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Abstract

Among bacterial metabolites, hydrogen sulfide (H_2S) has received increasing attention. The epithelial cells of the large intestine are exposed to two sources of H_2S. The main one is the luminal source that results from specific bacteria metabolic activity towards sulfur-containing substrates. The other source in colonocytes is from the intracellular production mainly through cystathionine beta-synthase (CBS) activity. H_2S is oxidized by the mitochondrial sulfide oxidation unit, resulting in ATP synthesis, and thus establishing this compound as the first mineral energy substrate in colonocytes. However, when the intracellular H_2S concentration exceeds the colonocyte capacity for its oxidation, it inhibits the mitochondrial respiratory chain, thus affecting energy metabolism. Higher luminal H_2S concentration affects the integrity of the mucus layer and displays pro-inflammatory effects. However, a low/minimal amount of endogenous H_2S exerts an anti-inflammatory effect on the colon mucosa pointing out the ambivalent effect of H_2S depending on its intracellular concentration. Regarding colorectal carcinogenesis, forced CBS expression in late adenoma-like colonocytes increased their proliferative activity, bioenergetics capacity, and tumorigenicity; while genetic ablation of CBS in mice resulted in a reduced number of mutagen-induced aberrant crypt foci. Activation of endogenous H_2S production and low H_2S extracellular concentration enhance cancerous colorectal cells proliferation. Higher exogenous H_2S concentrations markedly reduce mitochondrial ATP synthesis and proliferative capacity in cancerous cells, enhance glycolysis, but do not affect their ATP cell content nor viability. Thus, it appears that, notably through an effect on colonocyte energy metabolism, endogenous and microbiota-derived H_2S are involved in the host intestinal physiology and physiopathology.

Key words: hydrogen sulfide, energy metabolism, inflammatory bowel diseases, colorectal carcinogenesis

Introduction

The intestinal microbiota in different physiological and pathophysiological situations is characterized by its composition, diversity, and metabolic capacities (93). Regarding the catabolic side of the metabolic activities, the microbial species can produce a variety of compounds by degrading undigested (or not fully digested) alimentary and endogenous substrates (11). Among the substrates used by bacteria for catabolism, the undigested dietary and endogenous proteins are degraded through the protease and peptidase activities releasing amino acids (75). The amino acids are not absorbed to any significant extent by the colonic epithelium except in the neonatal period (90). They can then be used by the intestinal bacteria for their own protein synthesis, and in other various metabolic pathways leading to the production of numerous metabolic end products that are released in the large intestine luminal content (23).

Among these bacterial metabolites, H_2S has received increasing attention in the last decades as a compound that was initially considered as a toxic substance when present in excess in the environment (73), while acting as a gaseous mediator involved in numerous physiological functions when synthesized endogenously (37). The aim of this review is to present an overview of the experimental and clinical evidence that allow to consider H_2S as an important player in the communication between some bacterial species in the intestinal microbiota and the large intestine epithelial cells in different physiological and pathophysiological situations.

Production of luminal H_2S by the intestinal bacteria

H_2S is produced by different bacterial species from both dietary and endogenous compounds of organic and inorganic nature (13) (Figure 1). Recent data suggest that H_2S is mainly produced through cysteine catabolism, and to a lower extent by sulfate reducing bacteria (SRB) (36). Cysteine catabolic bacteria include *Fusobacterium*, *Clostridium*, *Escherichia*, *Salmonella*, *Klebsiella*, *Streptococcus*, *Desulfovibrio*, and *Enterobacter* which convert cysteine to H_2S, pyruvate, and ammonia by the enzymatic activity of cysteine desulfhydrase (8). SRB includes *Desulfovibrio*, *Desulfobacter*, *Desulfobulbus*, and *Desulfotomaculum*. *Desulfovibrio* is the dominant genera of SRB, and it includes *D.piger* and *D.desulfuricans* (72). The numerous microbes belonging to SRB possess the capacity to reduce several compounds including sulfate, sulfite, thiosulfate, elemental sulfur, and several thionates (13). The capacity of some

bacteria to cope with surrounding H_2S may be related to some specific metabolic characteristics. For instance in *Escherichia Coli*, cytochrome bd-type O_2-oxidases that are relatively insensitive to sulfide allow bacterial oxygen consumption and growth in the presence of this compound (30).

The concentrations of sulfide measured in the colon luminal content and in the feces are rather variable depending on the processing of the biological materials and on the technique used for the assay. Some techniques are specific for sulfide detection, like for instance the targeted GC-MS measurement (41); while others are rather unspecific and detect other sulfur-containing compounds. Thus, concentrations of sulfide measured in the large intestine content and in faecal material (that latter representing an approximation of the concentration in the rectum) in mammals ranges from 0.2 mM up to 2.0 mM (13). This order of magnitude difference, apart from the specificity of the techniques used for measurement, may also be explained by the fact that the luminal concentration of sulfide in the large intestine is dependent on the dietary status of the individuals. For instance, the consumption of a diet with a high protein content, where some dietary and endogenous proteins escape digestion in the small intestine (26) results in an increased amount of sulfide in the colon luminal content, and in the fecal material, when compared with the situation of lower protein intake (9, 59). Sulfide in the luminal content can exist in 3 forms: the H_2S gas that is partly dissolved in the aqueous phase, and is highly diffusible, hydrosulfide anion HS^-, and sulfide ion S^{2-}, this latter being present at a negligible level. Indeed, in aqueous phase, H_2S dissociate in HS^- and $S^{2-}$and H^+ with pKa values being 7.04 and 11.96 respectively (13). In healthy subjects, the pH at the colonic mucosal surface ranges between 7.2 and 7.5 in the descending colon and rectum (14). Thus, considering a pH of 7.4, approximately one third of sulfide is in the form of H_2S at equilibrium with two third being hydrosulfide anion. However, when the luminal pH is more acidic, the H_2S/HS^- ratio in the large intestine increases. Taking into account that H_2S, unlike HS^- easily penetrates biological membranes (14), lower pH will increase H_2S concentration, then amplifying its entry in the colonic epithelial cells. Lastly, measurement in human faeces showed that 8% of sulfide are in unbound form (45), representing a concentration around 60 µM, while measurement in the rat caecal content suggests that 0.2% of total luminal sulfide is in unbound form (55). Several compounds from dietary origin that are not fully absorbed by the small intestine have been shown to bind H_2S. These compounds include zinc (83), heme (43), and polyphenols (1).

Incidentally, by comparing both free H_2S and bound sulfane sulfur concentrations in blood plasma recovered from conventional and germ-free rodents, it was found that these concentrations are markedly lower in animals with no intestinal microbiota, thus suggesting that the circulating H_2S is largely originating from the microbiota metabolic activity (79). This result is in accordance with the fact that fecal H_2S synthesis in germ-free mice represents approximately half of that observed in colonized mice (28).

H_2S and the absorbing colonic epithelial cells

The colonic epithelium is a monolayer of cells that make the border between the outer and the inner media. This epithelium is structurally organized as colonic crypts and surface epithelium. This structure that is continuously renewed within few days, contains stem cells located at the crypt bottom, and differentiated cells with specialized functions including absorbing colonocytes, enteroendocrine cells, goblet cells, and Tuft cells (64, 89). These different cell phenotypes are polarized with a luminal side being protected by mucous layers that face the luminal content (44), and a baso-lateral side in close contact with the basal lamina, lamina propria and the capillaries (95). The fully mature colonocytes are finally exfoliated into the luminal content mainly by apoptosis (78). The renewal of the colonic epithelium is depending on a coordinated sequence of events that allow to maintain the epithelium homeostasis (24).

The absorbing colon epithelial cells, often referred as colonocytes, are responsible for water absorption, and electrolyte absorption and secretion (6). These cells are characterized by relatively high energy requirement due to intense anabolism and the catalytic activity of the Na/K ATPase involved in sodium absorption (12). The colonocytes are equipped with the so-called sulfide-oxidizing unit (SOU), a mitochondrial multi-enzymatic system responsible for the oxidation of H_2S (Figure 2). When the extracellular concentration of NaHS, a rapid H_2S donor, is below a threshold value, approximately equal to 50 µM, the colonocytes are able to oxidize sulfide to thiosulfate in three steps (33) allowing the production of ATP (16). The first enzyme of the SOU is sulfide quinone reductase (SQR) that injects electrons into the mitochondrial respiratory chain through quinone before transfer to complex III cytochrome c, and finally reduction of oxygen to water at the level of complex IV (cytochrome oxidase). The persulfide formed in the first step of SOU activity is oxidized by the sulfur dioxygenase ETHE1 to sulfite. Disruption of the mitochondrial ETHE1 in mice is lethal 5 weeks after birth, indicating sulfide

toxicity, and vital importance of ETHE1 for sulfide disposal (85). Finally, the sulfur transferase rhodanese converts sulfite into the metabolic end product thiosulfate (Figure 2) (56). Sulfide oxidation pathway is mainly localized apically in human colonic crypts (58). When the expression of the genes coding for the 3 enzymes of the SOU was measured in human biopsies recovered from different anatomical parts of the large intestine, the rhodanese was found to be much less expressed in the rectum than in more proximal parts (that are caecum, ascending, transverse and descending colon), suggesting a lower capacity for sulfide disposal in the rectum (60).

Thus, H_2S can be considered as an inorganic fuel for colonocytes in addition to the usual main organic energy substrates provided through the luminal and baso-lateral sides, and used by colonocytes (e.g. short-chain fatty acids at the luminal side, and glutamine, and glucose at the baso-lateral side). H_2S in fact represents the first inorganic energy substrate for mammalian cells (34). However, in *in vitro* studies, when the extracellular concentration of NaHS is above 50 µM, an inhibition of the colonocyte respiration is observed (9). This is due to an inhibition of the catalytic activity of the cytochrome c oxidase, one of the complex in the mitochondrial respiratory chain (complex IV) (65) (Figure 2). In addition, in isolated human colonocytes, H_2S inhibits butyrate oxidation (5). Thus overall, the capacity of colonocytes to oxidize limited amount of sulfide in mitochondria appears to represent a way to detoxify this compound while recovering energy from it. Such a capacity can be altered by other gaseous mediator like nitric oxide (NO). Indeed, the NO donor MAMA NONOate, which markedly inhibits oxygen consumption in rat colonocytes by inhibiting mitochondrial cytochrome c oxidase activity (9, 22), prevents H_2S detoxification in colonocytes.

Several processes have been described regarding the adaptive capacity of colonocytes to face an increased concentration of H_2S in the extracellular medium. By feeding rodents with high-protein diet, and thus by increasing the cysteine availability for H_2S production by the large intestine microbiota, an increased content of H_2S was recorded as expected in the caecal and colonic luminal fluids, and this increased concentration coincided with an increased expression in colonocytes of the *Sqr* gene that is coding for SQR, the first enzyme in SOU (9).

However, it is worth noting that colonocytes are not only exposed to H_2S from their luminal side, but can also synthesize this mediator intracellularly from

cysteine and other co-substrates. The major pathway in colonocytes, appears to be through the activity of cystathionine beta-synthase (CBS), a versatile enzyme that is able to convert cysteine to H_2S and serine, but also 2 molecules of cysteine into H_2S and lanthionine, and cysteine and homocysteine into H_2S and cystathionine (36). NO has been shown to interfere with H_2S endogenous synthesis through the binding to CBS heme, a process that results in inhibition of CBS catalytic activity (33, 91). This indicates that the cellular metabolism of H_2S may depend on the local concentration of NO.

Thus, the intracellular H_2S concentration in colonocytes depends on the diffusion of luminal H_2S through the brush-border membrane, on the intracellular synthesis from cysteine, and on the capacity of colonocytes for its oxidation.

H_2S and the enteroendocrine cells

Among intestinal epithelial cells, enteroendocrine cells (EECs) secrete gut hormones that regulate many functions in the host, including food intake, insulin secretion or intestinal motility and epithelial barrier (35). EECs express a high number of receptors and channels that enable them to secrete hormones in response to different stimuli. Secretion is highly stimulated in the small intestine by nutrients arriving in the lumen of the gut after food intake, but many other compounds, including the ones present in the bloodstream, have been shown to stimulate gut hormone release. Moreover, different EEC subtypes differ by their hormonal production, but also according to the receptors they express, enabling a fine control of the different hormone secretion (10). EECs in the colon have also been shown to be involved in the regulation of metabolic functions despite not being directly stimulated by nutrients (57, 82), but rather by microbial derived compounds (3, 52, 86).

However, little is known on the impact of H_2S on EEC physiology or secretion in the large intestine. In GluTag cells, a murine colonic L-cell model, Pichette and collaborators have showed that slow- and rapid-releasing sulfide donors dose-dependently increased GLP-1 secretion, with an effect dependent on p38 MAPK signalling (70). Interestingly, in this study, the authors by using the prebiotic chondroitin sulfate, that increases the abundance of the SRB *D-piger* and sulfate moiety in the distal intestine, measured an increased H_2S production. Such increased production was associated with metabolic improvements through increased GLP-1 secretion during oral glucose tolerance tests.

Using cells originating from a more proximal region of the gastrointestinal tract, Slade and collaborators showed that the slow-releasing sulfide donor GYY4137 dose-dependently reduced the secretion of ghrelin in rat gastric primary culture. Ghrelin represents the main gut orexigenic hormone that regulates adiposity and gastrointestinal motility. Its secretion pattern is opposite to most gut hormones, as its secretion is inhibited by food intake. As ghrelin producing cells are present in the stomach, it is likely that the main source of H_2S there is through endogenous production. In fact, these cells express high level of cystathionine-gamma lyase (CSE), one of the enzymes involved in H_2S endogenous production (81). Inhibition of endogenous H_2S production increased ghrelin secretion. Similar inhibitory effects of H_2S on EEC secretion has been described by Bala and collaborators (7). Sulfide donors at high concentration strongly inhibited glucagon-like peptide 1 (GLP-1) and peptide-YY (PYY) secretion induced by Takeda-G-protein-receptor-5 (TGR5) stimulation in STC-1 cells, a cellular EEC model of murine small intestine that are poorly differentiated (7). Interestingly, L-cysteine, which increases H_2S endogenous production, also inhibited GLP-1 and PYY stimulated secretion.

H_2S and colonic inflammation

Chronic inflammatory bowel diseases (IBD), mainly Crohn's disease and ulcerative colitis (UC) are characterized by alternating episodes of remission and relapse, resulting from inappropriate mucosal immune responses against luminal intestinal components in genetically predisposed individuals (48). Although Crohn's disease and UC have different clinical features (67), the inflammation of the intestinal mucosa is often observed in the distal parts of the intestine (32). Etiology of IBD, although still largely elusive, is related to genetic (50), and environmental factors including the ones from dietary origin (92). The concept that excessive concentration of H_2S in the intestinal luminal content, notably in a context of diminished capacity for sulfide disposal in the mucosa (2), may participate in the etiology of mucosal inflammation has been proposed more than 2 decades ago (54). Severity of mucosal inflammation in the colon and rectum of patients with active UC is believed to be related to the concentration ratio of deleterious vs. beneficial bacterial metabolites. This ratio is notably depending on the substrate availability for production of these metabolites. Following for one year UC patients in remission, it was found that patients with high consumption of meat, dietary protein and sulfur/sulfate experienced an increased rate of relapse when compared with patients with low consumption of these compounds (46). This indicates that increased intake

of dietary substrates known to be used by the intestinal microbiota for H_2S synthesis, is associated with increased risk of relapse. In anesthesized rats, after preliminary evacuation of the luminal content, intracolonic instillation of NaHS used for 1 hour at concentrations between 0.5 and 1.5 mM, increased in colonocytes the expression of inflammation-related genes, namely those coding for the inducible form of nitric oxide synthase (iNOS), and interleukin-6 (9). Overexpression of iNOS is measured in colonic samples originating from patients with UC (80), and NO production in excess, notably through increased peroxynitrite formation, appears to play a role in the process of mucosal inflammation.

Ijssennagger and collaborators have shown that microbiota-derived H_2S at high concentration destabilizes the protective mucus layer covering the intestinal epithelium through the reduction of disulfide bonds linking the mucin 2 network (42) (Figure 3), a process that would increase the interactions between bacteria and the epithelium. Mottawea and coworkers have studied the microbiota composition of new-onset pediatric Crohn's disease patients, and found that this microbiota is characterized by high abundance of *Atopobium*, *Fusobacterium*, *Veillonella*, *Prevotella*, *Streptococcus*, and *Leptotrichia*, several members of those genera being known to produce H_2S through the catabolism of sulfur-containing amino acids (63). Importantly, in this cohort, the abundance of H_2S producers from cysteine was correlated with the severity of mucosal inflammation. To document the possible causal link between H_2S production and intestinal inflammation, the authors colonized Interleukin-$10^{-/-}$ mice with the H_2S producer *Atopobium parvulum* and measured a worsening of colitis, such a deleterious effect being attenuated by the H_2S scavenger bismuth. In addition, in the same Crohn's disease pediatric cohort, the colonic mucosa biopsies displayed decreased expression of the mitochondrial enzymes involved in H_2S detoxification. Overall, the results from this study strongly suggest that diminished capacity for sulfide disposal in the colonic mucosa of pediatric Crohn's disease patients amplify the pro-inflammatory effect of H_2S overproduction by the intestinal microbiota (Figure 3).

Thus, evidence from both experimental and clinical studies have highlighted that high H_2S concentrations, i.e. above the capacity of the intestinal mucosa to detoxify this bacterial metabolite, increase the risk of inflammation. However, it appears from experimental studies using pharmacological inhibitors of H_2S synthesis or H_2S-releasing compounds, that a minimal amount of sulfide is likely necessary to limit the risk of colonic mucosal inflammation. Then, the concept

that H_2S is a double-edge sword for the intestinal epithelium has been proposed (15). Indeed, several *in vivo* studies found that inhibition of H_2S endogenous synthesis favors intestinal inflammation and delays colitis resolution (40, 94). The capacity for H_2S production was markedly elevated after colitis induction in rat, and inhibition of H_2S synthesis in colon exacerbated the colitis. Interestingly, H_2S has been shown to exert some antioxidant activity, notably by persulfidation of cysteine residues that protects these residues from oxidative damage (33). This action may contribute to the anti-inflammatory effect of low production of H_2S in colonocytes. Regarding colitis resolution, H_2S-releasing compounds have been shown to promote this process through Hypoxia-Inducible factor-1alpha signaling pathway (29) Endogenous synthesis of H_2S was found to offer some protection against dextran sodium sulfate (DSS)-induced colitis in rodents partly by inhibiting the activation of NLRP3 inflammasome pathway (71). Also, the slow-releasing sulfide donor GYY4137 was shown to reduce *in vitro* the lipopolysaccharide or TNF-alpha/IFN-gamma induced increased permeability in colonocyte monolayer (21), and the intraperitoneal injection of GYY4137 in endotoxemic animals protects against intestinal barrier dysfunction in vivo (100). In addition, this H_2S donor improves mesenteric perfusion and intestinal injury in an experimental model of necrotizing enterocolitis, and these beneficial effects appear to be mediated through constitutive endothelial nitric oxide synthase (e-NOS)-dependent pathways (25). Lastly, endogenous production of H_2S appears to contribute to mucus production thus favoring segregation between luminal bacteria and the intestinal mucosa (62). From the different available results, it appears that the effects of the diffusible compound H_2S on the inflammatory process in the intestinal mucosa depend both on the exogenous production by the microbiota, and on the endogenous production by the host colonic tissue.

H_2S and colon carcinogenesis

Some recent experimental and clinical studies suggest that both endogenous synthesis of H_2S in colonic epithelial cells and luminal exogenous H_2S are implicated in the process of colorectal carcinogenesis. Gene and protein expression has revealed that the H_2S-synthesizing enzyme CBS is increased in colonic tumors when compared with the surrounding mucosa (66, 84), suggesting an increased capacity for H_2S synthesis in the cancerous samples. However, since colonic tumors and surrounding tissues contained numerous different cell phenotypes, it would be important to determine the expression of

this enzyme in colonic epithelial cells and other cell types. Upregulation of CBS in human biopsies of precancerous adenomatous polyps has been measured, and forced upregulation of CBS in late adenoma-like colonic epithelial cells was associated with differences in the expression of genes involved in colorectal cancer development, notably genes involved in NFkappa B, KRAS, and p53 signaling (69). Also these cells overexpressing CBS were characterized by increased proliferative capacity, and enhanced cellular bioenergetics capacity in terms of respiration and glycolysis. This increased CBS expression reinforced cell tumorigenicity in athymic nude mice. Of equal importance, genetic ablation of CBS in mice resulted in a reduction of the number of mutagen-induced aberrant crypt foci (69). Thus, increased endogenous production of H_2S within adenomatous colonocytes increases their proliferative capacity (Figure 4) and their ability to promote tumor formation.

Increased supply of H_2S produced by the intestinal microbiota has been implicated in the process of colorectal carcinogenesis. Indeed, by collecting feces and tissues samples collected on and off the tumor site in the same individuals, and by using multiomic data and community metabolic models to assess H_2S production in colorectal cancer, Hale et al. obtained data indicating an increased H_2S production by the gut bacteria in colonic cancer samples compared to non- cancerous samples (38). The predicted increased H_2S production at the tumor site was associated with the relative abundance of *Fusobacterium nucleatum*, a known H_2S producer largely suspected to promote colorectal cancer (18, 51, 74, 96, 97). By using paired colon tumor and normal adjacent tissues from volunteers, it was found that sulfidogenic *Fusobacterium nucleatum* was enriched in the colon tumors of patient with deficient mismatch repair colorectal cancer (39). In accordance with these results, in African Americans, sulfidogenic bacteria abundance was higher in colonic tissue biopsies recovered from patients with colorectal cancer when compared with healthy subjects (99), thus further reinforcing the view that these bacteria represent a risk factor for colorectal cancer development. In addition, sulfur-containing compounds in the samples of flatus recovered from patients with colorectal cancer were more abundant when compared with samples recovered from healthy subjects (98).

In vitro studies on the effects of H_2S on different cancerous colonic epithelial cell lines have used different H_2S donors (either fast- or slow releasing donors) at different concentrations and for different period of times. The interpretation and comparison of the different studies with different experimental protocols

on that topic is complicated by the fact that H_2S in the culture medium is rapidly released in the gas phase in the culture flasks, thus decreasing within hours the H_2S concentration in the extracellular medium (53). In addition, the capacity of colonic epithelial cells to oxidize H_2S may affect, depending on their density in the flasks, the H_2S concentration in the culture media. Lastly, since some components of the culture media may bind H_2S, the concentration of bioavailable hydrogen sulfide may differ from the theoretical values. With these reservations in mind, from the available studies, it appears that low concentrations of H_2S (micromolar) generally increase cell growth while higher concentrations (low millimolar) inhibit it. In the HCT116 colonic carcinoma cells, the H_2S donor GYY4137, used at 300 µM concentration, enhanced cell respiration and glycolysis, as well as cell proliferation (87) (Figure 5). The stimulatory effect of H_2S on glycolysis was due to the persulfidation of lactate dehydrogenase, thus enhancing its catalytic activity. Using 200 µM NaHS on the same cell line, the H_2S donor stimulated cell proliferation through an increased Akt and ERK activation (19). This effect was associated with a decreased expression of the cell cycle inhibitor p21/waf1/cip1. In good accordance with these latter results, increased endogenous production of H_2S in colorectal carcinoma cells was associated with increased proliferative capacity, since the allosteric CBS activator S-adenosyl-L-methionine, which increases as expected H_2S production in HCT116 cells, increased oxygen consumption and cell proliferation (61). Of note, in the cancerous subpopulation of parental HCT116 cells that are resistant to the chemotherapeutic agent 5-fluorouracil, CBS is upregulated leading to increased capacity of these cells to produce H_2S (88). In addition, decreased expression of CBS in HCT116 cells reduces the cell growth, and the growth rate and size of HCT116 xenografts (84). In accordance with these results, pharmacological inhibition of CBS catalytic activity inhibits HCT116 oxygen consumption and glycolysis, an effect that was paralleled by a G0/G1 arrest responsible for a reduction of cell growth without loss of cell viability (20). *In vivo*, the silencing or pharmacological inhibition of CBS activity attenuate the growth of colon carcinoma cell xenografts in nude mice, as well as neovessel density, suggesting a role of endogenous H_2S in colorectal cancer cell growth and tumor angiogenesis (84).

Overall, the available studies suggest that an increased CBS activity and resulting increased H_2S production in colonic epithelial cells, or moderate supply of exogenous H_2S, may participate in the promotion of colon carcinogenesis. However, increased CBS activity may not be the unique way by

which colonocyte increase their H_2S production in the carcinogenesis process. Indeed, increased H_2S production in the CSE pathway is involved in the growth of colorectal cells. In fact, silencing or pharmacological inhibition of CSE in the colorectal cancer cells SW480 decreased the capacity of colorectal cancer cell to proliferate *in vitro*, and decreased tumor xenograft growth *in vivo* (27). Incidentally, the interpretation of the data are complicated by the fact that CBS and CSE pathways has been demonstrated as competing reactions for the production of H_2S (47).

In contrast, higher concentration of GYY4137 (3.0 mM) inhibited markedly the proliferation of HCT116 cells (66). In the cancerous HT-29 colonic epithelial cells, NaHS at 1 mM concentration inhibited cell respiration and oxidation of glutamine and butyrate (53). These H_2S effects that coincided with a marked reduction of the cell proliferative capacity without cell viability loss, was paralleled by a spectacular increase of the glycolytic pathway. In this latter study, since the cells maintained their ATP cell content, it was proposed that by reducing cell growth, and thus ATP-consuming anabolic metabolism, and by enhancing glycolysis, the cell maintained their viability under restriction of mitochondrial ATP production (Figure 6). Spontaneous or butyrate-induced differentiated HT-29 cells were characterized by an increased capacity for sulfide oxidation when compared to highly proliferative cells, suggesting that high concentration of sulfide would affect more severely oxidative energy metabolism in proliferative than differentiated cancerous colon epithelial cells (60).

The *in vitro* studies by Attene-Ramos et al (4) have shown that exogenous H_2S is able to induce *in vitro* DNA single and double breaks in mammalian cells. However, recent *in vivo* studies have shown that H_2S at concentration found in the colonic luminal content are unlikely to be genotoxic for colonocytes in short-term experiments with rats (9). Further work is required to determine in what condition and context H_2S may alter genomic and mitochondrial DNA in colonic epithelial cells.

Conclusion and perspectives

Among the numerous compounds released by the microbiota in the colon and rectum, H_2S has emerged as one typical example of a metabolic end product that have an impact on the host colonic mucosa energy metabolism. The

capacity of the colonocytes to cope with increasing concentration of unbound H_2S in the luminal content is dependent on its ability to oxidize this compound in the mitochondria by the SOU. This process allows to control the intracellular concentration within colonocytes and to use it as a rapid mineral energy substrate allowing it to participate to ATP production in a context of rapid epithelium renewal and associated intense anabolism. However, the capacity for sulfide disposal is intrinsically limited, and thus, in condition of increased unbound H_2S luminal concentration, the intracellular sulfide concentration may exceed the detoxifying capacity of colonocytes. In such a case, the surplus of H_2S concentration inhibits colonocyte respiration and ATP production in the mitochondria. However, colonocytes are not only exposed to H_2S originating from the lumen, but also from the one produced endogenously. Mostly due to inherent technical difficulties, there is still little information on the endogenous and exogenous origin of the intracellular H_2S concentration within colonocytes in different physiological and pathophysiological contexts. However, it is highly likely that the gut microbiota contributes much more than endogenous synthesis to H_2S intracellular concentration value (15).

Regarding the implication of H_2S in the process of chronic inflammatory bowel diseases, there are evidence from both clinical and experimental data that an excess of sulfide over the oxidative capacity of colonocytes participates in the inflammatory process. In addition, such an excessive luminal concentration of sulfide destabilizes the mucus layers, thus decreasing the protection of the colonic epithelium against this (and other) deleterious luminal compounds. However, restricting markedly the endogenous production of H_2S within colonocytes appears counterproductive as it favours the inflammatory process. Further work is needed to determine possible differences in the action of H_2S on colonic epithelial cells depending on its intracellular or extracellular origin.

Regarding the implication of H_2S in the process of colorectal carcinogenesis, recent studies have suggested that increased H_2S synthesis in adenoma-like colonic epithelial cells through higher CBS activity, increased their energy metabolism, proliferation, and tumorigenicity in animal models. From colonic biopsies recovered from volunteers, it appears that H_2S production is increased in colorectal cancer when compared with adjacent healthy tissues. The experiments performed *in vitro* with various cell lines recovered from colorectal cancer indicate that H_2S at micromolar concentration favours cell energy metabolism and proliferation. In such condition, cell energy metabolism is enhanced at the mitochondrial level, but also at the glycolytic level.

Interestingly, such an increased glycolytic capacity was due to the persulfidation of lactate dehydrogenase, thus enhancing its catalytic activity. The meaning of this increased glycolysis for cell growth remains unclear giving the low ATP production from this metabolic pathway when compared to the mitochondrial ATP production from the respiratory chain, but it may relate to the synthesis of ribonucleotides and NADPH in the pentose phosphate pathway that is derived from glycolysis (68).

The process of protein persulfidation in colonic epithelial cells, as shown for LDH (86), may play an important role for explaining some of the H_2S effects. Thus, it appears plausible, but remained to be tested, that other cellular targets in colonocytes would be modified by this post-translational biochemical process. Just to take a first and stimulating example, a study performed in non-colonic cells, namely macrophages, has shown that H_2S-linked persulfidation of NFkappaB is linked to an antiapoptotic signal in these cells (77). Also of interest, in colonic smooth muscle cells, H_2S acts through the persulfidation process as an allosteric modulator of the ATP-sensitive K^+ channels (31).

In contrast, higher H_2S concentrations in the low millimolar range inhibited cancerous colonocyte proliferation, an effect that may be seen at a first glance at a beneficious effect of H_2S in the context of the development of colorectal cancer. However, it appears that in such conditions, cancerous cells are able to maintain their ATP content by decreased proliferation and increased glycolysis, thus maintaining their viability. It is then tempting to propose from these experiments, that such processes would allow the cancerous cells to survive to an enhanced extracellular supply of H_2S. It is worth noting however that conditions in which these *in vitro* experiments were performed are obviously very different from the *in vivo* conditions, considering the great heterogeneity of cells in the colorectal tumors (76) and of their microenvironment (17, 49).

Regarding the effects of endogenous and exogenous H_2S on the process of stimulus-secretion coupling in enteroendocrine cells of the colon, the mechanisms involved in such a process remains poorly known. Advantages could be taken in future studies from some comparison with studies devoted to the role of H_2S on insulin secretion, because of some proximity between EEC and pancreatic beta cells. Also, taking into account that H_2S can either, depending on its concentration, stimulates or inhibits mitochondrial ATP production, it would be of interest to determine to what extent modulation of

energy metabolism in EEC may participate in the effect of H_2S on enteroendocrine hormone secretion in the colon.

In conclusion, the available data from clinical and *in vivo*/*in vitro* experimental works indicate that endogenous and microbiota-derived H_2S have major impact on the colonic epithelial cells, notably in terms of energy metabolism. This is observed in healthy situation but also in the context of inflammatory bowel diseases and colorectal carcinogenesis. Then, reduction of excessive luminal H_2S concentration and/or excessive endogenous production by targeted pharmacological approaches in colonic epithelial cells, may prove to have some efficacy in reducing its deleterious effects on the colonic mucosa. However, given the ambivalent nature of the effects of H_2S on these cells, either beneficial or deleterious according to its intracellular concentration in a given physiological or pathophysiological context, this strategy can be considered as risky. Thus, in condition of excessive production, reduction of the number of H_2S producing-intestinal bacteria and/or limitation by dietary means of the supply of S-containing substrates for H_2S production, may represent a more strategic approach.

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983 Figure legends

984

985 Figure 1. Production of hydrogen sulfide by the colonic microbiota and by the
986 colonic absorptive epithelial cells. This schematic representation recapitulates
987 the main luminal substrates that are used by the colonic microbiota for H_2S
988 synthesis, the different forms of H_2S in the luminal content, and the main
989 metabolic pathway for endogenous H_2S synthesis in colonocytes. CBS,
990 cystathionine beta-synthase.

991 Figure 2. Mitochondrial oxidation of hydrogen sulfide in mammalian cells. This
992 schematic representation recapitulates the sulfide oxidation unit that allows
993 the conversion of low concentrations of endogenous or exogenous H_2S into
994 thiosulfate, and the synthesis of ATP in the mitochondrial respiratory chain (left
995 part of the figure); and the inhibition of complex IV by high concentration of
996 exogenous H_2S (right part of the figure). ETHE1, persulfide dioxygenase; $H_2S_2O_3$,
997 thiosulfate; SQR, Sulfide Quinone Reductase.

998 Figure 3. Schematic representation of the inflammatory effect of luminal
999 hydrogen sulfide in excess on colonocytes. When the metabolic capacity for H_2S
1000 synthesis by the colonic microbiota, due to either changes in microbiota
1001 composition and/or increased metabolic capacity for conversion of cysteine to
1002 H_2S, and/or increased availability of cysteine as precursor for H_2S synthesis, the
1003 H_2S luminal content may increase. An increased H_2S luminal concentration may
1004 destabilize the mucous layer, and increase the H_2S concentration in
1005 colonocytes. In situation of decreased capacity of the colonocytes for H_2S
1006 disposal by the Sulfide oxidation Unit (SOU), the gaseous mediator intracellular
1007 concentration may increase, then reducing the mitochondrial ATP synthesis, a
1008 process that, together with an increased expression of the proinflammatory
1009 interleukin IL-6, and of the inducible form of Nitric Oxide Synthase (iNOS), likely
1010 participates in the inflammatory response in colonocytes. In contrast, complete
1011 inhibition of the tiny intracellular production of H_2S in colonocytes by the
1012 cystathionine beta-synthase (CBS) is counterproductive, as a minimal amount
1013 of H_2S is likely necessary to limit the risk of colonic mucosal inflammation,
1014 raising the view that H_2S, depending on its intracellular concentration, is a
1015 double-edge sword for the intestinal epithelium.

1016 Figure 4. Schematic representation of the effect of increased intracellular
1017 production of hydrogen sulfide on the development of late-adenoma colonic

epithelial cells. When cystathionine beta-synthase (CBS) activity is increased in late adenoma colonocytes, the endogenous production of H_2S from cysteine increases, thus stimulating mitochondrial oxygen consumption and thus ATP production. This increased ATP production from mitochondrial oxidative phosphorylation, together with increased glycolysis would favor the cell proliferative capacity.

Figure 5. Schematic representation of the effect of low luminal hydrogen sulfide concentration and increased endogenous production of H_2S in colonocytes originating from colorectal carcinoma. Low H_2S luminal concentration together with increased endogenous H_2S production through increased cystathionine beta-synthase (CBS) activity, are associated with H_2S oxidation by the sulfide oxidizing unit (SOU), and thus stimulation of mitochondrial oxygen consumption, and ATP production. When H_2S accumulates in cancerous cells, it modifies lactate deshydrogenase by S-sulfhydration, thus enhancing its catalytic activity and thus glycolysis. This increased ATP production in the mitochondria, and increased glycolysis would favor the proliferative capacity of cancerous colonocytes.

Figure 6. Schematic representation of the effects of high luminal hydrogen sulfide concentration in colonocytes originating from colorectal carcinoma. High H_2S luminal concentration above the capacity of the sulfide oxidizing unit (SOU) for H_2S disposal, markedly reduces mitochondrial oxygen consumption, and thus ATP mitochondrial synthesis. Meanwhile, high H_2S concentration inhibits the cell proliferative capacity, and thus ATP utilization in the anabolic pathways associated with cell growth. This process, together with increased glycolysis, allow to maintain the ATP cell content, thus allowing the cancerous cells to maintain their viability.