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1 **Production of hydrogen sulfide by the intestinal microbiota and epithelial**
2 **cells and consequences for the colonic and rectal mucosa**

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29 Abstract

30 Among bacterial metabolites, hydrogen sulfide (H₂S) has received increasing
31 attention. The epithelial cells of the large intestine are exposed to two sources
32 of H₂S. The main one is the luminal source that results from specific bacteria
33 metabolic activity towards sulfur-containing substrates. The other source in
34 colonocytes is from the intracellular production mainly through cystathionine
35 beta-synthase (CBS) activity. H₂S is oxidized by the mitochondrial sulfide
36 oxidation unit, resulting in ATP synthesis, and thus establishing this compound
37 as the first mineral energy substrate in colonocytes. However, when the
38 intracellular H₂S concentration exceeds the colonocyte capacity for its
39 oxidation, it inhibits the mitochondrial respiratory chain, thus affecting energy
40 metabolism. Higher luminal H₂S concentration affects the integrity of the
41 mucus layer and displays pro-inflammatory effects. However, a low/minimal
42 amount of endogenous H₂S exerts an anti-inflammatory effect on the colon
43 mucosa pointing out the ambivalent effect of H₂S depending on its intracellular
44 concentration. Regarding colorectal carcinogenesis, forced CBS expression in
45 late adenoma-like colonocytes increased their proliferative activity,
46 bioenergetics capacity, and tumorigenicity; while genetic ablation of CBS in
47 mice resulted in a reduced number of mutagen-induced aberrant crypt foci.
48 Activation of endogenous H₂S production and low H₂S extracellular
49 concentration enhance cancerous colorectal cells proliferation. Higher
50 exogenous H₂S concentrations markedly reduce mitochondrial ATP synthesis
51 and proliferative capacity in cancerous cells, enhance glycolysis, but do not
52 affect their ATP cell content nor viability. Thus, it appears that, notably through
53 an effect on colonocyte energy metabolism, endogenous and microbiota-
54 derived H₂S are involved in the host intestinal physiology and physiopathology.

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

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63 Introduction

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

77 Among these bacterial metabolites, H₂S has received increasing attention in the
78 last decades as a compound that was initially considered as a toxic substance
79 when present in excess in the environment (73), while acting as a gaseous
80 mediator involved in numerous physiological functions when synthesized
81 endogenously (37). The aim of this review is to present an overview of the
82 experimental and clinical evidence that allow to consider H₂S as an important
83 player in the communication between some bacterial species in the intestinal
84 microbiota and the large intestine epithelial cells in different physiological and
85 pathophysiological situations.

86 *Production of luminal H₂S by the intestinal bacteria*

87 H₂S is produced by different bacterial species from both dietary and
88 endogenous compounds of organic and inorganic nature (13) (Figure 1). Recent
89 data suggest that H₂S is mainly produced through cysteine catabolism, and to a
90 lower extent by sulfate reducing bacteria (SRB) (36). Cysteine catabolic bacteria
91 include *Fusobacterium*, *Clostridium*, *Escherichia*, *Salmonella*, *Klebsiella*,
92 *Streptococcus*, *Desulfovibrio*, and *Enterobacter* which convert cysteine to H₂S,
93 pyruvate, and ammonia by the enzymatic activity of cysteine desulfhydrase (8).
94 SRB includes *Desulfovibrio*, *Desulfobacter*, *Desulfobulbus*, and
95 *Desulfotomaculum*. *Desulfovibrio* is the dominant genera of SRB, and it includes
96 *D.piger* and *D.desulfuricans* (72). The numerous microbes belonging to SRB
97 possess the capacity to reduce several compounds including sulfate, sulfite,
98 thiosulfate, elemental sulfur, and several thionates (13). The capacity of some

99 bacteria to cope with surrounding H₂S may be related to some specific
100 metabolic characteristics. For instance in *Escherichia Coli*, cytochrome bd-type
101 O₂-oxidases that are relatively insensitive to sulfide allow bacterial oxygen
102 consumption and growth in the presence of this compound (30).

103 The concentrations of sulfide measured in the colon luminal content and in the
104 feces are rather variable depending on the processing of the biological
105 materials and on the technique used for the assay. Some techniques are
106 specific for sulfide detection, like for instance the targeted GC-MS
107 measurement (41); while others are rather unspecific and detect other sulfur-
108 containing compounds. Thus, concentrations of sulfide measured in the large
109 intestine content and in faecal material (that latter representing an
110 approximation of the concentration in the rectum) in mammals ranges from 0.2
111 mM up to 2.0 mM (13). This order of magnitude difference, apart from the
112 specificity of the techniques used for measurement, may also be explained by
113 the fact that the luminal concentration of sulfide in the large intestine is
114 dependent on the dietary status of the individuals. For instance, the
115 consumption of a diet with a high protein content, where some dietary and
116 endogenous proteins escape digestion in the small intestine (26) results in an
117 increased amount of sulfide in the colon luminal content, and in the fecal
118 material, when compared with the situation of lower protein intake (9, 59).
119 Sulfide in the luminal content can exist in 3 forms: the H₂S gas that is partly
120 dissolved in the aqueous phase, and is highly diffusible, hydrosulfide anion HS⁻,
121 and sulfide ion S²⁻, this latter being present at a negligible level. Indeed, in
122 aqueous phase, H₂S dissociate in HS⁻ and S²⁻ and H⁺ with pKa values being 7.04
123 and 11.96 respectively (13). In healthy subjects, the pH at the colonic mucosal
124 surface ranges between 7.2 and 7.5 in the descending colon and rectum (14).
125 Thus, considering a pH of 7.4, approximately one third of sulfide is in the form
126 of H₂S at equilibrium with two third being hydrosulfide anion. However, when
127 the luminal pH is more acidic, the H₂S/HS⁻ ratio in the large intestine increases.
128 Taking into account that H₂S, unlike HS⁻ easily penetrates biological membranes
129 (14), lower pH will increase H₂S concentration, then amplifying its entry in the
130 colonic epithelial cells. Lastly, measurement in human faeces showed that 8%
131 of sulfide are in unbound form (45), representing a concentration around 60
132 μM, while measurement in the rat caecal content suggests that 0.2% of total
133 luminal sulfide is in unbound form (55). Several compounds from dietary origin
134 that are not fully absorbed by the small intestine have been shown to bind H₂S.
135 These compounds include zinc (83), heme (43), and polyphenols (1).

136 Incidentally, by comparing both free H₂S and bound sulfane sulfur
137 concentrations in blood plasma recovered from conventional and germ-free
138 rodents, it was found that these concentrations are markedly lower in animals
139 with no intestinal microbiota, thus suggesting that the circulating H₂S is largely
140 originating from the microbiota metabolic activity (79). This result is in
141 accordance with the fact that fecal H₂S synthesis in germ-free mice represents
142 approximately half of that observed in colonized mice (28).

143 *H₂S and the absorbing colonic epithelial cells*

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

156 The absorbing colon epithelial cells, often referred as colonocytes, are
157 responsible for water absorption, and electrolyte absorption and secretion (6).
158 These cells are characterized by relatively high energy requirement due to
159 intense anabolism and the catalytic activity of the Na/K ATPase involved in
160 sodium absorption (12). The colonocytes are equipped with the so-called
161 sulfide-oxidizing unit (SOU), a mitochondrial multi-enzymatic system
162 responsible for the oxidation of H₂S (Figure 2). When the extracellular
163 concentration of NaHS, a rapid H₂S donor, is below a threshold value,
164 approximately equal to 50 μM, the colonocytes are able to oxidize sulfide to
165 thiosulfate in three steps (33) allowing the production of ATP (16). The first
166 enzyme of the SOU is sulfide quinone reductase (SQR) that injects electrons
167 into the mitochondrial respiratory chain through quinone before transfer to
168 complex III cytochrome c, and finally reduction of oxygen to water at the level
169 of complex IV (cytochrome oxidase). The persulfide formed in the first step of
170 SOU activity is oxidized by the sulfur dioxygenase ETHE1 to sulfite. Disruption of
171 the mitochondrial ETHE1 in mice is lethal 5 weeks after birth, indicating sulfide

172 toxicity, and vital importance of ETHE1 for sulfide disposal (85). Finally, the
173 sulfur transferase rhodanese converts sulfite into the metabolic end product
174 thiosulfate (Figure 2) (56). Sulfide oxidation pathway is mainly localized apically
175 in human colonic crypts (58). When the expression of the genes coding for the
176 3 enzymes of the SOU was measured in human biopsies recovered from
177 different anatomical parts of the large intestine, the rhodanese was found to
178 be much less expressed in the rectum than in more proximal parts (that are
179 caecum, ascending, transverse and descending colon), suggesting a lower
180 capacity for sulfide disposal in the rectum (60).

181 Thus, H₂S can be considered as an inorganic fuel for colonocytes in addition to
182 the usual main organic energy substrates provided through the luminal and
183 baso-lateral sides, and used by colonocytes (e.g. short-chain fatty acids at the
184 luminal side, and glutamine, and glucose at the baso-lateral side). H₂S in fact
185 represents the first inorganic energy substrate for mammalian cells (34).
186 However, in *in vitro* studies, when the extracellular concentration of NaHS is
187 above 50 μM, an inhibition of the colonocyte respiration is observed (9). This is
188 due to an inhibition of the catalytic activity of the cytochrome c oxidase, one of
189 the complex in the mitochondrial respiratory chain (complex IV) (65) (Figure 2).
190 In addition, in isolated human colonocytes, H₂S inhibits butyrate oxidation (5).
191 Thus overall, the capacity of colonocytes to oxidize limited amount of sulfide in
192 mitochondria appears to represent a way to detoxify this compound while
193 recovering energy from it. Such a capacity can be altered by other gaseous
194 mediator like nitric oxide (NO). Indeed, the NO donor MAMA NONOate, which
195 markedly inhibits oxygen consumption in rat colonocytes by inhibiting
196 mitochondrial cytochrome c oxidase activity (9, 22), prevents H₂S detoxification
197 in colonocytes.

198 Several processes have been described regarding the adaptive capacity of
199 colonocytes to face an increased concentration of H₂S in the extracellular
200 medium. By feeding rodents with high-protein diet, and thus by increasing the
201 cysteine availability for H₂S production by the large intestine microbiota, an
202 increased content of H₂S was recorded as expected in the caecal and colonic
203 luminal fluids, and this increased concentration coincided with an increased
204 expression in colonocytes of the *Sqr* gene that is coding for SQR, the first
205 enzyme in SOU (9).

206 However, it is worth noting that colonocytes are not only exposed to H₂S from
207 their luminal side, but can also synthesize this mediator intracellularly from

208 cysteine and other co-substrates. The major pathway in colonocytes, appears
209 to be through the activity of cystathionine beta-synthase (CBS), a versatile
210 enzyme that is able to convert cysteine to H₂S and serine, but also 2 molecules
211 of cysteine into H₂S and lanthionine, and cysteine and homocysteine into H₂S
212 and cystathionine (36). NO has been shown to interfere with H₂S endogenous
213 synthesis through the binding to CBS heme, a process that results in inhibition
214 of CBS catalytic activity (33, 91). This indicates that the cellular metabolism of
215 H₂S may depend on the local concentration of NO.

216 Thus, the intracellular H₂S concentration in colonocytes depends on the
217 diffusion of luminal H₂S through the brush-border membrane, on the
218 intracellular synthesis from cysteine, and on the capacity of colonocytes for its
219 oxidation.

220 *H₂S and the enteroendocrine cells*

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

234 However, little is known on the impact of H₂S on EEC physiology or secretion in
235 the large intestine. In GluTag cells, a murine colonic L-cell model, Pichette and
236 collaborators have showed that slow- and rapid-releasing sulfide donors dose-
237 dependently increased GLP-1 secretion, with an effect dependent on p38
238 MAPK signalling (70). Interestingly, in this study, the authors by using the
239 prebiotic chondroitin sulfate, that increases the abundance of the SRB *D-piger*
240 and sulfate moiety in the distal intestine, measured an increased H₂S
241 production. Such increased production was associated with metabolic
242 improvements through increased GLP-1 secretion during oral glucose tolerance
243 tests.

244 Using cells originating from a more proximal region of the gastrointestinal tract,
245 Slade and collaborators showed that the slow-releasing sulfide donor GYY4137
246 dose-dependently reduced the secretion of ghrelin in rat gastric primary
247 culture. Ghrelin represents the main gut orexigenic hormone that regulates
248 adiposity and gastrointestinal motility. Its secretion pattern is opposite to most
249 gut hormones, as its secretion is inhibited by food intake. As ghrelin producing
250 cells are present in the stomach, it is likely that the main source of H₂S there is
251 through endogenous production. In fact, these cells express high level of
252 cystathionine-gamma lyase (CSE), one of the enzymes involved in H₂S
253 endogenous production (81). Inhibition of endogenous H₂S production
254 increased ghrelin secretion. Similar inhibitory effects of H₂S on EEC secretion
255 has been described by Bala and collaborators (7). Sulfide donors at high
256 concentration strongly inhibited glucagon-like peptide 1 (GLP-1) and peptide-YY
257 (PYY) secretion induced by Takeda-G-protein-receptor-5 (TGR5) stimulation in
258 STC-1 cells, a cellular EEC model of murine small intestine that are poorly
259 differentiated (7). Interestingly, L-cysteine, which increases H₂S endogenous
260 production, also inhibited GLP-1 and PYY stimulated secretion.

261 *H₂S and colonic inflammation*

262 Chronic inflammatory bowel diseases (IBD), mainly Crohn's disease and
263 ulcerative colitis (UC) are characterized by alternating episodes of remission
264 and relapse, resulting from inappropriate mucosal immune responses against
265 luminal intestinal components in genetically predisposed individuals (48).
266 Although Crohn's disease and UC have different clinical features (67), the
267 inflammation of the intestinal mucosa is often observed in the distal parts of
268 the intestine (32). Etiology of IBD, although still largely elusive, is related to
269 genetic (50), and environmental factors including the ones from dietary origin
270 (92). The concept that excessive concentration of H₂S in the intestinal luminal
271 content, notably in a context of diminished capacity for sulfide disposal in the
272 mucosa (2), may participate in the etiology of mucosal inflammation has been
273 proposed more than 2 decades ago (54). Severity of mucosal inflammation in
274 the colon and rectum of patients with active UC is believed to be related to the
275 concentration ratio of deleterious vs. beneficial bacterial metabolites. This ratio
276 is notably depending on the substrate availability for production of these
277 metabolites. Following for one year UC patients in remission, it was found that
278 patients with high consumption of meat, dietary protein and sulfur/sulfate
279 experienced an increased rate of relapse when compared with patients with
280 low consumption of these compounds (46). This indicates that increased intake

281 of dietary substrates known to be used by the intestinal microbiota for H₂S
282 synthesis, is associated with increased risk of relapse. In anesthetized rats,
283 after preliminary evacuation of the luminal content, intracolonic instillation of
284 NaHS used for 1 hour at concentrations between 0.5 and 1.5 mM, increased in
285 colonocytes the expression of inflammation-related genes, namely those
286 coding for the inducible form of nitric oxide synthase (iNOS), and interleukin-6
287 (9). Overexpression of iNOS is measured in colonic samples originating from
288 patients with UC (80), and NO production in excess, notably through increased
289 peroxynitrite formation, appears to play a role in the process of mucosal
290 inflammation.

291 Ijssennagger and collaborators have shown that microbiota-derived H₂S at high
292 concentration destabilizes the protective mucus layer covering the intestinal
293 epithelium through the reduction of disulfide bonds linking the mucin 2
294 network (42) (Figure 3), a process that would increase the interactions between
295 bacteria and the epithelium. Mottawea and coworkers have studied the
296 microbiota composition of new-onset pediatric Crohn's disease patients, and
297 found that this microbiota is characterized by high abundance of *Atopobium*,
298 *Fusobacterium*, *Veillonella*, *Prevotella*, *Streptococcus*, and *Leptotrichia*, several
299 members of those genera being known to produce H₂S through the catabolism
300 of sulfur-containing amino acids (63). Importantly, in this cohort, the
301 abundance of H₂S producers from cysteine was correlated with the severity of
302 mucosal inflammation. To document the possible causal link between H₂S
303 production and intestinal inflammation, the authors colonized Interleukin-10^{-/-}
304 mice with the H₂S producer *Atopobium parvulum* and measured a worsening of
305 colitis, such a deleterious effect being attenuated by the H₂S scavenger
306 bismuth. In addition, in the same Crohn's disease pediatric cohort, the colonic
307 mucosa biopsies displayed decreased expression of the mitochondrial enzymes
308 involved in H₂S detoxification. Overall, the results from this study strongly
309 suggest that diminished capacity for sulfide disposal in the colonic mucosa of
310 pediatric Crohn's disease patients amplify the pro-inflammatory effect of H₂S
311 overproduction by the intestinal microbiota (Figure 3).

312 Thus, evidence from both experimental and clinical studies have highlighted
313 that high H₂S concentrations, i.e. above the capacity of the intestinal mucosa to
314 detoxify this bacterial metabolite, increase the risk of inflammation. However,
315 it appears from experimental studies using pharmacological inhibitors of H₂S
316 synthesis or H₂S-releasing compounds, that a minimal amount of sulfide is likely
317 necessary to limit the risk of colonic mucosal inflammation. Then, the concept

318 that H₂S is a double-edge sword for the intestinal epithelium has been
319 proposed (15). Indeed, several *in vivo* studies found that inhibition of H₂S
320 endogenous synthesis favors intestinal inflammation and delays colitis
321 resolution (40, 94). The capacity for H₂S production was markedly elevated
322 after colitis induction in rat, and inhibition of H₂S synthesis in colon
323 exacerbated the colitis. Interestingly, H₂S has been shown to exert some
324 antioxidant activity, notably by persulfidation of cysteine residues that protects
325 these residues from oxidative damage (33). This action may contribute to the
326 anti-inflammatory effect of low production of H₂S in colonocytes. Regarding
327 colitis resolution, H₂S-releasing compounds have been shown to promote this
328 process through Hypoxia-Inducible factor-1alpha signaling pathway (29)
329 Endogenous synthesis of H₂S was found to offer some protection against
330 dextran sodium sulfate (DSS)-induced colitis in rodents partly by inhibiting the
331 activation of NLRP3 inflammasome pathway (71). Also, the slow-releasing
332 sulfide donor GYY4137 was shown to reduce *in vitro* the lipopolysaccharide or
333 TNF-alpha/IFN-gamma induced increased permeability in colonocyte
334 monolayer (21), and the intraperitoneal injection of GYY4137 in endotoxemic
335 animals protects against intestinal barrier dysfunction *in vivo* (100). In addition,
336 this H₂S donor improves mesenteric perfusion and intestinal injury in an
337 experimental model of necrotizing enterocolitis, and these beneficial effects
338 appear to be mediated through constitutive endothelial nitric oxide synthase
339 (e-NOS)-dependent pathways (25). Lastly, endogenous production of H₂S
340 appears to contribute to mucus production thus favoring segregation between
341 luminal bacteria and the intestinal mucosa (62). From the different available
342 results, it appears that the effects of the diffusible compound H₂S on the
343 inflammatory process in the intestinal mucosa depend both on the exogenous
344 production by the microbiota, and on the endogenous production by the host
345 colonic tissue.

346 *H₂S and colon carcinogenesis*

347 Some recent experimental and clinical studies suggest that both endogenous
348 synthesis of H₂S in colonic epithelial cells and luminal exogenous H₂S are
349 implicated in the process of colorectal carcinogenesis. Gene and protein
350 expression has revealed that the H₂S-synthesizing enzyme CBS is increased in
351 colonic tumors when compared with the surrounding mucosa (66, 84),
352 suggesting an increased capacity for H₂S synthesis in the cancerous samples.
353 However, since colonic tumors and surrounding tissues contained numerous
354 different cell phenotypes, it would be important to determine the expression of

355 this enzyme in colonic epithelial cells and other cell types. Upregulation of CBS
356 in human biopsies of precancerous adenomatous polyps has been measured,
357 and forced upregulation of CBS in late adenoma-like colonic epithelial cells was
358 associated with differences in the expression of genes involved in colorectal
359 cancer development, notably genes involved in NFkappa B, KRAS, and p53
360 signaling (69). Also these cells overexpressing CBS were characterized by
361 increased proliferative capacity, and enhanced cellular bioenergetics capacity
362 in terms of respiration and glycolysis. This increased CBS expression reinforced
363 cell tumorigenicity in athymic nude mice. Of equal importance, genetic ablation
364 of CBS in mice resulted in a reduction of the number of mutagen-induced
365 aberrant crypt foci (69). Thus, increased endogenous production of H₂S within
366 adenomatous colonocytes increases their proliferative capacity (Figure 4) and
367 their ability to promote tumor formation.

368 Increased supply of H₂S produced by the intestinal microbiota has been
369 implicated in the process of colorectal carcinogenesis. Indeed, by collecting
370 feces and tissues samples collected on and off the tumor site in the same
371 individuals, and by using multiomic data and community metabolic models to
372 assess H₂S production in colorectal cancer, Hale et al. obtained data indicating
373 an increased H₂S production by the gut bacteria in colonic cancer samples
374 compared to non- cancerous samples (38). The predicted increased H₂S
375 production at the tumor site was associated with the relative abundance of
376 *Fusobacterium nucleatum*, a known H₂S producer largely suspected to promote
377 colorectal cancer (18, 51, 74, 96, 97). By using paired colon tumor and normal
378 adjacent tissues from volunteers, it was found that sulfidogenic *Fusobacterium*
379 *nucleatum* was enriched in the colon tumors of patient with deficient mismatch
380 repair colorectal cancer (39). In accordance with these results, in African
381 Americans, sulfidogenic bacteria abundance was higher in colonic tissue
382 biopsies recovered from patients with colorectal cancer when compared with
383 healthy subjects (99), thus further reinforcing the view that these bacteria
384 represent a risk factor for colorectal cancer development. In addition, sulfur-
385 containing compounds in the samples of flatus recovered from patients with
386 colorectal cancer were more abundant when compared with samples
387 recovered from healthy subjects (98).

388 *In vitro* studies on the effects of H₂S on different cancerous colonic epithelial
389 cell lines have used different H₂S donors (either fast- or slow releasing donors)
390 at different concentrations and for different period of times. The interpretation
391 and comparison of the different studies with different experimental protocols

392 on that topic is complicated by the fact that H₂S in the culture medium is
393 rapidly released in the gas phase in the culture flasks, thus decreasing within
394 hours the H₂S concentration in the extracellular medium (53). In addition, the
395 capacity of colonic epithelial cells to oxidize H₂S may affect, depending on their
396 density in the flasks, the H₂S concentration in the culture media. Lastly, since
397 some components of the culture media may bind H₂S, the concentration of
398 bioavailable hydrogen sulfide may differ from the theoretical values. With
399 these reservations in mind, from the available studies, it appears that low
400 concentrations of H₂S (micromolar) generally increase cell growth while higher
401 concentrations (low millimolar) inhibit it. In the HCT116 colonic carcinoma cells,
402 the H₂S donor GYY4137, used at 300 μM concentration, enhanced cell
403 respiration and glycolysis, as well as cell proliferation (87) (Figure 5). The
404 stimulatory effect of H₂S on glycolysis was due to the persulfidation of lactate
405 dehydrogenase, thus enhancing its catalytic activity. Using 200 μM NaHS on the
406 same cell line, the H₂S donor stimulated cell proliferation through an increased
407 Akt and ERK activation (19). This effect was associated with a decreased
408 expression of the cell cycle inhibitor p21/waf1/cip1. In good accordance with
409 these latter results, increased endogenous production of H₂S in colorectal
410 carcinoma cells was associated with increased proliferative capacity, since the
411 allosteric CBS activator S-adenosyl-L-methionine, which increases as expected
412 H₂S production in HCT116 cells, increased oxygen consumption and cell
413 proliferation (61). Of note, in the cancerous subpopulation of parental HCT116
414 cells that are resistant to the chemotherapeutic agent 5-fluorouracil, CBS is
415 upregulated leading to increased capacity of these cells to produce H₂S (88). In
416 addition, decreased expression of CBS in HCT116 cells reduces the cell growth,
417 and the growth rate and size of HCT116 xenografts (84). In accordance with
418 these results, pharmacological inhibition of CBS catalytic activity inhibits
419 HCT116 oxygen consumption and glycolysis, an effect that was paralleled by a
420 G0/G1 arrest responsible for a reduction of cell growth without loss of cell
421 viability (20). *In vivo*, the silencing or pharmacological inhibition of CBS activity
422 attenuate the growth of colon carcinoma cell xenografts in nude mice, as well
423 as neovessel density, suggesting a role of endogenous H₂S in colorectal cancer
424 cell growth and tumor angiogenesis (84).

425 Overall, the available studies suggest that an increased CBS activity and
426 resulting increased H₂S production in colonic epithelial cells, or moderate
427 supply of exogenous H₂S, may participate in the promotion of colon
428 carcinogenesis. However, increased CBS activity may not be the unique way by

429 which colonocyte increase their H₂S production in the carcinogenesis process.
430 Indeed, increased H₂S production in the CSE pathway is involved in the growth
431 of colorectal cells. In fact, silencing or pharmacological inhibition of CSE in the
432 colorectal cancer cells SW480 decreased the capacity of colorectal cancer cell
433 to proliferate *in vitro*, and decreased tumor xenograft growth *in vivo* (27).
434 Incidentally, the interpretation of the data are complicated by the fact that CBS
435 and CSE pathways has been demonstrated as competing reactions for the
436 production of H₂S (47).

437 In contrast, higher concentration of GYY4137 (3.0 mM) inhibited markedly the
438 proliferation of HCT116 cells (66). In the cancerous HT-29 colonic epithelial
439 cells, NaHS at 1 mM concentration inhibited cell respiration and oxidation of
440 glutamine and butyrate (53). These H₂S effects that coincided with a marked
441 reduction of the cell proliferative capacity without cell viability loss, was
442 paralleled by a spectacular increase of the glycolytic pathway. In this latter
443 study, since the cells maintained their ATP cell content, it was proposed that by
444 reducing cell growth, and thus ATP-consuming anabolic metabolism, and by
445 enhancing glycolysis, the cell maintained their viability under restriction of
446 mitochondrial ATP production (Figure 6). Spontaneous or butyrate-induced
447 differentiated HT-29 cells were characterized by an increased capacity for
448 sulfide oxidation when compared to highly proliferative cells, suggesting that
449 high concentration of sulfide would affect more severely oxidative energy
450 metabolism in proliferative than differentiated cancerous colon epithelial cells
451 (60).

452 The *in vitro* studies by Attene-Ramos et al (4) have shown that exogenous H₂S is
453 able to induce *in vitro* DNA single and double breaks in mammalian cells.
454 However, recent *in vivo* studies have shown that H₂S at concentration found in
455 the colonic luminal content are unlikely to be genotoxic for colonocytes in
456 short-term experiments with rats (9). Further work is required to determine in
457 what condition and context H₂S may alter genomic and mitochondrial DNA in
458 colonic epithelial cells.

459

460 *Conclusion and perspectives*

461 Among the numerous compounds released by the microbiota in the colon and
462 rectum, H₂S has emerged as one typical example of a metabolic end product
463 that have an impact on the host colonic mucosa energy metabolism. The

464 capacity of the colonocytes to cope with increasing concentration of unbound
465 H₂S in the luminal content is dependent on its ability to oxidize this compound
466 in the mitochondria by the SOU. This process allows to control the intracellular
467 concentration within colonocytes and to use it as a rapid mineral energy
468 substrate allowing it to participate to ATP production in a context of rapid
469 epithelium renewal and associated intense anabolism. However, the capacity
470 for sulfide disposal is intrinsically limited, and thus, in condition of increased
471 unbound H₂S luminal concentration, the intracellular sulfide concentration may
472 exceed the detoxifying capacity of colonocytes. In such a case, the surplus of
473 H₂S concentration inhibits colonocyte respiration and ATP production in the
474 mitochondria. However, colonocytes are not only exposed to H₂S originating
475 from the lumen, but also from the one produced endogenously. Mostly due to
476 inherent technical difficulties, there is still little information on the endogenous
477 and exogenous origin of the intracellular H₂S concentration within colonocytes
478 in different physiological and pathophysiological contexts. However, it is highly
479 likely that the gut microbiota contributes much more than endogenous
480 synthesis to H₂S intracellular concentration value (15).

481 Regarding the implication of H₂S in the process of chronic inflammatory bowel
482 diseases, there are evidence from both clinical and experimental data that an
483 excess of sulfide over the oxidative capacity of colonocytes participates in the
484 inflammatory process. In addition, such an excessive luminal concentration of
485 sulfide destabilizes the mucus layers, thus decreasing the protection of the
486 colonic epithelium against this (and other) deleterious luminal compounds.
487 However, restricting markedly the endogenous production of H₂S within
488 colonocytes appears counterproductive as it favours the inflammatory process.
489 Further work is needed to determine possible differences in the action of H₂S
490 on colonic epithelial cells depending on its intracellular or extracellular origin.

491 Regarding the implication of H₂S in the process of colorectal carcinogenesis,
492 recent studies have suggested that increased H₂S synthesis in adenoma-like
493 colonic epithelial cells through higher CBS activity, increased their energy
494 metabolism, proliferation, and tumorigenicity in animal models. From colonic
495 biopsies recovered from volunteers, it appears that H₂S production is increased
496 in colorectal cancer when compared with adjacent healthy tissues. The
497 experiments performed *in vitro* with various cell lines recovered from
498 colorectal cancer indicate that H₂S at micromolar concentration favours cell
499 energy metabolism and proliferation. In such condition, cell energy metabolism
500 is enhanced at the mitochondrial level, but also at the glycolytic level.

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

508 The process of protein persulfidation in colonic epithelial cells, as shown for
509 LDH (86), may play an important role for explaining some of the H₂S effects.
510 Thus, it appears plausible, but remained to be tested, that other cellular targets
511 in colonocytes would be modified by this post-translational biochemical
512 process. Just to take a first and stimulating example, a study performed in non-
513 colonic cells, namely macrophages, has shown that H₂S-linked persulfidation of
514 NFkappaB is linked to an antiapoptotic signal in these cells (77). Also of
515 interest, in colonic smooth muscle cells, H₂S acts through the persulfidation
516 process as an allosteric modulator of the ATP-sensitive K⁺ channels (31).

517 In contrast, higher H₂S concentrations in the low millimolar range inhibited
518 cancerous colonocyte proliferation, an effect that may be seen at a first glance
519 at a beneficial effect of H₂S in the context of the development of colorectal
520 cancer. However, it appears that in such conditions, cancerous cells are able to
521 maintain their ATP content by decreased proliferation and increased glycolysis,
522 thus maintaining their viability. It is then tempting to propose from these
523 experiments, that such processes would allow the cancerous cells to survive to
524 an enhanced extracellular supply of H₂S. It is worth noting however that
525 conditions in which these *in vitro* experiments were performed are obviously
526 very different from the *in vivo* conditions, considering the great heterogeneity
527 of cells in the colorectal tumors (76) and of their microenvironment (17, 49).

528 Regarding the effects of endogenous and exogenous H₂S on the process of
529 stimulus-secretion coupling in enteroendocrine cells of the colon, the
530 mechanisms involved in such a process remains poorly known. Advantages
531 could be taken in future studies from some comparison with studies devoted to
532 the role of H₂S on insulin secretion, because of some proximity between EEC
533 and pancreatic beta cells. Also, taking into account that H₂S can either,
534 depending on its concentration, stimulates or inhibits mitochondrial ATP
535 production, it would be of interest to determine to what extent modulation of

536 energy metabolism in EEC may participate in the effect of H₂S on
537 enteroendocrine hormone secretion in the colon.

538 In conclusion, the available data from clinical and *in vivo/in vitro* experimental
539 works indicate that endogenous and microbiota-derived H₂S have major impact
540 on the colonic epithelial cells, notably in terms of energy metabolism. This is
541 observed in healthy situation but also in the context of inflammatory bowel
542 diseases and colorectal carcinogenesis. Then, reduction of excessive luminal
543 H₂S concentration and/or excessive endogenous production by targeted
544 pharmacological approaches in colonic epithelial cells, may prove to have some
545 efficacy in reducing its deleterious effects on the colonic mucosa. However,
546 given the ambivalent nature of the effects of H₂S on these cells, either
547 beneficial or deleterious according to its intracellular concentration in a given
548 physiological or pathophysiological context, this strategy can be considered as
549 risky. Thus, in condition of excessive production, reduction of the number of
550 H₂S producing-intestinal bacteria and/or limitation by dietary means of the
551 supply of S-containing substrates for H₂S production, may represent a more
552 strategic approach.

553

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- 982

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 H₂S
988 synthesis, the different forms of H₂S in the luminal content, and the main
989 metabolic pathway for endogenous H₂S 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 H₂S 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 H₂S (right part of the figure). ETHE1, persulfide dioxygenase; H₂S₂O₃,
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 H₂S
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 H₂S, and/or increased availability of cysteine as precursor for H₂S synthesis, the
1003 H₂S luminal content may increase. An increased H₂S luminal concentration may
1004 destabilize the mucous layer, and increase the H₂S concentration in
1005 colonocytes. In situation of decreased capacity of the colonocytes for H₂S
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 H₂S in colonocytes by the
1012 cystathionine beta-synthase (CBS) is counterproductive, as a minimal amount
1013 of H₂S is likely necessary to limit the risk of colonic mucosal inflammation,
1014 raising the view that H₂S, depending on its intracellular concentration, is a
1015 double-edge sword for the intestinal epithelium.

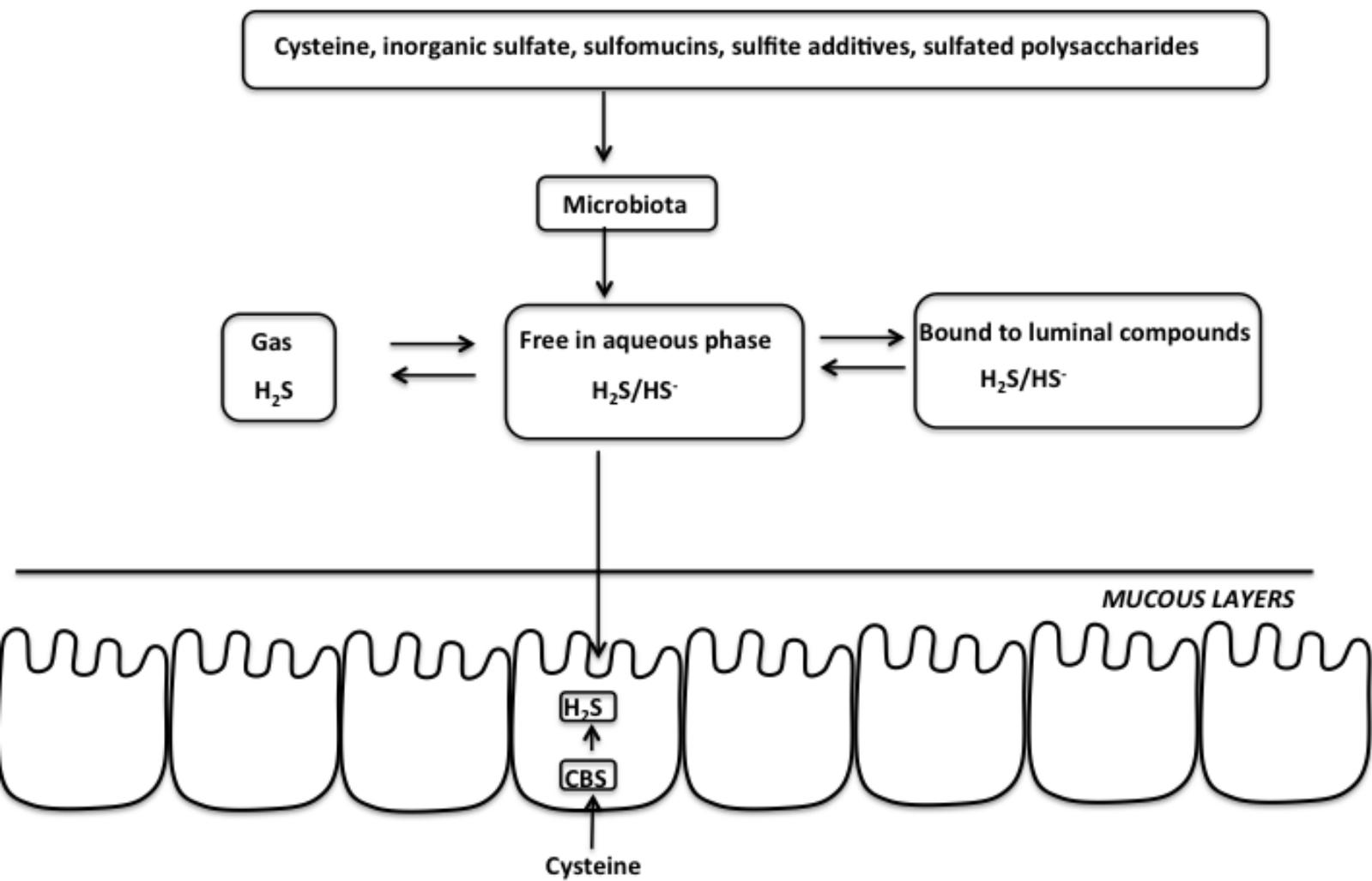
1016 Figure 4. Schematic representation of the effect of increased intracellular
1017 production of hydrogen sulfide on the development of late-adenoma colonic

1018 epithelial cells. When cystathionine beta-synthase (CBS) activity is increased in
1019 late adenoma colonocytes, the endogenous production of H₂S from cysteine
1020 increases, thus stimulating mitochondrial oxygen consumption and thus ATP
1021 production. This increased ATP production from mitochondrial oxidative
1022 phosphorylation, together with increased glycolysis would favor the cell
1023 proliferative capacity.

1024 Figure 5. Schematic representation of the effect of low luminal hydrogen
1025 sulfide concentration and increased endogenous production of H₂S in
1026 colonocytes originating from colorectal carcinoma. Low H₂S luminal
1027 concentration together with increased endogenous H₂S production through
1028 increased cystathionine beta-synthase (CBS) activity, are associated with H₂S
1029 oxidation by the sulfide oxidizing unit (SOU), and thus stimulation of
1030 mitochondrial oxygen consumption, and ATP production. When H₂S
1031 accumulates in cancerous cells, it modifies lactate dehydrogenase by S-
1032 sulfhydration, thus enhancing its catalytic activity and thus glycolysis. This
1033 increased ATP production in the mitochondria, and increased glycolysis would
1034 favor the proliferative capacity of cancerous colonocytes.

1035 Figure 6. Schematic representation of the effects of high luminal hydrogen
1036 sulfide concentration in colonocytes originating from colorectal carcinoma.
1037 High H₂S luminal concentration above the capacity of the sulfide oxidizing unit
1038 (SOU) for H₂S disposal, markedly reduces mitochondrial oxygen consumption,
1039 and thus ATP mitochondrial synthesis. Meanwhile, high H₂S concentration
1040 inhibits the cell proliferative capacity, and thus ATP utilization in the anabolic
1041 pathways associated with cell growth. This process, together with increased
1042 glycolysis, allow to maintain the ATP cell content, thus allowing the cancerous
1043 cells to maintain their viability.

1044



Low H₂S

High H₂S

