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The role of methane in mammalian physiology—is it a gasotransmitter?

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Abstract

Mammalian methanogenesis is widely considered to be an exclusive sign of anaerobic microbial activity in the gastrointestinal tract. This commonly held view was challenged, however, when *in vitro* and *in vivo* investigations demonstrated the possibility of nonmicrobial methane formation in aerobic organisms, in plants and animals. The aim of this paper is to discuss the available literature data on the biological role of methane. When we evaluate the significance of methane generation in the mammalian physiology, the question may be examined: is it a gas mediator? Overall the data do not fully support the gasotransmitter concept, but they do support the notion that methane liberation may be linked to redox regulation and may be connected with hypoxic events leading to, or associated with a mitochondrial dysfunction. In this respect, the available information suggests that hypoxia-induced methane generation may be a necessary phenomenon of aerobic life, and perhaps a surviving evolutionary trait in the eukaryote cell.

1. Introduction

Aerobic organisms have evolved a range of mechanisms through which to achieve the optimal utilization of atmospheric oxygen (O_2). In this well-controlled system, the availability of O_2 is the most critical issue, but it has become increasingly clear that other, less prominent components of the gaseous environment are also of importance to the cellular homeostasis. The discovery of nitric oxide (NO) as a signaling mediator radically altered the view of the roles and functions of gases in physiology, and the endogenous generation of carbon monoxide (CO) and hydrogen sulfide (H_2S) by the mammalian cell further deepened the knowledge on the *in vivo* significance of gaseous products. The research on 'gasotransmitter' candidates and derivatives has been intensified, and this is currently a topic of pronounced scientific interest. Not surprisingly, new family members with new effects were proposed, leading to the listing of four essential characteristics (simplicity, availability, volatility and effectiveness) and the definition of six criteria that make a gas physiologically important or irreplaceable [1]. The aim of this paper is to discuss the available literature data on methane (CH_4) from these aspects [2].

Criterion 1. *'Gasotransmitters are small gas molecules dissolved in biological milieu'*

Methane is a small, omnipresent, volatile molecule, the most hydrogen-substituted form of carbon. It plays a distinguished role in the tropospheric and stratospheric chemistry, where the bulk of the released CH_4 is oxidized to CO_2 through its reaction with hydroxyl radicals (formed by the photoreaction between ozone and water vapor) [3, 4]. It should be noted that the bioactivity or toxicity of the gas mediators NO, CO and H_2S is related to their tendency to react with biologically important molecules. Nevertheless, CH_4 is intrinsically nontoxic *in vivo*. The inhalation of normoxic air containing 2.5% CH_4 for 3 h has been shown to have no side-effects on the blood gas chemistry and not to influence the macrohemodynamics in unstressed animals [5]. It is a simple asphyxiant, which means that tissue hypoxia may occur when CH_4 displaces the air and hence the O_2 in a restricted space, and the concentration of O_2 is reduced to below approximately 18% in the internal milieu of the body. On the other hand, CH_4 can readily change the symbiosis with other gas molecules in closed spaces. The details and consequences of such *in vivo* relationships are basically unknown, because determination of the intracellular distribution of these gas molecules is technically limited.

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Only a few historical and contradictory data are available concerning the fate of CH_4 in nonbacterial biological systems. No detectable utilization of inhaled CH_4 was observed in healthy human volunteers [6], whereas 0.33% of intraarterially administered [^{14}C] CH_4 was converted to [^{14}C]CO_2 in the sheep [7]. The importance of these observations is uncertain, but a recent study with a comprehensive data set demonstrated high levels of oxidation and organic fixation of ^{14}C originating from [^{14}C]CH_4 in many organs, and especially the liver, in rats [8]. It was proposed that interactions with free radical reactions could lead to a higher level of fixation and perhaps the oxidation of CH_4 in a lipid environment, such as the mitochondrium membrane [8].

Criterion 2. *'Gasotransmitters are freely permeable to membrane. As such, their intracellular and intercellular movements do not exclusively rely on cognate membrane receptors or other transportation machineries'*

It is currently widely accepted that the bulk of the CH_4 produced by anaerobic fermentation in the mammalian intestine is excreted via the lungs, and breath testing has therefore become a tool for the diagnosis of certain gastrointestinal (GI) conditions [9–14]. Nevertheless, as a consequence of its physicochemical properties, endogenous CH_4 is distributed evenly across membrane barriers, and traverses the mucosa and enters the splanchnic microcirculation freely. Thus, it should be considered that the production of CH_4 is reflected not only in the exhaled air or in the flatus, but also in its passage through body surfaces. Indeed, the CH_4 concentration in the breath is usually > 1 ppm in only 30–60% of humans [15], but a recent study revealed the release of ~150 pg CH_4 cm^{-2} in 30 min through the skin in healthy individuals, corresponding to 313 fmol cm^{-2} min^{-1} [16]. This suggests that CH_4 transported by the circulating blood is excreted by the lungs only when a certain threshold is reached. For a perspective view of the release of CH_4, this can be compared with the release through skin emanations of acetone (median release of ~1100 fmol cm^{-2} min^{-1}), acetaldehyde (a median release of ~250 fmol cm^{-2} min^{-1}), 6-methyl-5-hepten-2-one (133 fmol cm^{-2} min^{-1}; tentatively originating from oxidative degradation of squalene), *n*-nonanal (a median release of ~60 fmol cm^{-2} min^{-1}; tentatively originating from the oxidative degradation of oleic acid) or isoprene (a median release of ~5 fmol cm^{-2} min^{-1}) [17].

Similarly, exogenous, inhaled CH_4 will move from the alveoli into the circulation, diffusing into the plasma, throughout which it is distributed rapidly and evenly [18]. The solubility of CH_4 in blood is rather low (a blood:air partition coefficient of 0.066) but the solubility in membrane bilayers is significantly higher (a partition coefficient of 0.20) [19, 20]. If there are no physical barriers to prevent its cellular entry, its concentration in all regions should be equal to the equilibrium concentration in the atmosphere (where it is normally 1–2 ppb), or to that in the inhaled air or that within the lumen of the GI tract, if these are the sole or predominant sources of CH_4. The fate of intracellular CH_4 is an open question, but there are many hydrophobic and hydrophilic interfaces in the cytoplasm and CH_4 may enter the hydrophobic nonpolar lipid tails of the phospholipid biomembranes [21]. This effect will be even stronger at high salt concentrations, because the hydrophobic interactions are enhanced as a result of the salting-out effect [22, 23]. This entry should be temporary, however, because, without a new supply, CH_4 will enter the circulation and then be excreted through the lungs if its partial pressure is higher than that in the atmosphere.

It follows that, if CH_4 is taken up by the cells in the organism, it is able to target the cytosol or cell organelles freely. Moreover, CH_4 may accumulate transiently at cell membrane interfaces, thereby transitorily changing the physicochemical properties or the *in situ* functionality of proteins, ion channels and receptors [24, 25] embedded within this environment, and in this case it may influence the function of membrane-bound structures.

Criterion 3. *'They are endogenously generated in mammalian cells with specific substrates and enzymes; more than the products of metabolism, their production is regulated to fulfill signaling messenger functions'*

Abiotic, purely chemical routes at high temperature and/or elevated pressure are known to lead to CH_4 formation [26], but these reactions are unlikely under ambient *in vivo* conditions. Nevertheless, in the complex ecosystem of the human GI tract, large amounts of CH_4 can be produced by carbohydrate fermentation, during which CO_2 is reduced to CH_4 by the anaerobic metabolism of methanogenic microorganisms [27]. The catalyzing enzyme of this pathway is methyl coenzyme M reductase, while the microorganisms are the Archae, a phylogenetically independent group, well distinguished from the usual bacteria and the eukaryotes. Their clinically important feature is the lack of peptidoglycan in the cell wall, which makes them susceptible to certain antibiotics [28]. As an obligate anaerobe, this taxon requires a redox potential of less than −300 mV for growth, a condition that is present in the GI tract of mammals and in other anoxic environments (underwater rice paddies or wetlands) [29]. The mammalian methanogens can be divided into two groups: H_2/CO_2- and acetate-consumers, which are members of a microbial consortium, where methanogens obtain substrates from higher levels, from H_2-producers or acetogens [30]. Methanogens are compelled to compete with other microorganisms, such as sulfate-reducing bacteria for the common substrates in the human colon [31].

The relevant human data are controversial, but CH_4 excretion is not detected in germ-free animals until shortly after they are contaminated with feces from a CH_4-producing animal [32, 33]. CH_4 breath testing in adults by means of lactulose ingestion reveals two distinct human populations, CH_4-producers and nonproducers, production usually being defined as a >1 ppm increase above the atmospheric CH_4 concentration [33]. The causes and consequences of this pattern are

subjects of debate and investigation; nevertheless, it is noteworthy that the proportion of CH_4-producers has remained relatively stable in the western population during recent decades, despite changes in GI medication and diets [34].

Although mammalian methanogenesis is widely considered to be an exclusive indicator of microbiological activity, *in vitro* and *in vivo* studies have also revealed the possibility of nonmicrobial CH_4 formation in eukaryote cells, in plants and in animals [35–39]. As long ago as 2003, it was shown that hypoxia can lead to the generation of measurable amounts of nonbacterial CH_4 in isolated mitochondria [40]. When the possible causes were explored, increasingly high amounts of CH_4 were reproducibly generated after the addition of ascorbic acid and hydrogen peroxide (H_2O_2), and the formation was related linearly to the quantity of mitochondria incubated, the amount of H_2O_2 added and the pH of the reaction mixture [40]. Catalase, which decomposes H_2O_2, abolished the increase in CH_4 production, which indicated that mitochondrial H_2O_2 is required for the hypoxic activation of the CH_4-generating reaction [40].

These findings were substantiated when Keppler *et al* provided direct evidence of aerobic CH_4 generation in plants [35]. This key paper was followed by many plant studies that either supported or disagreed with the initial findings, but aerobic nonbacterial CH_4 release was later clearly identified [41]. Many subsequent publications have confirmed the nonmicrobial CH_4 release in cell cultures under various stress conditions [42–44]. More importantly, it has been shown that oxido-reductive stress or even physical injury elicits aerobic CH_4 emission [45]. These works were followed by a comprehensive study in which CH_4 formation was detected in tobacco, grape vine and sugar beet cell cultures [38]. What was particularly interesting was that, under non-stress conditions, the cells produced only small amounts of CH_4, but the production was increased by one to two orders of magnitude when sodium azide (NaN_3) was added to the cultures [38]. The main effect of NaN_3 in plants is its direct and irreversible binding to the heme cofactor of cytochrome c oxidase, the final electron acceptor of the electron transport chain; thus, it can also be considered a tool via which to study mitochondrial oxido-reductive stress. Detailed overviews on CH_4 generation in plants were presented by Keppler *et al* [46], and in the recent review by Wang *et al* [45] the emission of CH_4 from plants is now clearly linked to a hitherto overlooked defense reaction.

In 2008, a study was undertaken which demonstrated aerobic CH_4 emission in cultured endothelial cells exposed to hypoxia and metabolic distress [47]. The latter included the inhibition of glucose uptake and anaerobic glycolysis, the application of site-specific inhibitors of the mitochondrial electron transport chain (NaN_3 and NaCN), alone or in combination with glycolysis inhibitors, the application of an uncoupling agent, and treatment of the cells with increasing concentrations of the hydroxyl radical-generating Udenfriend system (where the iron-catalyzed Fenton-type reaction between hydrogen peroxide and a transition metal is driven by ascorbate). These data provided clear evidence of stress-induced nonbacterial CH_4 production in eukaryotes [47]. The results revealed that a disturbance of the normal mitochondrial function led to significant CH_4 generation in endothelial cells (~2–23 nmol mg^{-1} range), depending on the nature and intensity of the metabolic distress, and a similarly high and dose-dependent level of CH_4 generation was measured after free-radical attack via the Udenfriend reaction.

In parallel with these studies, significant CH_4 release was also demonstrated in whole animals under hypoxic stress conditions [37, 48], where changes in exhaled or released CH_4 changes were detected *in vivo*. As a further step, CH_4 exhaled from the airways or discharged through the skin and body orifices was quantified by means of a whole-body CH_4 detection setup using photoacoustic spectroscopy [39, 49]. The *in vivo* CH_4 production profile was determined after the induction of mitochondrial distress by chronic NaN_3 administration, and the magnitude of the whole-body CH_4 emission was compared with that in rats treated with antibiotics to eradicate CH_4-producing intestinal bacteria. The emanation of endogenous CH_4 was detected throughout the 8 d experiments, and the inhibition of mitochondrial cytochrome c oxidase by chronic NaN_3 administration induced a significant level of CH_4 generation, independently of the methanogenic flora [39].

Thus, the overall evidence from these findings suggests that the excretion of CH_4 in the breath in mammals reflects not only intestinal bacterial fermentation, but also unidentified nonbacterial generation induced from target cells. In the setups involving NaN_3 administration, the inhibition of complex IV could cause a rearrangement of the electron transport and result in the increased production of reactive oxygen species (ROS) at complexes II and III. It is therefore possible that the formation and constant build-up of ROS in the mitochondria are components of a reaction that furnishes CH_4 in the living organism. As in plants, the release of CH_4 may be associated with ROS generation after transient intracellular O_2 deprivation [38, 39, 43, 47], and may be an integral feature of cellular responses to changes in oxidative status in all eukaryotes.

2. Chemical processes leading to aerobic CH_4 formation.

After the paper by Keppler *et al* describing the *in situ* formation of CH_4 in plants, the accompanying editorial commentary asked what mechanism could be involved in the production of the fully hydrogenated gas CH_4 in an oxidizing environment [35, 50]. Many publications are now available on the *in vivo* generation of CH_4 under distinct pathophysiological conditions, but the exact mechanism of mammalian aerobic CH_4

generation is basically unexplained, and at present a number of cellular sources are subjects of investigation. This is mainly due to the various possible sources and unknown reaction pathways that can be envisaged. However, as is evident from the following list, the common denominator of the different experimental setups has been hypoxia or ischemia and reperfusion with transient oxido-reductive stress.

- The first hypothesis proposed the possible role of electrophilic methyl (CH_3) group (EMG)-containing biomolecules, leading to CH_4 formation under reductive stress conditions [51]. It has been hypothesized that, if the sources of the CH_3 groups are molecules such as phosphatidylcholine or S-adenosylmethionine, the substitution will lead to the generation of CH_4. Mitochondrial experiments partially confirmed this theory (i.e. under highly reductive conditions, potentially electron acceptor biomolecules can be reduced, leading to the formation of CH_4).
- After the above hypothesis was forward, our research group identified CH_4 formation in animal mitochondria [40]. To clarify the possible mechanism, an experimental setup was established which involved substances ubiquitously available in biotic systems, but without biological structures. In a chemical model reaction, the formation of CH_4 from choline was demonstrated in the presence of H_2O_2, catalytic iron (Fe^{3+}) and ascorbic acid [40]. In this exothermic reaction, CO_2 and CO are formed in parallel with CH_4 generation. The components of the reaction mixture (other than uncomplexed iron) are known to be present in comparatively high concentrations in biological systems, but the *in vivo* significance of these chemical processes remained unknown. Thus, during the past decade other possible mechanisms have been proposed, with partial modification of the previous presumptions [44, 46, 47].
- Through the use of simple buffers with compounds thought to be involved in aerobic CH_4 formation, a ROS-generating Udenfriend system was established, which consisted of an EMG or CH_3-donating compound (MDC)/electron acceptor (e.g. choline chloride, betaine or phosphatidylcholine), reducing agents (e.g. ascorbate, NADH, NADPH, dithiothreitol or N-acetyl-L-cysteine), H_2O_2 and uncomplexed Fe^{3+} as catalyst. In this series of experiments, when the CH_4-generating capacity of choline metabolites was tested, the amount of CH_4 formed was generally observed to increase linearly with the number of CH_3 groups in the molecule (choline > 2,2-dimethylethanolamine > 2-methylethanolamine > ethanolamine) [47].
- Althoff *et al* proposed an alternative, but similar approach under more controlled conditions [52] for the aerobic formation of CH_4 by the oxidation of ascorbic acid with iron compounds and H_2O_2. On the basis of experimental results with MDCs, methionine and methionine sulfoxide were used, which exhibited the highest CH_4-formation ability, and the sulfur-bonded CH_3 group in methionine was unambiguously identified by stable isotope labeling techniques as the carbon precursor of the CH_4 molecule [53, 54]. These findings are in accordance with the concept of EMGs, which also explains the differences in CH_4-formation ability between similar molecules. For example, although choline readily produces CH_4, the analogous molecule betaine, which also contains an EMG, does not. In betaine, the nitrogen-bonded CH_3 group is sterically shielded and electrons are repelled by the electronegative N atom, reducing its reactivity.
- In these model experiments, the highest level of CH_4 formation was measured at around pH 3.0 under hypoxic conditions, which can be explained at least partially by chemical reasons. Deprotonation of ascorbate ($pK_a = 4.37$) leads to an acidic pH, at which the production of hydroxyl radicals is favored, unlike the situation at higher pH, where H_2O_2 produces nonreactive oxygen species [55]. A more thorough analysis of postulated reaction routes and detected and possible intermediates is to be found in Althoff's works [52–54].
- A very similar process was described in plants [35, 36, 46]. CH_4 release was reported from both fresh and dried foliage, in some cases after UV light irradiation. Later, pectin was shown to be the one of the origins of CH_4, the carbon atom arising from the methoxy (CH_3O) group. We refer here to an excellent review [45], in which the authors summarize the key characteristics of possible CH_4-generating compounds, and in particular those involved in methanogenesis in plant cells.
- The positive correlation between CH_4 production and temperature (30–70 °C), the possibility of CH_4 liberation from dried leaves and the heavily exothermic nature of the model reaction (up to 95 °C) suggested a chemical explanation rather than an enzymatic catalytic process [35, 40]. Nevertheless, a modified reaction using methionine sulfoxide as substrate led to significant CH_4 generation at ambient temperature [54]. In this chemical reaction, CH_4 is readily formed from the S-CH_3 groups of organosulfur compounds with tremendously varying yields, in a model system containing iron(II/III), H_2O_2 and ascorbate that uses organic compounds with heterobonded CH_3 groups for the generation under ambient (1000 mbar and 22 °C) and aerobic (21% O_2) conditions (figure 1).

It should be added here that nonmicrobial CH_4 release from methionine has been confirmed in fungi under aerobic conditions [56] and methionine is known to be a key factor in many biochemical reactions in plants, fungi and animals. Methionine residues in the surface of proteins are highly susceptibility to oxidation, with the product generally being methionine sulfoxide, and it has been suggested that the susceptibility to oxidation of proteins is proportional to the surface exposure of the methionine residues [57]. More importantly, the available data suggest that reversible methionine oxidation could be a novel mechanism in redox regulation, which involves the oxidation to methionine sulfoxide leading to an activated protein function [58]. The repair mechanism of methionine sulfoxide reductases (Msr) is capable of reducing the protein-bound methionine sulfoxide back to methionine, in a stereospecific manner. It is noteworthy that cells lacking MsrA and MsrB genes are respiratory-deficient due to lower levels of mitochondrial cytochrome c, and can have a shorter lifespan [59]. The *in vivo* generation of CH_4 in association with the non-enzymatic methionine-methionine sulfoxide pathway or the methionine-Msr system has never been investigated, though it might be strongly relevant to the biochemistry and the role of biotic CH_4 formation under oxidative conditions (figure 2). The *in vivo* role of choline or choline metabolites in endogenous methane formation is substantiated by the results showing that exogenous PC can suppress the methanogenic reaction [37]. Similarly, when L-alpha-glycerylphosphorylcholine (GPC), a water-soluble, deacylated PC derivative was administered in NaN_3-induced chemical hypoxia, the extent of CH_4 generation was reduced [39].

In summary, no direct enzymatic route of aerobic, nonbacterial biotic CH_4 generation has yet been proven. Perhaps there is no need for it. If we consider the whole scale of the published *in vitro* findings, basically two postulations can be made. (1) A prerequisite of CH_4 formation under ambient conditions is the availability of a donor compound containing one or more EMGs or MDCs or other related functional groups, and (2) a further prerequisite is the presence of oxidative or reductive stress (elevated reducing potential and radical reactions). It seems that various organic compounds can serve as the source of the liberated CH_4. The common property of these molecules is the presence of a CH_3 [54] or related group: CH_3O [42, 46], CH_3CO [44] or $HOCH_2$ [42]. Practically ubiquitous compounds such as pectin, lignin, phospholipids, amino acids or even proteins may serve as depots of these functional groups. Another common feature of the chemical processes explored so far is the presence of a highly reactive radical. However, the expanding literature on aerobic CH_4 generation has not gone hand in hand with an understanding of the complexities of intracellular redox chemistry. Hydroxyl radicals can be produced in the iron-catalyzed Fenton or Haber–Weiss reactions during hypoxia and reoxygenation, and by virtue of its *in situ* reactivity this radical is able to break intramolecular bonds in membranes, leading to the formation of CH_4. Nevertheless, the data of Althoff *et al* suggested the critical role of a ferryl species ($[Fe^{IV}=O]^{2+}$) and methyl radicals, leading to the *in vitro* generation of CH_4 from the starting methionine molecule [54]. This reaction route uses ferrihydrite, a biomimetic iron compound which releases Fe^{2+}, maintaining a steady-state ion concentration. Most importantly, the process is theoretically relevant *in vivo*, since the inorganic core of ferritin is formed from ferrihydrite, and the generation of ferryl species is commonly mediated by iron-containing oxygenases [60].

(4) *'Gas mediators have well-defined specific functions at physiologically relevant concentrations,* ***(5)*** *the functions of endogenous gases can be mimicked by their exogenously applied counterparts and* ***(6)*** *they are involved in signal transduction and have specific cellular and molecular targets'.*

In a discussion of these aspects of CH_4 biology it should be born in mind that there is a conceptual difference between the baseline or physiological generation of a gas (e.g. NO, CO and H_2S) and that after *de novo* induction or discordant alteration by inducer factors, and the affected processes or evolving responses may therefore be dependent on the number of molecules and/or the reactivity of the microenvironment. In this sense, the physiological levels of CH_4 in the human body have not yet been determined. In general terms, about one-third of healthy individuals emit gaseous CH_4 identified by breath testing [34], but the significance of endogenous CH_4 generation in the human body is still an open question.

Once generated by anaerobe microbes, or released by a nonbacterial process, CH_4 is widely considered to be biologically inactive. However, some data do suggest an association with the small bowel motility as the whole gut transit time is longer in human subjects who produce more CH_4 than in those defined as nonproducers [11]. More directly, diarrheal conditions such as inflammatory bowel disease are negatively associated with CH_4 production, and there is evidence that the production of CH_4 as determined by breath testing is associated with delayed intestinal transit and constipation [13, 61]. Nevertheless, there are many inconsistencies in human clinical investigations, and we refer here to a recent review in which the authors summarize the key characteristics of CH_4-caused GI motility changes in humans, and in particular those which are clearly linked to bacterial methanogenesis [11].

Somewhat more straightforward data are available from experimental animal models. Using a physiological model of peristalsis, Pimentel *et al* explored the effects of CH_4 on the neuromuscular function in the guinea pig ileum [14]. Gassing the bath with CH_4 significantly increased the amplitude of contraction both orally and aborally in response to a stimulus. In a similar setup, luminal CH_4 infusion reduced the transit time by an average of 59% in dogs, and thus it was there-

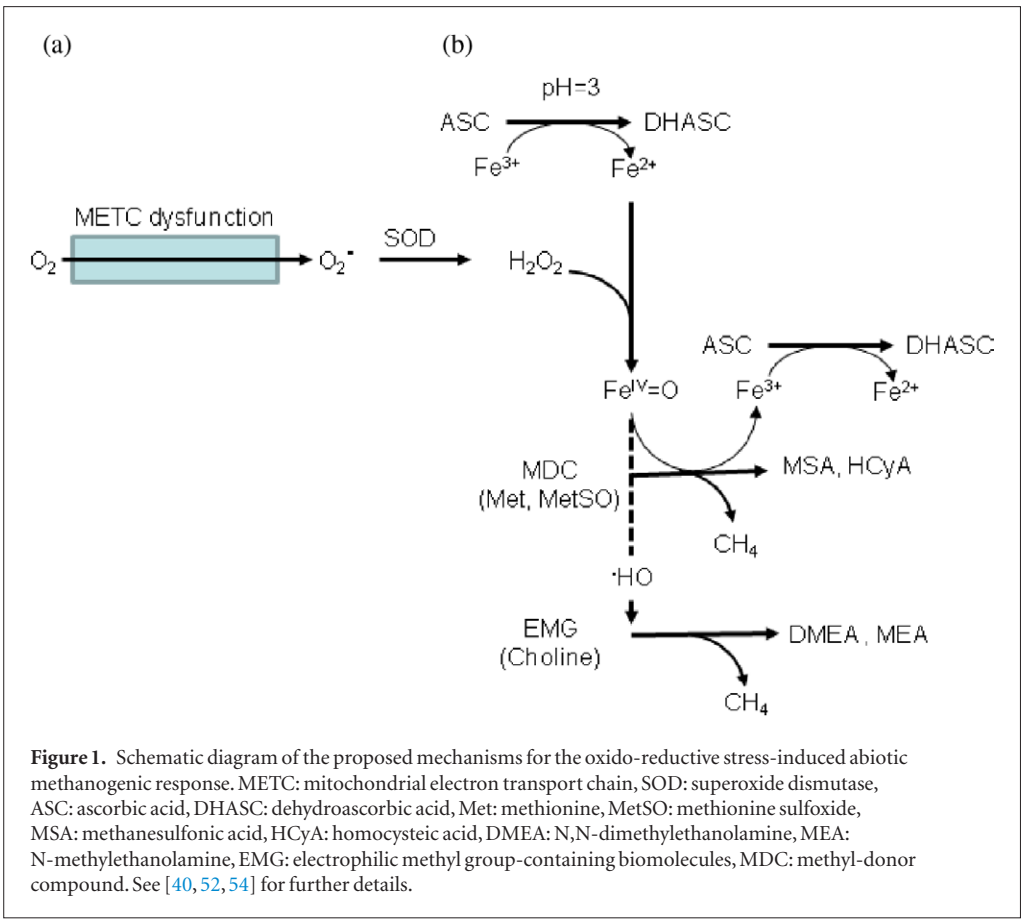

Figure 1. Schematic diagram of the proposed mechanisms for the oxido-reductive stress-induced abiotic methanogenic response. METC: mitochondrial electron transport chain, SOD: superoxide dismutase, ASC: ascorbic acid, DHASC: dehydroascorbic acid, Met: methionine, MetSO: methionine sulfoxide, MSA: methanesulfonic acid, HCyA: homocysteic acid, DMEA: N,N-dimethylethanolamine, MEA: N-methylethanolamine, EMG: electrophilic methyl group-containing biomolecules, MDC: methyl-donor compound. See [40, 52, 54] for further details.

fore hypothesized that CH_4 activates a reflex pathway that leads to slowing in the proximal intestinal segment. Finally, it was concluded that CH_4 may act as a neuromuscular transmitter, resulting in reduced propagation of the peristaltic movement in the intestine [14]. Another supportive study demonstrated that gaseous CH_4 delayed the contraction velocity of peristalsis and increased the amplitude of peristaltic contractions in the guinea pig ileum *in vitro* [62]. Whether the infusion of gases adequately mimics the physiologic setting remains unknown, but another comprehensive study showed that the contractility of the intestinal muscles and/or their contraction rhythm were influenced by CH_4-induced decreases in the postprandial serotonin level [63]. Moreover, high levels of methanogen-produced CH_4 were found in rats that consumed high-fat chow and also in obese human subjects, and the extent of colonization of methanogens in the GI tract of animals and humans was positively correlated to the development of obesity [64, 65]. Furthermore, a report is available where CH_4 inhibited the contractile activity of the proximal colonic longitudinal muscle by activating the voltage-dependent potassium channels and increasing the voltage-dependent potassium current of colonic smooth muscle cells *in vitro* [66]. Taken together, these data strongly suggest that CH_4 might modulate the signaling activity of the enteric nervous system in both health and pathologies.

Nevertheless, exogenously applied CH_4 has been shown to have other biological and/or signaling functions *in vitro* and *in vivo*. Information on the cardiovascular effects of CH_4 is sparse, but a historical paper reported an increased survival time in hemorrhaged rats after treatment with a CH_4-air mixture [67]. It has additionally been shown that normoxic ventilation with 2.5% CH_4 supplementation protects the tissues by mitigating the effects of an ischemia-reperfusion insult [5]. In this animal model, the levels of tissue ROS generation were reduced, the mesenteric vascular resistance changes were only moderate, and the intestinal pCO_2 gap (a marker of the microcirculation) tended to normalize after reperfusion. As decreased tissue and plasma granulocyte activities were also found, the effects of CH_4 on the polymorphonuclear (PMN) leukocyte functions were further investigated by using isolated cells. The *in vitro* results substantiated the *in vivo* findings, and established that CH_4 exposure specifically decreases the ROS production of activated PMN leukocytes in a hitherto unrecognized reaction pathway [5]. More importantly, the inhalation of 2.5% CH_4 decreased the signs of oxidative and nitrosative stress, with reduced structural damage [5].

3. Summary

In an evaluation of the role and the significance of CH_4 in physiology, it seems reasonable to proceed via previously defined points [1]. CH_4 has a long evolutionary history on Earth, but the observation that CH_4 formation occurs in many hypoxic systems has opened up new, interesting and challenging research avenues. Is it a waste product or is it still bioactive?

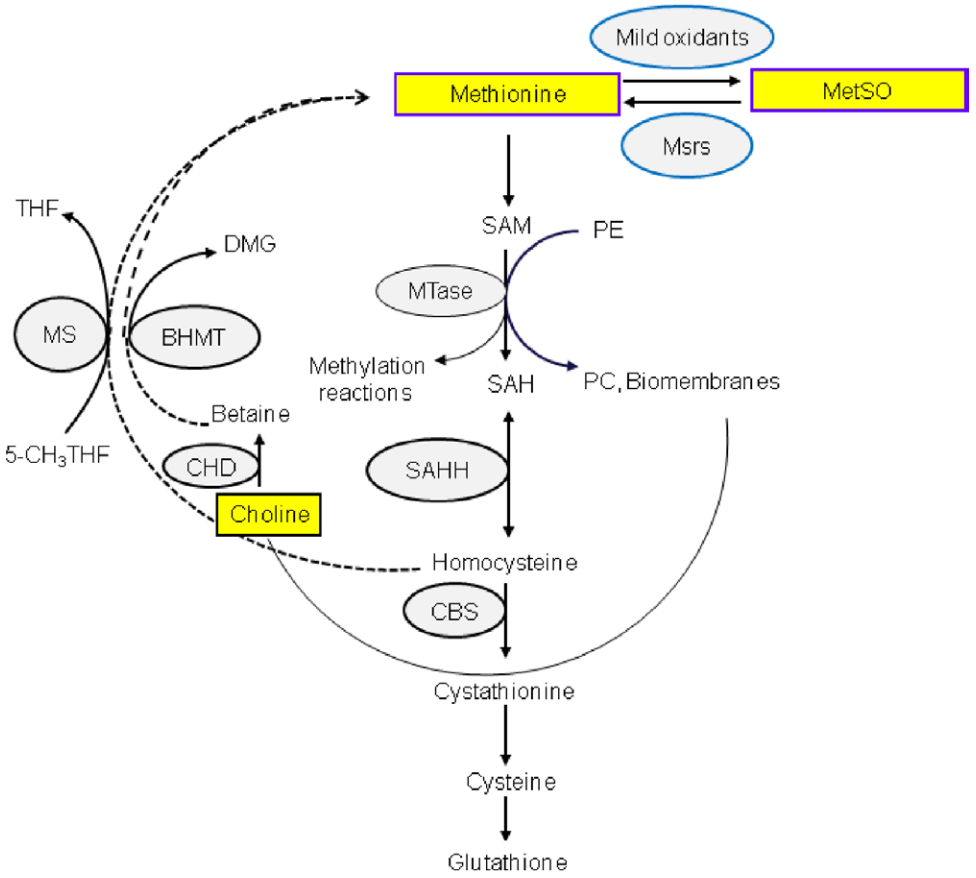

Figure 2. The metabolic routes and the possible links between the most relevant substrates (i.e. methionine sulfoxide generation, choline and the folate-methionine cycle) involved in biotic methane generation. THF: tetrahydrofolate, BHMT: betaine-homocysteine methyltransferase, SAM: S-adenosyl methionine, SAH: S-adenosyl homocysteine, SAHH: SAH hydrolase, MS: methionine synthase, CBS: cystathione-beta-synthase, DMG: dimethylglycine, CHD: choline dehydrogenase, PE: phosphatidylethanolamine, PC: phosphatidylcholine, Mtase: SAM-dependent methylases, MetSO: methionine sulfoxide, Msrs: methionine sulfoxide reductases.

Various data suggest that the excretion of CH_4 in the breath of mammals may predominantly reflect intestinal bacterial fermentation, but a variable amount is possibly linked to a mitochondrial dysfunction. If nonbacterial CH_4 is added to the bacterial production, this addition could occur at a time and rate that is impossible to be detected by the conventional techniques that have thus far been utilized to look for it.

In a consideration of aerobic biotic CH_4 generation, the origin of the emission and the underlying mechanisms are still not known with certainty, and the immediate challenge is therefore to test the proposed hypotheses for aerobic CH_4 formation from different biomolecules and cellular/tissue structures, and to construct a comprehensive picture of the specific importance of these reactions in mammals. Although the results presented to date establish a bioactive role for CH_4, it is not obvious whether it originates from bacterial, external or endogenous sources. As an analogy, other gaseous compounds, such as NO, H_2S or CO, were previously thought to be toxic or to be without effects on the function of the living aerobic organisms. If CH_4 bioactivity is acknowledged, it is tempting to speculate that a low, but stable proportion of intrinsic CH_4 is required to keep the inflammatory signals in resting conditions in the GI tract. Indeed, in contrast with other organs, the gut wall is persistently exposed to bacterial toxins, products of phagocytic cells and non-bacterial antigens that cross the mucosal epithelium, and this presupposes the action of a system which tunes or modulates the constant pro-inflammatory activity. Hypoxic events could have additional effects in the way in which they enhance inflammatory activation, leading in parallel to CH_4 generation.

Whether there is a cellular membrane 'receptor' for such events needs to be elucidated. The subcellular molecular targets are not known either. Thus, overall these data do not fully support the gasotransmitter concept, but they do support the notion that CH_4 liberation may be linked to redox regulation. In this respect, the available information suggests that hypoxia-induced CH_4 generation may be a necessary phenomenon of aerobic life, and perhaps a surviving evolutionary trait in plants and animals.

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