracellular ATP content (71); a depletion that is likely to cause cell necrosis. Interestingly, it was found that endogenous NO production or exogenous NO is necessary for the chemotherapeutic drug doxorubicin to exert its cytotoxic effect on HT-29 cells (72). The NO-donor glyceryl trinitrate was found to induce apoptosis in human colon cancer cells by a mechanism which imply caspase cascade and down regulation of beta-catenin i.e. a protein which is involved in colonic cell malignant transformation in case of inappropriate activation (73,74). The pro-apoptotic effect of NO is likely due –in part at least- to its capacity to react with superoxide to form peroxynitrite ($ONOO^-$) (75). Indeed, peroxynitrite is able to induce apoptosis in HT-29 cells (76). Le Goffe *et al.* (77) have demonstrated that when HT-29-Cl.16 E were incubated with the NO donor PAPANONOate, NO exerted cytotoxic effect on these cells followed by apoptosis associated with peroxynitrite formation, intracellular glutathione depletion and nitrotyrosine formation. In this latter study, it was shown that increasing the availability of reducing equivalents formed in the pentose phosphate pathway, it was possible to maintain the intracellular concentration of glutathione and to protect cells from the effect of the NO donor. Ho *et al.* (78) have shown that the antioxidant N-acetyl-L-cysteine can protect the cancerous human colonic cells COLO 205 from apoptosis induced by gaseous NO. Williams *et al.* (79) have tested NO-releasing nonsteroidal anti-inflammatory drugs (NSAIDs) for their inhibiting effects on the growth of HT-29 cells, and have found a better effectiveness than the one measured with traditional NSAIDs. More recently, it has been demonstrated that the NO-donating aspirin compound NO-ASA inhibits the growth of SW-480, HT-29, HCT-15, LoVo and HCT-116 human colonic cancerous cells and the binding of NF-kappa B to DNA leading the authors to propose that the growth inhibitory effect of NO-ASA on colonic cancerous cells may be due to inhibition of NF-kappa B activation (80).

3.6. Effects of nitric oxide on ornithine decarboxylase catalytic activity: the channelling hypothesis

Nitric oxide is a strong inhibitor of ODC activity. Indeed, at one micromolar concentration, sodium nitroprusside is able to markedly inhibit ornithine decarboxylase activity in HT-29 $Glc^{-/+}$ cells (66). This inhibition which is depending on the dose of SNP can be partly reverted by the NO trap haemoglobin indicating that NO release from SNP plays a role for the inhibition of ODC activity. Other NO donors like MAHMA/NO and DEA/NO were found to be effective for ODC activity inhibition in colonic cancerous epithelial cells although these latter compounds were less potent that SNP (81). Using the human colon carcinoma cell line Caco-2, Buga *et al.* (82) have shown that N^G-hydroxy-L-arginine (NOHA i.e. the main intermediate in the synthesis of NO from L-arginine) is an inhibitor of Caco-2 cell proliferation likely (in part at least) through the inhibition of arginase activity. They have confirmed that NO causes cell cytostasis through inhibition of ODC activity. Since arginase and ODC are the two metabolic steps involved in polyamine synthesis from L-arginine, these results have strongly reinforced the idea that the metabolism of L-arginine in the NO synthase pathway may be involved in the control of colon cancerous epithelial cell growth (see Figure 5).

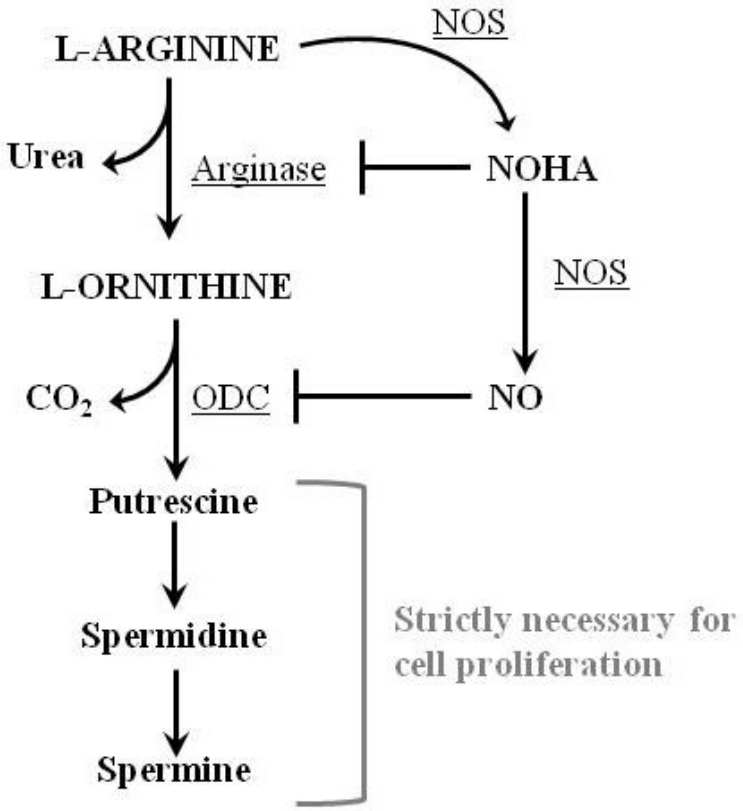

Figure 5. The L-arginine channelling hypothesis. L-ARG: L-arginine; L-ORN: L-ornithine; NOHA: N^G-hydroxyl-L-arginine; NO: nitric oxide; ODC: ornithine decarboxylase; NOS: nitric oxide synthase.

Further works in the late 1990 have documented that NO inhibits ODC activity by S-nitrosylation (83). The residue of ODC protein which is S-nitrosylated was identified as the cysteine 360 in the active site of the enzyme (84). Furthermore, cysteine 82 in S-adenosylmethionine decarboxylase (i.e. the enzyme which allows the synthesis of decarboxylated-S-adenosylmethionine involved in spermidine and spermine synthesis) reacts also with NO. Thus, NO inactivates this enzyme which is also involved in polyamine biosynthesis (85). Much interestingly, the cysteine 360 residue on ODC protein which was S-nitrosylated by NO was found to be the same residue that the one which forms covalent adducts with the ODC inhibitor alpha-difluoromethylornithine DFMO (86); reinforcing the view that cysteine 360 in its reduced form is mandatory for ODC full catalytic activity and explaining why the presence of thiol-reducing agents in the assay buffer are necessary for the measurement of ODC activity in biological samples.

Further works are needed to determine in which experimental condition the endogenous production of NO from arginine would be sufficient for efficient inhibition of polyamine endogenous synthesis from either arginine (through ornithine synthesis) or directly from ornithine in colonic preneoplasic and neoplasic lesions. Also, the use of NO-donating compounds administered via the luminal route may represent a valuable strategy for inhibiting endogenous polyamine synthesis in such lesions as long as no major deleterious effects of NO are observed on the healthy mucosa.

3.7. Metabolism of agmatine in normal and cancerous colonic epithelial cells

Agmatine is another polyamine which can be produced from L-arginine through the activity of arginine decarboxylase in bacteria and in plants. Cloning of arginine decarboxylase in humans has been reported (87) and there is ample evidence for the presence of agmatine in mammalian tissues (88). However, the metabolic capacity of mammalian cells for agmatine biosynthesis remains much controversial (89). Although agmatine was found at concentration between 130 and 360 micromolar concentrations in the segments of rat large intestine (90), there is no information on agmatine uptake by normal colonic epithelial cells. In contrast, several research groups have examined metabolism of agmatine in cancerous colon epithelial cells (Figure 6). In HT-29 $Glc^{-/+}$ cells, agmatine accumulate six fold inside cells without binding to any significant extent to the cell surface (91). These cells have no metabolic capacity for agmatine synthesis from L-arginine and do not degrade agmatine through agmatinase (91). This capacity for agmatine accumulation appears to be a general property of colon carcinoma cells since six human cancerous colonic epithelial cell lines exhibit capacity for such an uptake (92).

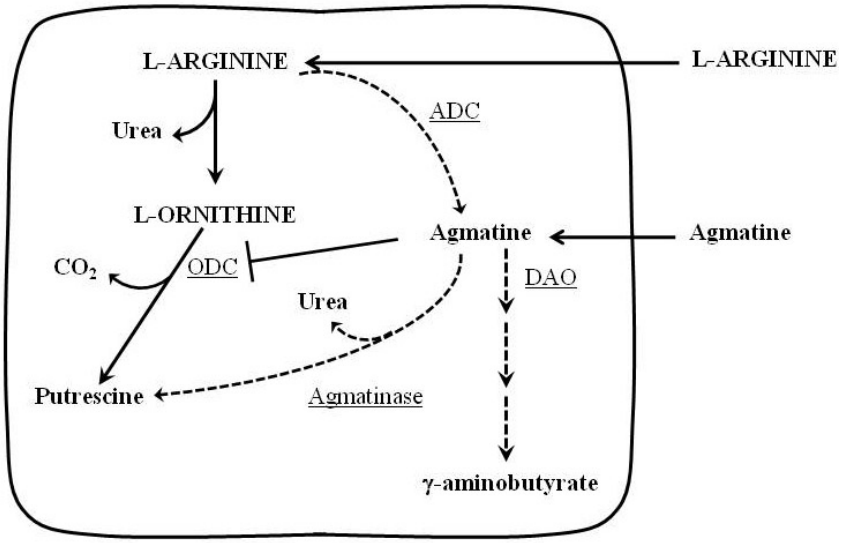

Figure 6. Schematic view of the metabolism of agmatine in proliferative cancerous colonic epithelial cells (HT-29 cell line). L-ARG: L-arginine; L-ORN: L-ornithine; ADC: arginine decarboxylase; DAO: diamine oxidase; ODC: ornithine decarboxylase. Dotted lines indicate quantitatively minor metabolic pathways.

3.8. Effects of agmatine on the growth of cancerous colonic epithelial cells

Agmatine used at concentrations between 1 and 5 mM dose-dependently inhibits HT-29 $Glc^{-/+}$ and Caco-2 colonic adenocarcinoma cell proliferation (91). Agmatine was found to decrease the rate of L-ornithine decarboxylation and to induce a 70% down-regulation of ODC expression. In addition, agmatine caused a marked decrease of putrescine and spermidine HT-29 $Glc^{-/+}$ cell content and an increase in the N_1-acetylspermidine level. Agmatine is able to cause an accumulation of these cancerous cells in the S and G2/M phase of the cell cycle as well as reducing the DNA synthesis (91). Agmatine appears to inhibit the growth of all human colonic cancerous epithelial cells tested since this bacterial metabolite inhibited Colo 205E, Cx1, Colo 320, HT-29, Caco-2 and SW 480 cell growth with IC_{50} values (i.e. concentration inhibiting the cell proliferation by 50%) ranging from 0.6 to 5 mM (93).

4. CONCLUSION

Colonic epithelial cells can import L-arginine from blood and metabolize this amino acid in the NO and polyamine pathways. Polyamines which are much more produced by cancerous colonic epithelial cells than by normal colonocytes are strictly necessary for cell proliferation. Polyamines can be supplied to colonic epithelial cells by endogenous synthesis and/or imported from polyamines present in the lumen, a part of it being likely produced by the microbiota metabolic activity. In cancerous colonic epithelial cells, nitric oxide appears to represent a strong inhibitor of polyamine synthesis and of cell proliferation raising the view that NO and polyamines (endogenously produced and/or exogenously supplied) can modulate proliferation of these cells. In addition, exogenous agmatine which is produced by the intestinal microbiota from L-arginine and which exerts strong antimitotic effect against colonic cancerous epithelial cell growth is able to accumulate inside these cells and to strongly reduce ornithine decarboxylase activity and polyamine endogenous synthesis. It therefore appears that the metabolism of L-arginine by colonic epithelial cells and by the microbiota leads to the production of metabolites with marked opposite effects on epithelial cell growth. Since most of these experiments were done using an *in vitro* experimental design, additional works are required in order to test in *in vivo* experimental studies the efficiency of treatments which will associate compounds with inhibiting effects upon polyamine endogenous synthesis together with decreased supply of polyamines from dietary and microbiota origins. This is likely to represent an efficient strategy for polyamine depletion in preneoplasic or neoplasic colonic lesions and thus reduction of their proliferative capacity. In that respect, nitric oxide (or nitric oxide-donating agents) supplied luminally together with agmatine may represent a good association for the inhibition of ornithine decarboxylase activity and thus polyamine endogenous synthesis in such lesions.

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