Gasotransmitters

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Preface

Gasotransmitters are real and exist everywhere in our body. Gasotransmitters are unique and specifically regulate biological functions. Gasotransmitters are novel signaling gas molecules we cannot live without.

Since its inception in 2002 (R. Wang, Two's company, three's a crowd—Can H_2S be the third endogenous gaseous transmitter? *FASEB J.*, 2002, **16**, 1792–1798), the concept of 'gasotransmitters' has been widely accepted and applied in different life-science disciplines. The initial members of the gasotransmitter family included nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S). This concept has continuously evolved and been refined with the inclusion of new members, such as ammonia (NH_3) (R. Wang, Gasotransmitters: Growing pains and joys, *Trends Biochem. Sci.*, 2014, **39**, 227–232[†]). The birth and growth of the gasotransmitter framework have deepened our understanding of cellular signaling processes and led to the discovery of new pathogenic mechanisms and therapeutic strategies for related diseases. Research on gasotransmitters has also gone beyond the boundaries of mammalian biology and medicine. The production and function of gasotransmitters in plant and bacteria, for example, have attacted the attention and stimulated the interest of numerous research teams and researchers worldwide.

Over the last 15 years, the gasotransmitter literature has exponentionally grown. Each year, about 15 000 papers are published on gasotransmitters. A recent study revealed that, before 2004, H_2S biology-related publications were less than 100 per year. By 2015, the annual publications on H_2S biology-related research had increased by about seven-fold (G. Yang and L. Wu, Trends in H_2S biology and medicine research—A bibliometric analysis,

[†]Note: This seminar paper framing the concept and establishing the qualification standards for gasotransmitters has been reprinted as an appendix of this book.

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CHAPTER 8

Production and Signaling of Methane

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8.1 Introduction

Many gases are biologically active *in vivo*, but contrary to 'classical' pathways of signal transduction, the exact roles of gaseous compounds in the mediation of extra- or intracellular events are to date not completely understood. These molecules are also at the forefront of medical research due to the anti-inflammatory effects of nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S). Methane (CH_4) has a long evolutionary history on Earth, and it is also part of the gaseous environment that maintains the metabolism within the aerobic eukaryote cells but, in fact, the role of CH_4 in physiology is largely unmapped. Conventionally believed as physiologically inert, studies cited in this review suggest that it can modulate the pathways involved in oxidative and nitrosative stress responses and key events of inflammation.

This chapter is divided in two parts, the first one being devoted to the biogenesis of CH_4 in eukaryotes and the interactions of CH_4 with other biological gases *in vivo*, while the second part deals with well-documented biological responses and their potential physiology- and pathology-related implications.

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8.2 Physico-chemical Properties and Toxicity of CH_4

The bioactivity or toxicity of gas mediators NO, CO, and H_2S is related to their tendency to react with biologically important molecules. Despite this, CH_4 is intrinsically non-toxic *in vivo*; rabbits can inhale a mixture of one volume of oxygen and four volumes of CH_4 for any length of time without showing any obvious side-effects. It is a simple asphyxiant, which means that tissue hypoxia might occur when an increasing concentration of CH_4 displaces the inhaled air in a restricted area, and the concentration of oxygen is reduced to below approx. 16–18% in the internal milieu. With a density of 0.716 $g\,L^{-1}$ under standard conditions of temperature and pressure, CH_4 is lighter than air; nevertheless, CH_4 intoxication can occur in open fields as well.[1] In such cases, respiratory arrest is not due to the chemical specificity of the gas, but to the decreased O_2 content. CH_4 will displace oxygen down to 18% in air when present at about 14% (or 140 000 parts per million by volume, ppmv). It should be mentioned that CH_4 is combustible and forms explosive mixtures with air at concentrations between 5% (lower explosive limit) and 15% (upper explosive limit) at room temperature. In the early days of colonoscopy, an accumulation of colonic gas and the use of electrosurgical devices sometimes led to intracolonic explosions.[2] It is expected to cause unconsciousness due to central nervous system (CNS) depression when it reaches high concentrations (30% or so), well above the lower explosive limit and level for asphyxiation.

The inhalation of normoxic air containing 2.5% CH_4 for 3 h or CH_4-rich saline (MRS) treatments for several days had no side-effects on the blood gas chemistry (pH, PaO_2, $PaCO_2$), in unstressed animals.[3,4] When the effect of exogenous CH_4 on the respiratory activity of the mitochondrial oxidative phosphorylation (OxPhos) system was investigated by high-resolution respirometry, the incubation of 2.2% CH_4 did not affect the activity of OxPhos complexes of intact rat liver mitochondria.[5] MRS treatment had no significant effect on the redox system of normal retinal tissue and did not affect the cytochrome c release or activation of caspase-9 and caspase-3 in rats.[4] When 10 $mL\,kg^{-1}$ MRS was applied intraperitoneally and micromolar CH_4 levels were detected in the circulation, the plasma glucose and hematocrit levels did not change. Data on human cardiovascular effects are sparse, but in a case report with a 45 min CH_4 exposure to liquid manure, the unconscious patient presented spontaneous breathing with an arterial pH value of 7.26 and made a full recovery later.[6]

8.3 Methanogenesis – Biotic and Abiotic Sources in the Environment

In the atmosphere, which currently contains over 1.8 ppmv, CH_4 is an important greenhouse gas. The atmospheric concentration of CH_4 has increased dramatically since pre-industrial times from about 715 parts per billion by volume (ppbv) to 1800 ppbv in the year 2010.[7,8] The current global

budget of atmospheric CH_4 is of the order of 550–650 Tg (million tonnes) of CH_4 per annum.[8] The global atmospheric CH_4 budget is determined by many natural and anthropogenic terrestrial and aquatic surface sources, balanced primarily by one major sink (hydroxyl radicals) in the atmosphere. However, a large fraction of the emissions is mainly the result of environmental microbial processes, such as archaeal methanogenesis in wetlands, rice fields, and ruminant and termite digestive systems.

8.3.1 Abiotic Sources of CH_4 (Including Thermogenic Degradation of Organic Matter)

About a quarter of all CH_4 sources are associated with chemical processes, including emissions from mining and combustion of fossil fuels, and the burning of biomass or geological sources such as volcanoes and geothermal systems. The largest fraction of this chemically formed CH_4 comes from thermal degradation of organic matter and it is sometimes not classified as abiotic because the precursor substance was delivered from biological compounds.

Strictly speaking, abiotic (or abiogenic) CH_4, formed by chemical reactions, does not directly include organic matter and it is produced in much smaller amounts on a global scale. These reactions occurs on Earth in several specific geological environments and they may be produced by either high-temperature magmatic processes in volcanic and geothermal areas, or *via* low-temperature (<100 °C) gas-water-rock reactions in continental settings. For example, these reactions might involve the hydrogenation of CO_2, also known as the 'Sabatier reaction'. For more details regarding the abiotic formation of CH_4 on Earth, we refer the reader to a review by Etiope and Sherwood Lollar.[9]

8.3.2 Microbial Methanogenesis – Formation of CH_4 by Archaea

Usually, biogenic methanogenesis is regarded as a microbial process carried out by a unique class of prokaryotes (archaea). Methanogens do not use oxygen to respire and the terminal electron acceptor is carbon. The two best-described pathways involve the use of acetic acid and inorganic carbon dioxide (CO_2) as terminal electron acceptors. Methanogenic archaea and CH_4 production are typically found in ruminants or termites or in the environment in wetlands and landfills, and at those sites where organic matter is decomposing in the absence of oxygen or other oxidants, such as nitrate, sulfate, or ferric iron. In all methanogenic archaea, CH_4 is formed from methyl-coenzyme M (CH_3–S–CoM) by reduction, where the methyl coenzyme M reductase catalyzes the reaction between thioether methyl coenzyme M and thiol *N*-(7-mercaptoheptanoyl)threonine 3-*O*-phosphate to give CH_4 and a mixed disulfide.[10,11]

In both rumen and the hindgut of insects (termites, cockroaches, scarab beetles), CH_4 seems to be exclusively produced by hydrogenotrophic methanogens that utilize hydrogen gas (H_2) and CO_2 and their activity is dependent on the availability of H_2. CH_4 production from acetate plays no obvious role and acetoclastic methanogens are usually not detected in such environments.[7] In the rumen, CH_4 production largely occurs in the gut lumen where it reduces H_2 to concentrations that are not permissive for acetogenesis. In the insect hindgut, however, methanogenesis is restricted to the gut wall or other gut structures, or it is associated with gut flagellates, while in the gut lumen acetogenesis is often the main H_2-consuming process. The microbiology and methanogenic processes in ruminants and termites have been reviewed in detail.[12–15]

In contrast to ruminants, where all animals produce and emit substantial amounts of CH_4, only around one third of humans are considered to emit CH_4 at measureable rates. CH_4 is produced in the human large intestine by microbial activity, with *Methanobrevibacter smithii* being the predominant archaeon that utilizes H_2 and CO_2. Human CH_4 production will be further discussed later (Section 8.5).

8.3.3 Non-archaeal CH_4 Formation in Eukaryotes

Until recently, biological CH_4 formation had been associated exclusively with anoxic environments and microbial activity (prokaryotes – archaea). However, recent studies have unambiguously confirmed direct (endogenous) CH_4 release from eukaryotes, including plants, fungi, lichens, marine algae, and animals, even in the absence of microbes and in the presence of oxygen (Figure 8.1).[16–26] Thus, the recently found formation of CH_4 in eukaryotes is often termed 'aerobic' or 'non-microbial' CH_4 formation. However, in this chapter, we prefer to use the term 'non-archaeal CH_4 formation'.

Various *in vitro* and *in vivo* experimental data have established the possibility of biotic, non-archaeal generation of CH_4 under various conditions. Precursor compounds such as pectin, cellulose, lignin, ascorbic acid, leaf waxes, and methionine have been shown to deliver methyl groups for CH_4 production.[18,21,27–30] Furthermore, it was found that aerobic CH_4 release may be stimulated in plants by increasing the temperature, physical injury, reactive oxygen species (ROS) and UV radiation, and inhibition of cytochrome c oxidase by sodium azide (NaN_3).[30–33] Along these lines, significant *in vivo* CH_4 release was demonstrated in a rodent model of chemical asphyxiation, after chronic inhibition of the activity of mitochondrial cytochrome c oxidase by NaN_3.[34] However, at this time, the biochemical reactions leading to CH_4 production in plants and mammals and the potential enzymes involved have not been identified yet, even though some experimental data seem to indicate that CH_4 formation may be linked to redox regulation and be connected with hypoxic events.[35] In this respect, it was hypothesized that hypoxia-induced CH_4 generation may be a necessary phenomenon of aerobic life, and perhaps a surviving evolutionary trait in eukaryote cells.[17]

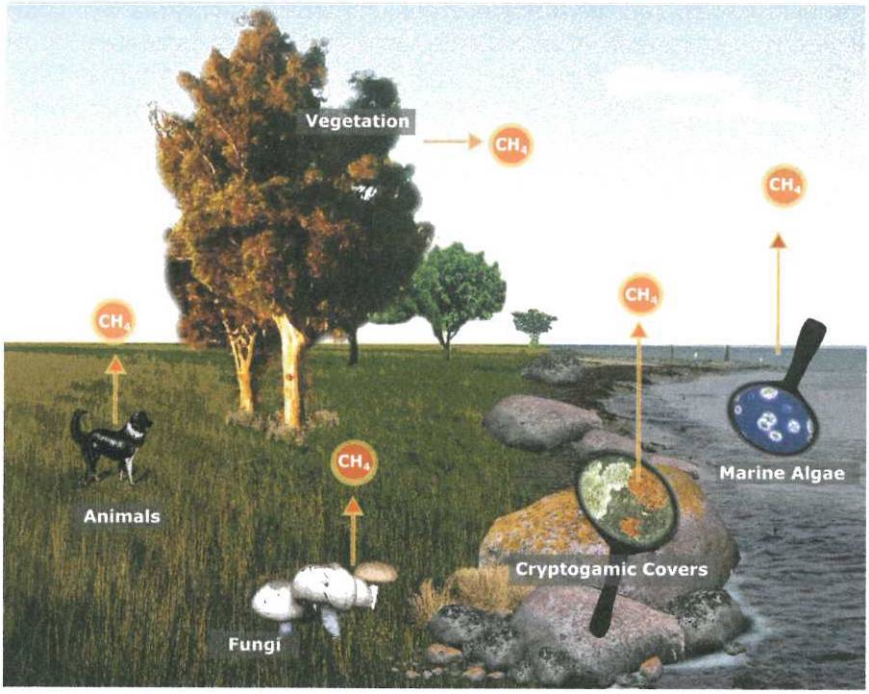

Figure 8.1 Conceptual scheme showing the recently identified novel sources of non-archaeal CH_4 in the biosphere (designed by Thomas Klintzsch, Heidelberg University, and reproduced with permission).

Future research should particularly seek to answer the question of whether CH_4 generation is a by-product of the chemical degradation of biomolecules, *e.g.*, induced by UV irradiation, increased temperatures, or hypoxia, or whether it also plays a more general physiological role (see below). It appears that, in plants, high rates of CH_4 generation may be linked to environmental stress. Similar mechanisms might be active in animals, and probably humans as well, producing CH_4 when the organisms are under external or internal (*e.g.*, inflammation) stress.

8.4 Potential Pathways of CH_4 Formation in Eukaryotes

While the biochemistry of methanogenesis in archaea (procaryotes) has been well described, pathways of aerobic CH_4 generation from eukaryotes including plants, mammals, fungi, and algae have yet to be elucidated and its precursor compounds identified.[36] However, some studies are available proposing potential reaction schemes that may possibly occur in eukaryotes.[17,21,33,37]

It has been hypothesized that the hetero-bonded methyl groups of biomolecules, such as the sulfur-containing amino acid methionine or the

ammonium salt choline, might be carbon precursors of CH_4 in living cells. In this context, it was proposed that electrophilic methyl groups (EMGs) bound to positively-charged nitrogen moieties (such as the choline molecule) may potentially act as electron acceptors, and that these reactions may entail the generation of CH_4 in animal cells.[38] A continuous lack of the electron acceptor O_2 will maintain an elevated mitochondrial NADH/NAD^+ ratio, causing the formation of a nucleophilic hydride ion that is transferred to the EMG (see Figure 8.2). Such an anomalous increase in reducing power also occurs in pathologies involving the interruption of electron flow down the mitochondrial electron transport chain (ETC). This may be supported by the observation that non-microbial CH_4 generation from animal cells was observed when endothelial rat liver cells were exposed to site-specific inhibitors of the ETC.[25]

It has been argued that similar mechanisms might occur in plant mitochondria and chloroplasts. The ETC in plant mitochondria consists of four multi-subunit complexes, respiratory complexes I–IV, assisted by ubiquinone and cytochrome c (see Figure 8.3). The mitochondrial electron transport chain is a potential source of ROS such as superoxide and H_2O_2, with complexes I and III being their main sites of production. Complex IV receives electrons from reduced cytochrome c and transfers them to an oxygen molecule. The complex functionality can be inhibited by NaN_3, which blocks the electron transport.[39] Whiskerman *et al.* investigated the possibility of non-microbial CH_4 formation in heterotrophic plant cell cultures such as tobacco BY-2 (*Nicotiana tabacum*), grape vine (*Vitis vinifera*), and sugar beet (*Beta vulgaris* L.).[33] They also examined the disturbance of mitochondrial functionality using ETC inhibitors such as NaN_3, rotenone, and salicylhydroxamic acid (SHAM). Under non-stress conditions, the plant cell cultures produced trace amounts of CH_4, but these could be increased by one to two orders of magnitude when NaN_3 was added to the cell cultures. The addition of other ETC inhibitors did not result in significant CH_4 formation, indicating that a site-specific disturbance of the ETC at complex IV causes CH_4 formation in plant cells.[33]

Figure 8.2 The proposed reaction scheme for CH_4 formation in hypoxic animal cells. The nucleophilic hydride-ion (H^-) is transferred to an electrophilic methyl group. This is followed by separation of the methyl group and the formation of CH_4.
Adapted with permission from F. Keppler, M. Boros, C. Frankenberg, J. Lelieveld, A. McLeod, A. M. Pirttila, T. Rockmann and J. P. Schnitzler, *Environ. Chem.*, 2009, **6**, 459 © The Authors.

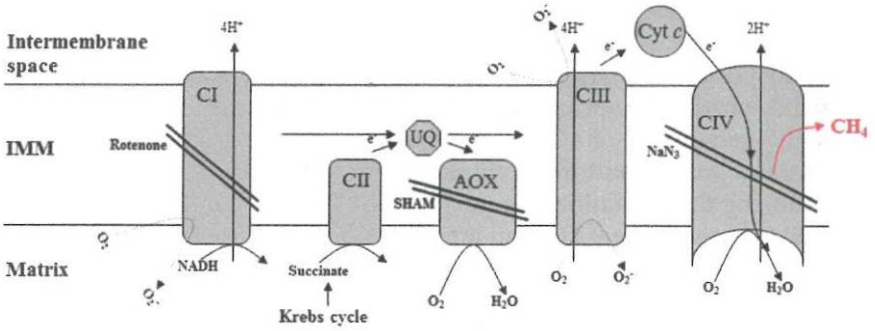

Figure 8.3 Electron transport chain in the inner membrane of plant mitochondria (after Møller 2007, modified). CI–CIV stand for complexes I–IV, UQ – ubiquinone, Cyt c – cytochrome c. Inhibitors: rotenone, SHAM, and NaN_3. Methane is formed when sodium azide, a compound known to disrupt the electron transport flow at cytochrome c oxidase (complex IV) in plant mitochondria, is added to cell cultures.
A. Wishkerman, S. Greiner, M. Ghyczy, M. Boros, T. Rausch, K. Lenhart and F. Keppler, *Plant Cell Environ.*, 2011, **34**, 457, John Wiley and Sons, © 2010 Blackwell Publishing Ltd.

Furthermore, a reaction of ROS with methoxyl groups (OCH_3) of pectic polysaccharides was suggested as a possible route to CH_4 formation in plants exposed to UV radiation.[32] This study concluded that UV radiation evokes CH_4 production from pectic methyl groups by interacting with UV photosensitizers to generate hydroxyl radicals, but it was also suggested that other diverse processes might generate hydroxyl radicals and contribute to CH_4 emissions independently of UV irradiation.

Recently, Althoff *et al.* presented a novel chemical reaction that readily forms CH_4 from organosulfur compounds, such as methionine, dimethylsulfide (DMS), and dimethylsulfoxide (DMSO), under highly oxidative conditions, ambient atmospheric pressure and temperature.[21] In the first phase of the reaction, methyl sulfides are oxidized to the corresponding sulfoxides.[21] Then, in the next phase, demethylation of the sulfoxide *via* homolytic bond cleavage leads to methyl radical formation and finally to CH_4. In this reaction, the oxidant for both phases was proposed to be a ferryl species (see Figure 8.4). However, other oxidants (including ROS) are also conceivable. Because sulfoxidation of methyl sulfides is ubiquitous in the environment, they suggested that this novel chemical route might be involved in CH_4 formation in living aerobic organisms. Thus, it could be envisaged that these thioethers and sulfoxides might be a direct precursor of the CH_4 formed from eukaryotes. The highest CH_4 formation rates might be expected from aerobic organisms, especially when oxygen availability is limited or the organisms are under hypoxia. This conclusion is in broad agreement with previous results, which demonstrated enhanced CH_4 formation in animal cells under reduced oxygen content.[25]

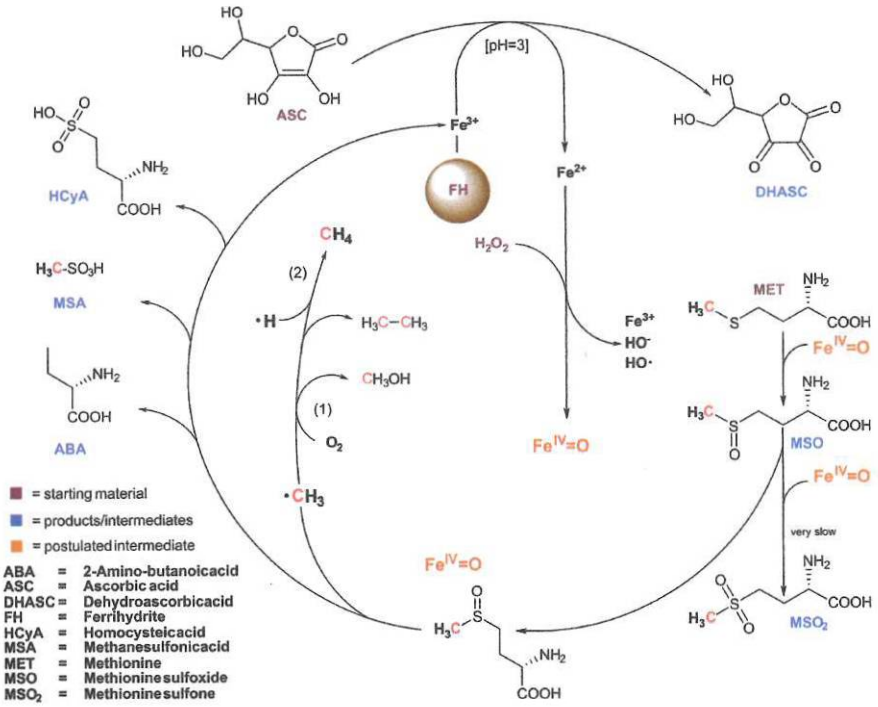

Figure 8.4 Potential route for CH_4 formation using amino acid methionine as the methyl precursor. The carbon atom present in the CH_4 molecule is highlighted in red.
Adapted with permission from F. Althoff, K. Benzing, P. Comba, C. McRoberts, D. R. Boyd, S. Greiner and F. Keppler, *Nat. Commun.*, 2014, 5, 4205. © The Authors.

8.5 Human CH_4 Production – Archaeal and Non-archaeal Sources

In humans, several gaseous products are formed as a result of various eukaryotic (human) and prokaryotic (bacterial) activities by enzymatic or non-enzymatic processes. Methanogenesis in humans has long been considered an exclusive attribute of methanogenic archaea, a group quite distinct from the usual bacteria and eukaryotes.[40–42] These strictly anaerobic inhabitants of the gastrointestinal tract produce CH_4 from decomposing organic matter through hydrogenotrophic, methylotrophic, and acetotrophic classes of methanogenesis. From a breath analysis, approximately 30–60% of adults were classed as CH_4 producers when production was defined as a >1 ppmv increase above the ambient air level.[43] CH_4 on breath testing is associated with higher levels of *Methanobrevibacter smithii* in stool and the proportion of *M. smithii* in stool also correlates well with the amount of breath CH_4.[44] Some conditions are thought to increase the CH_4 production within the colon, such as excessive intracolonic anaerobiosis and elevated intracolonic pH.

In regard to CH_4 production, gender, age, and ethnic differences have been observed.[42,45,46] Moreover wide day-to-day (inter-day) variations and reduced emission after physical exercise have also been reported.[47,48] However, the fundamental factors influencing the number of methanogens and the amount of CH_4 produced are still not known. Many studies have reported correlations between breath CH_4 levels and afflictions such as irritable bowel syndrome, large bowel cancer, and constipation.[42,43,49–51] However, the findings of these studies remain controversial and the impact of endogenous microbial CH_4 generation still has not been determined.

The relationship between age and breath CH_4 concentration is crucial when interpreting the results from studies investigating the correlation between breath CH_4 levels and specific diseases such as diverticulosis and large bowel cancer.[43,52] Interestingly, two recent studies showed that, in a German population with an age range from 4 to 95 years, the percentage of breath CH_4 producers greatly varied with age.[46,53] When subjects were divided into age groups of 15 years, a significant increase in the percentage of breath CH_4 producers with age was observed. The incidence of many diseases increases with age and, if this is not taken into account, the concurrent increase observed in the percentage of breath CH_4 producers with age might easily lead to a misinterpretation of the data arising from supposed correlations.[46] For more detailed information regarding the occurrence of CH_4 in man, and its possible link to certain diseases, we refer the reader to reviews by de Lacy Costello.[42,43]

Quite significantly, a recent study using a stable carbon isotope and high precision concentration measurements provided evidence that the exhaled CH_4 levels of all volunteers investigated ($n = 112$) in an age range from 1 to 80 years were above (on average ~118 ppbv) the inhaled CH_4 concentration.[53] Based on their data, the authors hypothesized that next to microbial sources in the gastrointestinal tracts there might be other, as yet unidentified, non-archeal processes involved in CH_4 formation, which supports the idea that humans might also produce CH_4 endogenously in cells. In this sense, the physiological levels of CH_4 in the human body have not yet been determined.

In the case of the gastrointestinal tract, various data suggest that the excretion of CH_4 in the breath of mammals may predominantly reflect intestinal archeal production, but a variable amount is possibly linked to a mitochondrial dysfunction. Significant CH_4 formation was detected in unrestrained rats treated with NaN_3, which led to the selective and stable inhibition of mitochondrial cytochrome c oxidase activity.[54] The NaN_3-induced global mitochondrial dysfunction was evidenced by hepatic ATP depletion, and a systemic inflammatory reaction. CH_4 exhaled from the airways, together with the amounts discharged from the skin and body orifices, was quantified by means of whole-body photoacoustic spectroscopy. Through the determination of the amount of CH_4 released from the animals at different times, this study demonstrated that chronic NaN_3 administration was accompanied by increasing emanation of endogenous CH_4 throughout the

entire duration of the experiments, irrespective of the concomitant antibiotic treatment targeting the potential CH_4-producer gastrointestinal microbial flora.[26] To sum up, it has been shown that CH_4 excretion reflects the intestinal microbial fermentation along with an unknown and variable amount of generation induced from target cells, and if non-archaeal CH_4 is added to the constitutive low-level CH_4 steady-states or microbial production, this addition could occur at such a low rate that it is impossible to detect by any conventional technique.

Apart from the above considerations, the designation of subjects as 'producers' or 'non-producers' based on CH_4 breath testing only accounts for one way of escape, but not the large amount that is passed directly as flatus (between 50% and 80% of the total). Indeed, there is a release of $\sim$150 pg CH_4 cm^{-2} in 30 min through the skin in healthy individuals, corresponding to a 313 fmol cm^{-2} min^{-1} discharge.[55] It should be added that a recent study using highly sensitive open-circuit respiration chambers (which account for the total CH_4 emission) found that natural differences in CH_4 yields between individual sheep were not due to naturally differing densities of methanogenic archaea in the rumen, *i.e.*, greater densities of methanogens in high CH_4 animals and lower densities in low CH_4 animals, suggesting that other – genetic, epigenetic, or environmental – factors are present in the background.[56]

Another significant issue is that the large differences in breath gas analysis data and the rather wide day-to-day (interday) variations[47] are presumably not only due to variations in the personal background, bacterial strains, sampling, and analysis techniques, but have hemodynamic and microcirculatory causes as well. It should be borne in mind that intraluminally generated CH_4 traverses the gastrointestinal mucosa and enters the splanchnic microcirculation freely. Thereafter, owing to its physicochemical properties, CH_4 is transported by the circulating blood and, when reaching the lungs, it is partially released into the breath if the partial pressure is higher than that in the atmosphere. In this context, it may be presumed that exhaled CH_4 levels will vary in association with gastrointestinal perfusion changes and, in this way, the breath CH_4 output may also be regarded as a marker for mesenteric microcirculatory alterations. However, the correct characterization of this association warrants the dynamic measurement of breath gases. Indeed, in a recent human study with CH_4-producing volunteers, the on-line measured alveolar breath CH_4 level decreased dramatically (from 11.4 to 2.8 ppmv) during treadmill exercises, while the lung ventilation–perfusion ratio increased by a factor of 2–3. Based on mass balance equations and a three compartment model, the dynamics of CH_4 profiles were described and it was found that the breath CH_4 concentration was affected not only by changes in the ventilation–perfusion ratios, but also by changes in the fractional large intestinal blood flow.[48]

A great deal of effort has been devoted toward the measurement of breath CH_4 levels in humans, but the clinical utility of CH_4 measurement methods is controversial.[57] The intra- and intersubject variabilities are usually very

large, and the interpretation of the results is often difficult. CH_4 producers may stop excreting, non-producers may start to excrete CH_4, and occasionally the CH_4-producing status does not change after antibiotic treatments targeting the intestinal methanogenic flora.[58–60] Indeed, it has recently been suggested that the clinical implications of breath CH_4 analyses should themselves undergo an in-depth revision.[61] It should be mentioned that, in clinical laboratory practice, breath CH_4 levels are usually determined by sampling of breath air in gastight bags, which are then analyzed by means of gas chromatography (GC) equipped with either flame ionization (FID), thermal conductivity, or mass spectrometry detectors. Thus, the sampling frequency of these traditional methods is rather limited. The major problems are that discontinuous detection methods cannot accurately reflect the overall profile of *in vivo* CH_4 generation and that, because CH_4 distributes itself evenly across membrane barriers, the production is manifested not only in the exhaled air but also through other body surfaces.

Recent advances in analytical methods for high temporal resolution measurements of CH_4 might further improve our understanding of CH_4 formation in humans. Tuboly *et al.* applied a near-infrared laser technique-based photoacoustic spectroscopy (PS) system for *in vivo* studies, which had previously been validated for real-time whole-body and single breath measurements of CH_4 emissions in human and animal studies.[26,34] With this technique, a daily CH_4 production profile can be determined and stress-caused changes or treatment effects may be accurately evaluated with reproducible results. In addition, Keppler *et al.* used high resolution optical spectroscopy (Cavity Ring Down Spectroscopy, CRDS) for the investigation of CH_4 in breath samples.[53] This method is capable of precisely determining the concentration and the stable carbon isotope composition of CH_4 with high temporal resolution.

8.6 Intestinal Gases and the Influence of CH_4 on Gastrointestinal Motility

In a pioneering study, pulmonary CH_4 excretion ranged from undetectable to 0.66 mL min^{-1}, and 20% of the total CH_4 produced was excreted *via* the lungs.[40] There are two major sources of gas in the gut; namely, swallowed air and gas produced locally during the fermentation of colonic contents or independently from the gut microbiome. A third component (CO_2) is generated by an acid–base reaction in the duodenum, but this is rapidly diffused back into the circulation and exhaled. At rest, the intestines receive about 15% of the total blood flow, which is completely adapted to meet the transport and metabolic needs of gut tissue. In fact, the volume of intestinal gas is fairly stable because expeditious gas transit and evacuation prevent gas pooling; hence, gaseous distension of the bowel does not occur so long as absorption by blood or elimination as flatus keeps pace with production.[62] According to previous studies, values for intra-intestinal gas

volumes range from 176 to 199 mL. More recently, CT image analyses gave similar results (median volumes were 155–220 mL) and the endogenous gas production or composition was not that different between patients with gas-related complaints and healthy subjects.[63,64] Despite this, it is likely that the quantitative equilibrium of gas concentrations in the intestinal compartments is influenced by qualitative changes. While the breathing of N_2O causes the expansion of air in the bowel lumen and additionally augments the accumulation of intestinal gases, it has been demonstrated in a canine study with CH_4- or CO_2-filled bowel segments that, when dogs breathed oxygen, the bowel gas volume decreased, while breathing N_2O just increased the volume of the CH_4-containing segment. Breathing oxygen after 30 min of breathing N_2O reduced the volume of the CH_4-containing segment toward control volumes.[65] In brief, alterations in one or other gas components in the intestinal lumen can affect the other gases and the net result of these processes determines the final composition of the gaseous environment.

Gas molecules in this milieu are likely to have access to a variety of ion channels and the receptor components of the neuromuscular apparatus that are involved in gastrointestinal motility regulation and, in this respect, the association between CH_4 production (again, defined as more than 1 ppm increase for breath testing above the atmospheric CH_4 level) and intestinal transit seems to be well established: CH_4 production is usually associated with a constipation-related phenotype.[66,67] It has been shown not just in patients, but also in healthy volunteers that the total colonic transit times are significantly more prolonged in CH_4-producers than in non-CH_4 producers; those who present CH_4 in exhaled air during the lactulose H_2 breath test have a delayed orocecal transit compared to those with no CH_4 production.[68,69] Other reports also support the observation that diarrheal conditions such as inflammatory bowel disease (IBD) are negatively associated with CH_4 production and a link was suggested between the low gastrointestinal transit time and the CH_4 production capacity (*i.e.*, a higher abundance of methanogenic archaea) in IBS patients.[67,70,71] It has also been shown that a constipation phenotype is associated with a higher abundance of methanogenic archaea. Based on these observational data, Gottlieb *et al.* proposed a causative role for CH_4 in constipation-related gastrointestinal disorders (which supports the CH_4-first hypothesis). In this case, a feedback loop exists where CH_4 produces better survival conditions for methanogen archaea, and an increase in the gastrointestinal transit time promotes the growth of archaea until a steady state is reached.[72] Nevertheless, there are still 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-linked gastrointestinal motility changes in humans, especially those linked to microbial methanogenesis.[67]

Data are also available from *in vitro* models of intestinal peristalsis. There are obvious limitations of perfusion of gases to mimic the actual physiological setting of the small bowel or colonic environment, but with a constant concentration of 980–1010 ppmv of infused CH_4 and constant pH in

the organ bath, regardless of the rate of flow, Pimentel *et al.* demonstrated that CH_4 exposure significantly augmented the contractile force of the guinea pig ileal segments.[73] Based on these data sets, it was proposed that CH_4 predisposes one to constipation because it slows the transit process and promotes segmental (non-propagating) contractions.[73] These results are consistent with another series of studies involving dog ileal segments; here, CH_4 infusion at a rate that corresponded to an increase of 50 ppmv in exhaled air induced a 59% slowing down of the intestinal transit, whereas room air had no effect. In another report, Liu *et al.* using circular and longitudinal muscle strips after pre-treatment with tetrodotoxin to distinguish between the direct action of CH_4 on smooth muscle cells and the indirect action mediated by intrinsic nerves and *N*-nitro-L-arginine methylester to inhibit nitrergic mechanisms, demonstrated that CH_4 significantly attenuated the spontaneous contractile amplitude of longitudinal muscle strips isolated from rat intestines.[74] Thereafter, Jahng *et al.* used a similar setup to detect velocity changes in peristaltic contractions using isolated guinea pig ileum and right and left colon segments gassed with a control (95% O_2–5% CO_2), H_2, or CH_4, and again, the velocity of ileal peristaltic contraction decreased while the amplitude of peristaltic contraction increased after CH_4 infusion.[75]

Taken together, these findings strongly suggest that CH_4 might modulate the peristaltic activity and signaling mechanisms of the enteric nervous system. Although there is no direct evidence that CH_4 influences gastrointestinal mediator levels, in key *in vitro* studies, CH_4 infusion augmented the intestinal contractions of isolated intestinal segments in both the orad and aborad directions relative to a stimulus. Freely diffusing through membranes, CH_4 could affect any of the neuromuscular elements participating in a reflex mechanism of the enteric nervous system without the involvement of the brain–gut axis. Indeed, there is some pertinent data in the literature that suggests CH_4 may influence the gastrointestinal membrane structures embedded in the lipid bilayer. In a recent study, Liu *et al.* demonstrated that adding a 3% CH_4 solution significantly increased the density of voltage-dependent potassium channels (IKV) (from 13.3 ± 1.0 pA/pF to 18.5 ± 1.4 pA/pF at +60 mV) in an isolated single colonic smooth muscle cell system.[74] Furthermore, Pimentel *et al.* showed that CH_4 production is associated with a lower postprandial serotonin (5-hydroxytryptamine, 5-HT) response in CH_4-producing IBS subjects with constipation.[76] Serotonin is a potent stimulator of gut peristalsis, and 95% of all serotonin secreted by enterochomaffin cells is found in the gastrointestinal tract. It has been demonstrated that the baseline serotonin levels were not different between CH_4 producers and non-producers but, after a carbohydrate challenge, the serum serotonin concentration was significantly lower in the CH_4-producing IBS subjects compared to those who were H_2-producers. Interestingly, an early study with isolated, perfused, and ventilated rat lung preparations demonstrated the potency of halogenated methane to inhibit the uptake of 5-HT.[77]

8.7 Effects of CH_4 on the Metabolism

More attention is being paid to the role of gastrointestinal microflora in association with the energy metabolism of the human body. The physiological effects of endogenous CH_4 levels on extra-intestinal systems have not yet been evaluated, but lots of data suggest that CH_4 itself may play a specific role in the metabolism and energy homeostasis in humans. The energy balance is defined as the equilibrium between the ingested meal energy and the energy excreted as waste products, the energy being used for metabolism, and the energy incorporated into the body as growth, reproduction, and fat stores. Quite surprisingly, higher exhaled CH_4 levels are associated with greater body mass indexes (BMIs) among obese human subjects; and humans with increased concentrations of exhaled CH_4 exhibit increased levels of obesity compared to individuals with lower breath CH_4 concentrations.[78,79] Elevated breath CH_4 in humans is associated with a higher increase in the absolute glucose levels when undergoing an oral glucose challenge than their non-CH_4 producing counterparts, independent of the BMI.[80] If CH_4 *per se* is slowing the intestinal transit, this may increase the duration of the postprandial nutrient absorption (with a direct effect on the gastrointestinal motility), and the slowing of the transit process could result in higher levels of methanogenic microflora as well (the indirect effect on motility); hence, both of these effects could lead to increased weight gain and the development of obesity.[78]

The coexistence of H_2-producing bacteria with H_2-utilizing methanogenic archaea in obese individuals leads to the hypothesis that H_2 transfer between bacterial and archaeal species is an important mechanism for increasing the energy uptake by the human large intestine in obese persons.[81] Indeed, using real-time polymerase chain reaction, Zhang *et al.* detected significantly higher numbers of H_2-utilizing methanogenic archaea in the gastrointestinal tract of obese population than in normal-weight individuals.[81]

An increased abundance of methanogen strains has been observed in the cecal flora of Ob/Ob mice as well. It has been suggested that methanogens affect the caloric harvest by increasing the capacity of polysaccharide-consuming bacteria to digest polyfructose-containing glycans, which leads to increased weight gain in mice.[82] Studies of gnotobiotic normal mice colonized with the principal methanogenic archaeon in the human gut, *Methanobrevibacter smithii*, and/or *B. thetaiotaomicron* revealed that co-colonization not only increases the efficiency but also changes the specificity of bacterial polysaccharide fermentation, leading to a significant increase in adiposity compared to mice colonized with either organism alone.[83]

In a study, the colonization of the rat gut with *M. smithii* was not limited to the large bowel, but rather extended to the small bowel, including the ileum, jejunum, and duodenum. Therefore, it was suggested that obese human subjects may have increased numbers of methanogens in the small bowel, rather than in the colon, thus exerting slowing effects in the small bowel while preserving the colonic transit.[84]

Rats that had gained more weight had higher stool levels of *M. smithii* than rats that had gained less weight, and the extent of colonization of the bowel with *M. smithii* colonization also corresponded with weight gain in these rats, irrespective of the diet. Taken together, these findings support the opinion that the level and extent of colonization of the intestinal tract with *M. smithii* is predictive of the degree of weight gain in this animal model.

We need to ask whether these changes are causally linked to the presence of methanogenic microorganisms, and more precisely, to the presence of their product. The direct link between CH_4 and energy metabolism needs further elucidation, but chronic oral administration of bromochloromethane (BCM), a compound that reduces the activity of the methanogen populations, induced an obese trend in Sprague Dawley rats. What is more, the expression of peroxisome proliferator-activated receptor gamma (PPAR-γ), lipoprotein lipase, protein phosphatase 2A, and adiponectin genes was universally upregulated, and the expression of the fasting-induced adipose factor (Fiaf) gene, a target of PPAR-α, was downregulated.[85] After termination of BCM treatment and followed either with or with no re-incubation with a faecal methanogen mixture, blood parameters and gene expression returned to the original levels only in rats with faecal methanogen populations. These results suggest a transient, direct effect of CH_4 on energy homeostasis and imply that that influencing the CH_4 production in either direction might influence the energy homeostasis. Moreover, PPAR-γ, originally identified as a key regulator of lipid metabolism, inhibits the activation of the nuclear factor NF-κB, while the upregulation of the macrophage/Kupffer cell PPAR-γ leads to the attenuation of the pro-inflammatory tumor necrosis factor (TNF-α) response and endothelial dysfunction.[86–88]

It should be mentioned that a shift in the energy balance may alter the inflammatory status as well and emerging evidence strongly supports an anti-inflammatory role for CH_4.[3] Also, it is worth noting that physical exercise, particularly endurance training, has been shown to produce substantial amounts of ROS in skeletal muscle from both mitochondrial and non-mitochondrial sources, which include xanthine oxido-reductase (XOR), NADPH oxidases, and phospholipase A_2, among others. In a rat model of one-time exhaustive exercise, treadmill running induced a weight loss, a decrease in blood glucose levels, and an increase in blood lactate, creatine kinase (CK), and urea nitrogen (UN) concentrations (parameters of muscle injury and protein metabolism, respectively); in addition to structural muscle changes, the signs of inflammatory activation, including leukocyte accumulation (evidence *via* myeloperoxidase activity), increased plasma levels of interleukins (IL-1β, IL-6, IL-10), and the TNF-α were present.[89] In this model of intense endurance exercise where the main sources of energy were aerobic oxidation and glycolysis, exogenous CH_4 administration (intraperitoneal injection of MRS) prolonged the treadmill running time by 27 min, normalized the changes in blood lactate and glucose, reduced the elevations in CK and UN, and the parameters of exercise-induced pro-inflammatory activation.[89] These findings partly suggest that exogenous CH_4

may improve the skeletal blood flow, which increases the oxygen supply and the percentage of aerobic oxidation (not only was the production of lactate in muscle reduced, but the metabolic clearance of blood lactate was also higher), and they partly indicate that there is an anti-inflammatory activity for MRS as well. Along these lines, it has recently been shown that the nuclear factor-erythroid2 p45-related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1) pathway is one of the major cellular defence mechanisms that operates during acute exercise stress in the skeletal muscle and, more significantly, it has also been demonstrated that the anti-inflammatory activity of CH_4 is partially mediated by the activation of Nrf2 signaling.[4,90]

8.8 Interaction with Other Biological Gases: CO, NO, and H_2S

Biological gases form complex extra- and intracellular pathways and gas mediators may regulate a great many processes in an antagonistic or synergistic way. The gastrointestinal lumen also contains a range of potentially bioactive gas metabolites such as CO, H_2, NH_3, and H_2S. The gas composition of the stomach is actually quite similar to that of inhaled air, but the composition of the ileal and colonic gas environment is different. In the complex ecosystem of the gastrointestinal tract, methanogens are compelled to compete with other microorganisms such as sulfate-reducing bacteria for their common substrates in the colon; hence, CH_4 is present in the intestinal atmosphere in variable amounts, in close symbiosis with other gas molecules. The details and consequences of such *in vivo* relationships are nevertheless basically unknown, because detection of the *in vivo* dynamics and distribution of these gas molecules is technically limited. However, the reciprocal and synergistic relation among gasotransmitters with diverse effects on basal cell functions has recently been experimentally demonstrated.[91] It was shown that CO induces an elevation in H_2S production and NO, one of the most important biological transmitters, and that it can interact with H_2S, which may determine the final biological effects of both gaseous transmitters.[92]

H_2 can also act as an electron donor for dissimilatory sulfate reduction. The major end product of this process is sulfide, which is rapidly hydrolyzed to H_2S, defined as a gasotransmitter. Sulfate-reducing bacteria can also get energy by oxidizing molecular hydrogen while reducing sulfate to H_2S. Hydrogen may pass through the gut wall into the blood and be transported to the lungs, where large excretion rates have been found.[93,94]

The volume of H_2 in the bowel of healthy subjects varies from 0.06 to 29 mL and H_2 production, which averages 0.24 mL min^{-1} in the fasting state, increased by seven-fold after intestinal instillation of lactose. Indeed, methanogens are unique in that their metabolism increases in the presence of gaseous products as they use molecular H_2 to reduce CO_2 to CH_4.[94,95] The conversion of H_2 into CH_4 is a reaction associated with the reduction of

intestinal gas volume, because the reaction reduces five volumes of gas to one ($CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$).[96]

To date, the interplay of CH_4 with H_2, NO, CO, or H_2S in mammals has not yet been investigated systematically. Yet, on the one hand, it has been shown that the inhibition of mitochondrial cytochrome c oxidase (complex IV), an important target of NO under hypoxia, leads to CH_4 generation.[33,34] On the other hand, it was demonstrated that normoxic CH_4 ventilation decreases the tyrosine nitrosylation after ischemia–reperfusion (IR) injury, a process that involves NO and peroxynitrite formation.[3] Notably, CH_4 can influence the ROS (mainly superoxide) production of activated immune cells or ROS-producing enzymatic and non-enzymatic pathways in parenchymal cells. In principle, the diffusion-limited reaction between superoxide and NO leads to the formation of peroxynitrite (ONOO) and nitrotyrosine (3-NT), the latter being widely recognized as a biochemical marker of the post-translational modification of proteins. The nitrotyrosine generation process involves a covalent modification, but it may also be a dynamic and reversible process.[97] In our *in vivo* animal model, nitrotyrosine formation was significantly suppressed by increasing the CH_4 input prior to stress induction, which means the removal of peroxynitrite from the reaction. In a recent study, CH_4 also suppressed 3-NT production in the cortex and hippocampus, as compared to controls in a rat model of CO poisoning.[98] This further suggests that the effect of an increased CH_4 input on tissue nitrosative stress is linked to a process that depends on the tissue superoxide and/or NO generation. Moreover, some of the effects exerted by CH_4 in model systems of inflammation can be accounted for by the indirect modulation of functions of NO. It is also likely that the two gases are able to modulate the effect of each other at membrane interfaces, where their concentration is at its peak.[99] NO can directly inhibit mitochondrial functions *via* several pathways, and if the effects of CH_4 are NO-influenced or mediated, the NO-mediated inhibition could be reversed by CH_4-containing gas mixtures.

Somewhat clearer data are available on the links of CH_4 with other gas messengers in plant pathophysiology. The effects of CH_4 supplementation to CO and NO biology were repeatedly observed in adventitious root formation, during the adaptation to abiotic stress and germination inhibition by NaCl or copper.[100–104] The administration of CH_4-rich water (MRW, *ca.* 0.021 g $CH_4\,kg^{-1}$ water, *i.e.*, *ca.* 1 mM CH_4) increased the adventitious root formation partly through a pathway including heme oxygenase-1 (HO-1).[100] Dose-response experiments demonstrated differences in the most effective doses of CH_4 among the species, but a 24-h pre-treatment with MRW not only induced the gene expression of target genes of adventitious root formation, but also the increased expression of CsHO1 and protein levels of HO-1. Pre-treatment with zinc protoporphyrin, a specific inhibitor of HO-1, abolished both CH_4-triggered root formation and the expression of genes involved in the process. Of note, by direct application of CO (the product of HO-1 thought to mediate the above effects), the CH_4-mediated rooting could be partially restored. Moreover, the involvement of Ca^{2+} was demonstrated

in the effects of CH_4 as the removal of Ca^{2+} from the incubation medium by chelation abolished the beneficial effects of CH_4.

Interestingly, adventitious root formation is reported to be partly mediated by NO and H_2S, and, in a recent paper, it has been shown that NO is a downstream signaling molecule involved in the CH_4-induced adventitious root formation of cucumber plants.[101,105,106] In these studies, the same concentration of CH_4 as a pre-treatment did indeed trigger increased NO generation through a mammalian NOS-like enzyme-dependent and a diamine-oxidase-dependent pathway. cPTIO and PTIO, specific NO scavenger compounds, managed to abolish the effects of CH_4, much like the NOS inhibitor L-NAME and diamine oxidase inhibitor β-HEH. In contrast, the inhibition of nitrite reduction, which is an alternative source of NO, did not influence the root formation or NO levels. Furthermore, CH_4 treatment did indeed increase the protein *S*-nitrosylation, an important post-translational modification mediated by NO. Interestingly, similar effects were observed in a similar model with CO treatment and hydrogen gas treatment, where NO also played a downstream mediator role for CH_4.[107,108]

In a salinity toxicity model with 100 mM NaCl-treated alfalfa seeds, sustained endogenous CH_4 production was detected during the germination process, in general agreement with previous reports of the research group of Keppler.[16,102] MRW (30%) alleviated the NaCl toxicity and, interestingly, further increased the endogenous CH_4 production, similar to the NaCl-induced stress. MRW re-established the altered ion homeostasis upon NaCl stress, especially by increasing the K^+/Na^+ ratio. MRW led to an increase of the antioxidative capacity of ascorbate peroxidase, superoxide dismutase (SOD), and guaiacol peroxidase isoforms, while the superoxide anion production and oxidative damage decreased. The expression of HO-1 was elevated during salt stress, and MRW pre-treatment further increased its expression. By blocking HO-1 with the zinc protopophyrin inhibitor, the beneficial effects of CH_4 were partly diminished, which demonstrates the contribution of HO-1 and CO to CH_4-induced protection, like that for adventitious root formation. Hence, it was suggested that HO-1 might be a central enzyme of NO and CH_4-linked responses for salinity stress in plants.

A copper (Cu) overdose also induces the inhibition of seed germination and, as a redox active metal, elevated Cu levels directly increase ROS formation. Like other stress factors, Cu treatment did significantly increase the CH_4 formation in alfalfa, and exogenous CH_4 was able to hinder Cu accumulation.[103] Furthermore, MRW prevented membrane lipid peroxidation, as indicated by the reduced thiobarbituric acid reactive-substance levels. An assessment of plasma membrane integrity using Evans blue and Schiff's reagent also supported the above findings. Interestingly, CH_4 treatment in the absence of Cu also caused slightly increased staining, which may demonstrate the direct effects of CH_4 on the plasma membrane fluidity.[109] Besides showing the beneficial effects on antioxidant enzyme systems, the authors were able to demonstrate pathologically increased proline accumulation under Cu stress, which was prevented using MRW. In conclusion,

exogenous CH_4 in plants is able to reduce oxidative stress and promotes the activation of antioxidant defence systems, much like in mammals.

8.9 Bioactivity of Exogenous CH_4

The oxidative or reductive by-products of cells can influence the physiological responses but, once released, CH_4 is widely regarded as biologically inactive. Although a historical paper reported an increased survival time in hemorrhaged rats after treatment with a CH_4–air mixture, such paradigms are deeply rooted and not easy to modify.[110] Apart from studies on the biological role of endogenously generated CH_4, several studies have demonstrated explicit effects of exogenous CH_4 in eukaryotic biological systems. In the case of NO or H_2S, the administration of precursors may stimulate endogenous release or the enzymatic synthesis of the compounds can be induced.[111,112] However, with CH_4, direct delivery is a feasible option for increasing the *in vivo* concentration up to the theoretical upper limit (*i.e.*, 5%). In our studies, gas inhalation was used with artificial air containing 21% O_2 and variable amounts of CH_4. Increasing the CH_4 concentration up to 2.5% in normoxic artificial air did not influence the blood gas chemistry and hemodynamics in mechanically ventilated anesthetized dogs or in rodents. In this way, the inspired CH_4 is quickly transported from the lungs to the organs by the circulating blood and attains levels of 2–3-fold or more over basal concentrations in tissues, which is sufficient to modulate the local ROS and RNS production (*i.e.*, peroxynitrite and superoxide generation).[109] Another possible way of administration is MRW in plants or MRS in mammals, using pure CH_4 dissolved in distilled water or a 0.9% saline solution under 0.4 MPa for 3 to 8 h to reach a supersaturation level. These solutions are stored under atmospheric pressure at 4 °C in γ-radiation-sterilized aluminium bags with no dead space, and freshly prepared to ensure that the CH_4 concentration exceeds a minimum of 1.5 mmol L^{-1} before administration. Chen *et al.* reported that the average CH_4 concentration one day after preparation was 1.6 mmol L^{-1}, which remained relatively stable over four weeks, and that the reduction in CH_4 concentration was $\leq$15%.[113]

The kinetics of CH_4 was studied after the inhalation of normoxic CH_4–air mixtures and ip MRS treatment in rat models. A 15 min inhalation of a normoxic CH_4–air mixture at a flow rate of 300 mL min^{-1} resulted in 6.6 ppm mg^{-1} tissue CH_4 in ileal tissue (detected by the PS technique) and 1.5 ppm mL^{-1} CH_4 in the caval vein.[109] Substantially elevated concentrations of CH_4 were measured in spinal cords as early as 10 min after a 10 mL kg^{-1} ip MRS injection and the levels remained high after 12, 24, 48, and 72 h of treatment (117.3 ± 14.1, 105.1 ± 12.3, 93.5 ± 11.6, 90.2 ± 10.2 μmol g^{-1}, respectively).[4] Chen *et al.* detected the CH_4 concentration in myocardial tissue 10 min after a 10 mL kg^{-1} ip injection and the tissue CH_4 level was about six times higher than that in the control group, demonstrating that ip-injected CH_4 reaches the target tissues.[113]

When CH_4 is absorbed into the mesenteric veins from the peritoneal cavity or the intestinal lumen, it is transferred by circulation to the lungs. In the lungs, it may be exhaled and the remaining fraction of CH_4 will be further circulated (which does not interfere with the oxygen saturation of the blood) and may again diffuse into tissues with a CH_4 concentration gradient. It should be added that, in a clinical study with non-pregnant gynaecological patients with closed-system mechanical ventilation, a progressive accumulation of CH_4 was found at the end of the ventilation (with a mean CH_4 concentration of 941 ppmv).[114]

The solubility of CH_4 in blood is rather low (with a blood/air partition coefficient of 0.066), so 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 or that within the lumen of the gastrointestinal tract, if these are the sole or predominant sources of CH_4. If there is no additional exogenous administration, the CH_4 concentration will decrease over time due to exhalation, falling to a low level.

Only a few historical and contradictory findings are available concerning the fate of CH_4 in non-archaeal biological systems. No detectable utilization of inhaled CH_4 was observed in healthy human volunteers, whereas 0.33% of intraarterially administered $[^{14}C]CH_4$ was converted to $[^{14}C]CO_2$ in sheep. The significance 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. It was proposed that interactions with free radical reactions might lead to a higher level of fixation and perhaps the oxidation of CH_4 in lipid environments, such as the mitochondrium membrane.[115,116]

Although the fate of extra- or intracellular CH_4 is an open question, there are many hydrophobic and hydrophilic interfaces in the cytoplasm and CH_4 may enter the hydrophobic non-polar lipid tails of phospholipid biomembranes. This effect will be even stronger at high salt concentrations, because the hydrophobic interactions are enhanced as a result of salting-out effects. 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. In this round rotation, there is a close association with CH_4 emanations, gastrointestinal perfusion, and the cardiac output. In a recent human study with CH_4-producing volunteers and dynamic online breath CH_4 measurements, the alveolar breath CH_4 data revealed substantial changes under exercise and non-exercise conditions, and apart from an increased dilution of breath CH_4 within the lungs (due to a rise in the ventilation–perfusion ratio), exercise also altered the fractional perfusion of the intestine, which represents a production site of CH_4 in the body. With this line of reasoning, it may be inferred that, under constant resting or workload conditions, the breath CH_4 concentration is affected by changes in the intestinal blood flow.[48,117]

8.9.1 CH_4 Effects in Sterile and Infectious Inflammation

Hypoxia inhibits the von Hippel–Lindau protein, thereby stabilizing the hypoxia-inducible factor 1 alpha (HIF-1α), which translocates to the nucleus. HIF-1α and the transcriptional factor NF-κB are involved in regulating the expression of cytokines and other mediators that participate in acute inflammatory responses, many of which are associated with the increased generation of ROS. Toll-like receptors (TLRs) are the main components of the innate immune system, and the TLR–ligand binding induce a number of responses, including NF-κB activation and the production of pro-inflammatory cytokines such as TNF-α, interleukin-6 (IL-6), and many others. Although the TLR-mediated increased TNF-α, IL-1β, and IL-6 responses might provide a protective function for controlling infection, the enhanced pro-inflammatory response could potentially become detrimental; therefore, negative regulatory signaling including the interleukin (IL)-10 pathway is critical for maintaining homeostasis or for antagonizing excessive or aberrant inflammatory activation.

The anti-inflammatory potential for CH_4 was first reported by our laboratory in experimental intestinal IR and, thereafter, many studies have explored the relationship between CH_4 actions in the context of inflammatory cell biology in various animal models.[3] Interestingly, CH_4 treatment can significantly reduce the level of TNF-α and IL-1β in IR injuries and elevate the levels of IL-10 both *in vitro* and *in vivo*.

Most publications have addressed and revealed four aspects of therapeutic activity. These are (1) modulation of pro-inflammatory cytokine release (TNF-α, IL-6, and IL-1b); (2) anti-apoptotic effects evidenced by a reduced number of apoptotic cells, normalized caspase-3 and caspase-9 activity, decreased Bax, and increased Bcl-2 levels and/or gene expression; (3) suppressed generation of oxidative stress biomarkers (malondialdehyde (MDA), 8-oxo-2'-deoxyguanosine, 4-hydroxynonenal) with concurrent potentiation of endogenous antioxidant systems (SOD, catalase, glutathione peroxidase); and (4) improved organ function.

8.9.2 Endotoxemia

Lipopolysaccharide (LPS)-linked cellular reactions are mediated by TLRs and, as part of the stress response, the generation of both superoxide and NO is one of the main consequences of LPS exposure in various TLR4-expressing cell types. Pre-treatment with CH_4 (using an MRS solution) inhibits the expression of LPS-induced TNF-α and IL-6 proteins in a dose-dependent manner in peritoneal macrophages and murine bone marrow-derived macrophages.[118] It has also been shown that LPS-induced NF-κB/MAPKs signals are inhibited by MRS solutions. Interestingly, a post-treatment MRS regime was also effective, and the IL-6 mRNA levels were reduced by approximately 95% 6 h after LPS stimulation, suggesting a long-lasting and strong inhibitory effect.

CH_4-treated mice produced significantly less TNF-α and IL-6 than control animals in response to an *in vivo* LPS challenge, and the PI3K/AKT/GSK-3β-mediated IL-10 expression was enhanced. Consistent with the *in vitro* observations, the serum levels of TNF-α and IL-6 of CH_4-treated mice were significantly reduced during *E. coli* bacteraemia, and the bacterial load in the blood was also reduced after MRS administration.[118]

8.9.3 Autoimmune Inflammation

Concanavalin A (ConA)-induced hepatic injury is primarily driven by the activation and recruitment of T-cells, and hence this model has many similarities with immune-mediated human hepatitis, such as autoimmune hepatitis and acute viral hepatitis. He *et al.* showed that MRS protected against ConA-induced autoimmune hepatitis in C57BL/6 mice.[119] CH_4 treatment suppressed the secretion of pro-inflammatory cytokines including TNF-α, interferon-γ (IFN-γ), IL-6, and IL-1β, and increased the anti-inflammatory cytokine IL-10 production. Furthermore the MDA and 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels were reduced, while the activities of SOD and catalase were increased in the liver after CH_4 administration compared to control mice with Con A-induced hepatitis. Furthermore, the data indicated that the phosphorylated IκB, NF-κB, and P38 MAPK levels were significantly downregulated in the liver.[119]

8.9.4 Experimental Colitis

Dextran sulfate sodium (DSS)-induced colitis is a commonly used murine IBD model in preclinical efficacy studies. CH_4 treatments (6 mL kg^{-1} bw MRS on three consecutive days) during DSS administration significantly reduced the elevated IL-6 plasma levels by about 85%, decreased the macroscopic mucosa damage and the microscopic signs of injury, the infiltration of inflammatory cells, and also the disease activity index. Consistent with these findings, CH_4-treated mice exhibited prolonged survival 72 h after the insult.[118]

8.9.5 Ischemia–Reperfusion

The maintenance of the tight redox balance of the intracellular milieu is a prerequisite for the functioning of biological processes and hypoxia perturbs this homeostasis. As an initial step, the lack of electron acceptor oxygen leads to decreased ATP generation; reduced ATP availability in turn limits ion pumps in cell membranes, resulting in a calcium overload, structural disorganization, and apoptotic and necrotic cell death. In addition, ischemia induces conformational changes in cellular enzymes such as XOR, thus paradoxically replenishment of the oxygen supply further amplifies the cell damage by generation of ROS and reactive nitrogen species (RNS) and lipid peroxidation products. Lipid peroxidation is a rapid chain reaction between

free radicals and fatty acids that leads to the breakdown of biomembranes, decompartmentalization, loss of cellular integrity, and, ultimately, cell death.

The survival of an aerobic cell after an acute hypoxia–reoxygenation episode comes at the price of an increasing prevalence of sterile inflammation-associated reactions. In hypoxic/ischemic conditions, at least two factors contribute to the pathology: the ischemic/hypoxic phase itself, and the return of blood perfusion with the reintroduction of molecular oxygen to the previously ischemic tissues. The prolonged lack of oxygen during ischemia is accompanied by a decrease in ATP production and an increase in ATP hydrolysis, while the overproduction of ROS and RNS during the reoxygenation phase leads to oxidative and nitrosative stress and membrane function failure. In addition to the oxidative damage, the IR-induced increased activity of the main lipolytic enzymes also results in modified biomembrane structures, leading to the loss of essential membrane-forming glycerophospholipids and functional derangements. IR is strongly associated with electrical membrane breakdown, a second-wave response critical for cell integrity and survival; while the arrival of polymorphonuclear (PMN) leukocytes is accompanied by further ROS formation in the reperfused tissues. These antigen-independent responses interact and amplify each other, finally leading to impaired microhemodynamics, functional and structural cell damage, and remote or systemic inflammatory complications. IR events are major determinants of mortality and morbidity in many areas of clinical practice, such as shock situations, thrombolytic therapy, and transplantation surgery; experimental IR studies are usually conducted to analyze ROS-induced reactions and the *in vivo* effectiveness of anti-inflammatory or antioxidant therapies on the tissue integrity and function.

CH_4 has a well-documented effect on this cascade. Firstly, it reduces the increased superoxide production and effectively reverses the hydrogen-peroxide (H_2O_2) production.[3,5] Secondly, it attenuates the IR-induced elevation of the MDA level, which is the end-product of lipid peroxidation.[122] In the following paragraphs, selected examples from the literature are presented that show different aspects of CH_4-linked effects in experimental IR models.

8.9.5.1 Intestines

The single-cell epithelial layer of the gastrointestinal mucosa is the most important barrier between the internal milieu and the hostile external environment. In certain pathologies, this 'thin red line' is rapidly deranged and the influx of luminal foreign material leads to acute immune stimulation and inflammation. Intestinal mucosal injury may be the result of partial or complete occlusion of the arterial perfusion or might be a complication of systemic low-flow states. The latter, non-occlusive mesenteric ischemia, is a highly lethal consequence of circulatory disturbances associated with a period of decreased cardiac output or hypovolemia, and it is thought to result mainly from excessive splanchnic vasoconstriction. The tissue damage

is characterized by a progressive shortening of the villus height, loss of villus epithelium, especially at the tips, and the invasion of inflammatory cells, mainly at the level of the crypts. Interestingly, normoxic ventilation with 2.5% CH_4 was found to significantly protect the intestinal tissues and mitigate the biochemical effects of an IR lesion.[3] The levels of tissue ROS generation were reduced, the mesenteric vascular resistance changes were only moderate, and the intestinal pCO_2 gap (a difference between local tissue and arterial pCO_2 levels being a reliable index of local tissue perfusion in the gastrointestinal tract) tended to normalize after reperfusion. Decreased tissue and plasma granulocyte activities were also observed, the effects of CH_4 on the PMN leukocyte functions were further investigated 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. This agrees with another finding that CH_4-treatment reduces the XOR activity *in vitro*.[3] Being the most important ROS producing enzyme in the postischemic gut, the inhibition of XOR contributes to a diminished superoxide production during the reperfusion phase.

Recent experiments have revealed another *in vivo* phenomenon possibly related to the protective effects of CH_4 in the gastrointestinal system. Confocal laser scanning endomicroscopy based on tissue fluorescence makes use of local contrast agents to produce very high-resolution images relative to conventional histopathology. In these studies, the IR-induced structural damage was evidenced by *in vivo* endomicroscopy, and direct intravital data were also obtained for deranged intestinal microcirculation. Exogenous normoxic CH_4 inhalation maintained the superficial mucosal structure, and the reperfusion-induced epithelial hyperpermeability was significantly alleviated. Moreover, the direct assessment of the intestinal macro- and microcirculation revealed that CH_4 treatment prevents the flow reduction in postischemic tissue. The latter observation might be connected to a lower activation level of circulating PMN leukocytes and a direct effect of CH_4 on erythrocyte deformability and aggregability.[109]

8.9.5.2 Skin

The first *in vivo* data on CH_4 bioactivity in an IR-associated animal model were later validated through independent experiments.[120] IR injury is an important cause of skin flap failure in microsurgical transplantations. Song K *et al.* reported that, in a rat model of abdominal-island skin-flap, MRS treatment (5 mL kg^{-1} ip, 15 min before and after reperfusion, then repeated every 12 h) nearly doubled the average blood perfusion (measured by laser Doppler flowmetry and laser speckle contrast analysis) and the viable skin flap area with a decreased inflammatory infiltration 72 h after surgery compared to positive controls.[120] It was one of the first reports that demonstrated the anti-apoptotic effect of CH_4. As a result of MRS treatment, the number of apoptotic cells was significantly reduced in the transplanted skin

flaps. These findings were substantiated by the decreased expression of Bax and increased expression of Bcl-2, key proteins in apoptosis. The mammalian mitogen-activated protein kinase (MAPK) family consists of extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun NH2-terminal kinase (JNK), while apoptosis signal-regulating kinase 1 (ASK-1) is a member of the MAP3K family, which is responsive to oxidative stress and inflammatory cytokine-induced cell damage. The activation of ASK-1 may determine the cell fate by regulation of both the MKK4/MKK7-JNK and MKK3/MKK6-p38 MAPK signaling cascades.[121] Song *et al.* provided evidence in their study that MRS treatment significantly decreases the expression levels of activated ASK-1 and JNK during skin IR injury.[120]

8.9.5.3 Heart

In another IR study, ip treatment with 10 mL kg^{-1} MRS (where the CH_4 concentration in the solution was about 1.5 mmol L^{-1}) significantly prolonged the survival time of rats with myocardial ischemia induced by ligation of the left anterior descendent coronary artery.[113] In this model, CH_4 exerted dose-dependent myocardial protection (0.6–10 mL kg^{-1}), characterized by a reduced infarct area and serum levels of myocardial necroenzymes. The pro-inflammatory activation (evidenced by TNF-α, IL-1β, and MPO content) and oxidative damage of DNA was significantly alleviated by CH_4. MRS treatment reduced the protein expression of the pro-apoptotic Bax, decreased the cytoplasmic cytochrome c content, and cleaved caspase-3 and caspase-9 levels, but markedly increased the levels of Bcl-2 and mitochondrial cytochrome c, indicating an anti-apoptotic effect here as well. Besides the early life-threatening condition caused by an ineffective left ventricular function upon myocardial infarction, heart failure developing later is a major cause of mortality of ischemic heart diseases. Quite significantly, CH_4 treatment maintained a satisfactory cardiac function measured at four weeks post-infarction with echocardiography, showing, among others, improved left ventricular ejection fraction, diastolic volume, and contractility compared to non-CH_4-treated rats. Myocardial remodeling and fibrosis is a maladaptive mechanism of the cardiac tissue upon infarction. When it was evaluated four weeks after the ischemic challenge, the MRS group had a significantly better structural condition with attenuated left ventricular remodeling.

8.9.5.4 Liver

CH_4 appears to exert a protective effect on experimental partial liver IR as well.[5,122] As an inherent and undesirable consequence of various liver surgeries, partial hepatic IR is accompanied by parenchymal necrosis, elevated levels of hepatocellular damage marker enzymes, and biochemical signs of inflammation. As was shown by Ye *et al.*, the increased alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels after 60-min

ischemia and 6-h reperfusion were reduced in a dose-dependent manner upon MRS administration (1–40 mL kg^{-1} MS with a CH_4 content of about 1 mmol L^{-1}), but CH_4 did not influence the lactate dehydrogenase (LDH) activity.[122] Tissue necrosis was reduced as well, with a concomitant decrease of lipid peroxidation evidenced by MDA measurements and enhanced levels of the antioxidant enzyme SOD.[113] In addition to its anti-apoptotic properties, MRS treatment prevented the gene expression and production of early inflammatory cytokines TNF-α, IL-1β, and IL-6, and reduced the infiltration of inflammatory CD68 positive cells in the liver tissue.

At a subcellular level, IR-induced mitochondrial dysfunction may occur because of damage to ETC proteins or organelle membranes, and similar to the brain and heart, the mitochondrial oxidative metabolism of the liver is especially active. In a recent study, the efficacy of liver mitochondrial ETC was assessed using high resolution respirometry.[5] Partial hepatic IR resulted in a lower oxidative phosphorylation capacity of rat liver mitochondria (complex II-coupled state III respiration) compared to controls, and the inhalation of normoxic CH_4 preserved the respiratory capacity in the first 30 min of reperfusion. At the same time, IR-induced cytochrome c release and hepatocyte apoptosis were also reduced. In the same study, higher ROS production rates measured in whole blood samples of IR animals were significantly inhibited upon CH_4 treatment, which suggests that there is a significant contributing role of circulating leukocytes and perhaps a link to the so-called ROS-induced ROS production.[123]

8.9.6 Neuroprotection

Like other organs discussed above, the CNS is also frequently affected by acute circulatory disturbances that lead to transient brain hypoxia. Moreover, similar pathophysiological processes underlie other disorders associated with secondary hypoxia and inflammation as well. Since the solubility of CH_4 is high in lipids compared to that in the water phase, a relative enrichment of the molecule in lipid-rich tissues (*e.g.*, brain and spinal cord) is expected. Accordingly, a number of studies have been performed to demonstrate the neuroprotective effects of CH_4, the results of which are summarized below.

8.9.6.1 Retina

Among the various retinal neurons, retinal ganglion cells (RGCs) are thought to be the most vulnerable to IR, caused by glaucoma or other vascular diseases.[124] RGCs share the final common neurons that collate vision signals in retinas and transmit them to brain visual centers through the optic nerve. As the primary cellular population affected by glaucoma, the loss of RGCs leads to irreversible visual impairment. Consequently, the prevention or reduction of cellular damage to RGCs is a major goal in neuroprotection studies. In a commonly used glaucoma model with transient elevation of intraocular

pressure, IR is known to cause neuronal necrosis and apoptosis and thinning in multiple layers of the retina. The study of Liu *et al.* provided evidence that a single treatment with MRS (25 mL kg^{-1}, administered after the end of a 60-min ischemia event) attenuated the IR-induced RGC loss and retinal thinning in rats measured one week after the IR challenge. What is more, the visual function was also preserved, as demonstrated by the measurement of visually evoked potentials.[125] The same study confirmed the anti-apoptotic and anti-oxidative effects of CH_4, which may play a substantial role in the prevention of the loss of neurons in this model.

Diabetic retinopathy is the leading cause of visual loss among the adult human population. In a streptozotocin-induced rat diabetic retinopathy model, decreased retinal thickness, RGC loss and blood-retinal-barrier (BRB) breakdown were all significantly suppressed by CH_4 treatment (MRS, 5 mL kg^{-1} once daily for 8 weeks).[126] It was shown earlier that the abnormally elevated production of the vascular endothelial growth factor (VEGF) contributed to the development of diabetic retinopathy. The diabetes mellitus-induced retinal overexpression of TNF-α and IL-1β, and the abnormal expression of glial fibrillary acidic protein (GFAP) and VEGF were also significantly ameliorated by MRS supplementation. Likewise, aberrant micro-RNA expression profiles have been recently shown to play a key role in the development of the disease. CH_4 treatment substantially upregulate retinal levels of miR-192-5p (related to apoptosis and the tyrosine kinase signaling pathway) and miR-335 (related to proliferation, oxidative stress, and leukocyte orchestration), thus defining novel directions for mechanistic studies with CH_4 in the future.

8.9.6.2 Spinal Cord

In a recent study by Wang *et al.*, rats experienced a brief spinal cord ischemia event induced by the occlusion of the descending thoracic aorta plus systemic hypotension, followed by a single MRS treatment (10 mL kg^{-1}, ip) and 72 h of reperfusion.[4] Substantially elevated concentrations of CH_4 were measured in spinal cords as early as 10 min after the MRS injection compared to non-treated animals, which is consistent with the findings of Mészáros *et al.* in other tissues.[109] Quite surprisingly, the CH_4 levels remained high throughout the 72 h reperfusion.

CH_4 supplementation attenuated both the motor and sensory deficits elicited by spinal cord IR. Upon MRS treatment, the increased expression and transcriptional activity of Nrf2 was demonstrated in neurons, microglia, and astrocytes in the ventral, intermediate, and dorsal grey matter of lumbar segments. The Nrf2-Keap1 pathway is a key orchestrator of various anti-oxidant systems and it might be a central mediator of gas messengers as well.[127,128] Along these lines, various anti-oxidant enzymes like HO-1, SOD, catalase, and glutathione peroxidase were upregulated, while the oxidative stress markers glutathione disulfide, superoxide, hydrogen peroxide, MDA, 8-hydroxy-2-deoxyguanosine, and 3-nitrotyrosine were reduced in the spinal cord of MRS-treated animals.

The redox-sensitive transcription factor Nrf2 is a key regulator of redox signaling events. Nrf2 is a short-lived protein continuously targeted for ubiquitination and proteasomal degradation. Keap1 forms an anchor complex with Nrf2, which dissociates in response to ROS and electrophiles. The released Nrf2 then binds to the nuclear antioxidant response elements (AREs) and coordinates the transcription of multiple antioxidant and detoxifying enzymes to counteract the oxidative stress. In addition to the predominant cytoplasmic and nuclear pool of Nrf2, Keap1 and Nrf2 have also been detected in the outer mitochondrial membrane. Interestingly, CH_4 induces the time-dependent nuclear translocation of the Nrf2 protein, while downregulation with Nrf2 siRNA blocks the anti-inflammatory effects of CH_4.[4] In addition, the increased nuclear Nrf2 was accompanied by downregulation of the Nrf2 inhibitor Keap1 in the cytoplasmic fraction.

MRS treatment reduced neuronal apoptosis and prevented the activation of microglia and astrocytes in grey matter zones, which was consistent with the suppression of inflammatory cytokines. MRS treatment attenuated the blood-spinal cord barrier dysfunction as well by preventing the activation of matrix metallopeptidase-9 and preserving the tight junction proteins. Although spinal cord ischemia is not as common as ischemic stroke of the brain, the mechanisms delineated in this model of spinal cord injury may have some relevance in circulatory disturbances in the cerebral cortex as well.

8.9.6.3 Brain

Carbon monoxide (CO) poisoning is often associated with hypoxic injury of the brain. Inhaled CO binds to hemoglobin (Hb) to form carboxyhemoglobin (COHb) and the easy displacement of O_2 from Hb reduces the amount of Hb available to carry O_2, causing hypoxaemia. At the same time, COHb shifts the oxyhemoglobin dissociation curve to the left, further decreasing the amount of the O_2 released and worsening the histanoxia. The central cause of injury due to CO poisoning is hypoxia, and the key pathophysiological mechanism is oxidative stress. One of the important mechanisms of brain injury in CO poisoning is ROS formation, partly by XOR, which results in neuronal death, thereby causing delayed neuropsychological sequelae. In two similar studies, MRS (0.99 mmol L^{-1}, 10 mL kg^{-1} ip 3 times every 8 h: 0, 8, and 16 h after CO poisoning) exerted long-term brain protection in rats after CO poisoning, and protected the acute consequences of CO poisoning as well.[98,129] In this model, the animals were exposed to 1000 ppmv CO at a rate of 4 L min^{-1} for 40 min, followed by 3000 ppmv CO for another 20 min until they lost consciousness. CH_4 suppressed the production of oxidative stress markers and inflammatory mediators in the cortex and hippocampus 24 h after CO poisoning and prevented neuronal apoptosis. Furthermore, CH_4 protected against CO poisoning-induced learning and memory deficits, as demonstrated by the Morris water maze test carried out on the sixth day after CO overdose. In the longer run, reduced levels of lipid peroxidation, DNA

oxidation, and protein nitration products, as well as increased anti-oxidative enzyme levels and decreased levels of inflammatory mediators, were present in the cortices and hippocampi of CH_4-treated rats. Based on the above data, it can be assumed that the protecting effect of CH_4 might be potentiated in tissues with high lipid content, which maintain a longer-lasting pharmacological profile.

8.9.7 Mitochondrial Effects

In aerobic organisms, mitochondria integrate the oxidation of substrates with the reduction of molecular oxygen; and it has been shown that exogenous CH_4 affects many aspects of mitochondrial physiology. On the one hand, dysfunction of the mitochondrial ETC is associated with mitochondrial CH_4 release, which can be provoked either by inhibitors of the electron transport or deprivation of the final electron acceptor oxygen molecule.[25,26] On the other hand, CH_4-containing normoxic artificial air preserves the oxidative phosphorylation after a period of tissue ischemia.[5]

Mitochondria are both targets and sources of oxido-reductive stress. Hypoxia is inseparable from mitochondrial dysfunction, and ROS formation is especially pronounced in the inner mitochondrial membrane. Upon hypoxia, the complex IV activity is inhibited and, as a consequence, the oxygen molecule is not able to accept the flow of electrons.[130,131] The mitochondrial ETC contains several redox centers that may leak electrons to molecular oxygen, serving as the primary source of superoxide production in most tissues.[132] Yet, the traditional view of ROS as being invariably harmful and unwanted byproducts is strongly debated and today it is accepted that physiological levels of ROS regulate a multitude of signaling pathways (*e.g.*, NF-κB, Nrf-2, STAT3) directly and indirectly.[128,133,134] The rate of ROS production strongly depends on the metabolic state of the cells and it has been suggested that, *in situ*, mitochondria might be more like sinks than sources of ROS, if the high antioxidative capacity of mitochondria is taken into account.[135–137] The mitochondrial ROS-detoxifying mechanisms among others include membrane lipid peroxide removal systems, phospholipid hydroperoxide glutathione peroxidase, MnSOD, cytochrome c, catalase, glutathione, glutathione-*S*-transferase, glutathione reductase, glutathione peroxidase, and peroxiredoxins. This suggests that ROS production is tightly regulated and secured by several lines of antioxidant defence systems intra- and extra-mitochondrially.

According to current views, the gasotransmitters NO, CO, and H_2S all readily inhibit mitochondrial oxygen consumption by cytochrome c oxidase. The inhibition by NO and CO is dependent on the oxygen concentration, but that of H_2S is not.[138]

Here, it should be noted that the *in vitro* incubation of isolated mitochondria in a respiration medium with normoxic CH_4 does not affect the activity of OxPhos complexes compared to room air.[5] However, the *in vivo* inhalation of CH_4-containing normoxic artificial air preserved the oxidative

phosphorylation after a period of tissue ischemia, and significantly improved the basal mitochondrial respiration state after the onset of reperfusion. In agreement with this, the cytochrome c oxidase activity together with ROS production and hepatocyte apoptosis were also significantly reduced in this model of liver IR. Although the downstream effectors of transiently increased ROS levels, which include Nrf-2 and P38 MAP kinase, mitochondria-related apoptotic events, and NF-κB activation, have been confirmed both *in vitro* and *in vivo,* it has not been clarified which molecular sensors become directly activated by CH_4. It seems that CH_4 may have a fundamental role in the individual mitochondrial effects of distinct interventions (see Figure 8.5), providing an explanation of why CH_4 supplementation may interfere with the consequences of diverse conditions associated with hypoxia, physical exercise, inflammation, and ROS-inducing compounds, which give rise to an increase in stress defence in model experiments.

As an example, IR injury is an antigen-independent stimulus that initiates the intrinsic signaling pathways of apoptosis, which is a mitochondria-related event. Upon damage of the mitochondrial membrane, the cytochrome c released from the inner membrane to the cytoplasma leads to the activation of the apoptotic caspase cascade. The apoptosis-inducing proteins may affect the mitochondria in different ways, namely by the opening of ion channels or the membrane permeability transition pore (mPT), which results in an outflow of cell death-activating molecules (such as cytochrome c and a second mitochondrial derived activator of caspases (SMAC)) from the organelle.[139] The mPT depolarizes the mitochondrion and dissipates the electrochemical H^+ gradient, and the increased permeability of the inner mitochondrial membrane causes mitochondrial swelling and the prevention of OxPhos, which leads to apoptosis and cell death.

It should be noted that the mechanism of IR-induced cell apoptosis involves many overlapping signal pathways. Mitochondrial ROS (generated by TNF-α) can oxidise the reduced thioredoxin-apoptosis signal-regulating kinase 1 complex ($Trx(SH)_2$-ASK-1), then activate ASK-1 and its downstream stress signaling targets, such as JNK, and initiate the apoptosis. The anti-apoptotic proto-oncogene protein B cell leukemia/lymphoma-2 (Bcl-2) and the pro-apoptotic protein Bcl-2 associated X protein (Bax) can combine to form a heterodimer and its ratio determines the fate of the cells, the ratio of Bax to Bcl-2 being a good predictive indicator of long-term cell survival. Bcl-2, regarded as a mitochondrial anchoring protein, may prevent a ROS-induced step by acting like an antioxidant partner, and it may inhibit Bax relocalization, mitochondrial membrane depolarization, cytochrome c release, and caspase activation. Caspase-3 may be activated by many factors, such as ROS and a lower expression of Bcl-2. Activated caspase-3 can target poly(ADP-ribose)polymerase (PARP) and increase the activity of Ca^{2+}/Mg^{2+}-dependent endonuclease to help destroy DNA molecules.

The study of Ye *et al.* was the first in a series of analyses to show that CH_4 protects against IR injury through antiapoptotic actions.[122] These authors demonstrated significantly reduced caspase-3 activity and hepatocyte

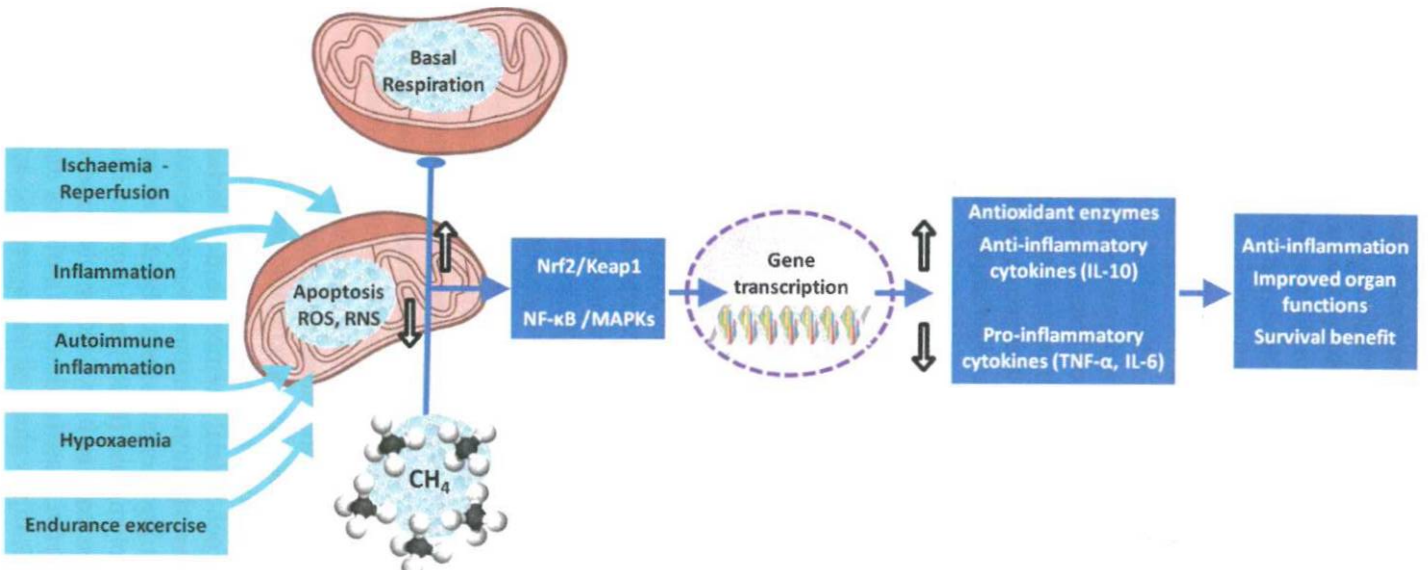

Figure 8.5 Simplified scheme of the central role of mitochondrial stress reactions in excessively amplified inflammation. CH_4 indirectly activates the cellular defense systems by influencing the NF-κB and Nrf2/Keap1-mediated gene transcription.

apoptosis after MRS treatment in rats with hepatic IR. In an accompanying study with retinal IR, the upregulation of pro-apoptotic factors including Bax, caspase-9, and caspase-3 was reversed by CH_4 treatment; while Bcl-2 was significantly upregulated. These findings were later reinforced in other tissues, such as skin, retina, heart, and spinal cord with IR injury and MRS treatment.[4,113,120,122,125] CH_4 inhalation can also effectively attenuate the apoptosis of hepatocytes. In a recent study with *in vivo* imaging using confocal laser scanning endomicroscopy, we demonstrated that normoxic CH_4 inhalation was able to effectively attenuate the apoptosis-linked morphological changes in a rat liver IR model.[5]

Based on the above findings, it seems to be well established that exogenous CH_4 confers cellular protection by restoration of the mitochondrial function, and probably the membrane integrity through the expression of the Bcl-2 family of anti-apoptotic proteins, decreasing the release of cytochrome c and deactivating the caspase signaling cascade.

8.10 Mechanism of Action

Theoretically, at least two different (but not mutually exclusive) possibilities should be taken into account when explaining the mechanism of CH_4 effects. We should also consider the redundancy of gaseous signals and the pleiotropism of influenced molecular pathways during *in vivo* inflammatory and oxido-reductive stress conditions.

8.10.1 Theory of a Membrane-associated Mechanism of Action

Several studies have demonstrated and evaluated the modulator effect of CH_4 on cell–cell junctions and plasma membrane integrity under oxido-reductive stress conditions. The rise of paracellular epithelial permeability is a well-recognized consequence of intestinal ischemia. Earlier, we showed that mucosal hyperpermeability was almost absent in CH_4-treated rats with 45 min ischemia, followed by 40 min reperfusion.[140] These preliminary findings were reinforced in other IR and inflammatory conditions with exogenous, CH_4-enriched saline solutions. Wu *et al.* demonstrated that the function of the blood-retinal barrier (BRB) was significantly disrupted in streptozotocin-treated diabetic rats, but the increase in BRB permeability was significantly improved and prevented after MRS treatment.[126]

In another series of rat experiments, Wang *et al.* demonstrated the disruption of the blood-spinal cord barrier (BSCB) after spinal cord IR injury with oedema and PMN leukocyte infiltration 72 h after the injury.[4] The severely diminished BSCB integrity was ameliorated by CH_4 treatment in an Nrf2-dependent manner, while the intrathecal injection of Nrf2 siRNA counteracted the beneficial effects of CH_4 (the CH_4-dependent increase in Nrf2 expression was absent).[4]

Recently, a more detailed study was conducted to demonstrate the disruption of the intestinal epithelial barrier after IR and the effects of CH_4 inhalation on the structural integrity of the mucosa in the early and later phases of reperfusion.[109] CH_4 once again proved to be protective. However, it remains to be determined what role the transmembrane proteins of the epithelial TJs play, and through what steps CH_4 is able to modulate the intercellular connections.

Apart from barriers formed by a network of cells, the modulation of single cell membrane characteristics may also have an important impact on organ functions. Lipid peroxidation, a common consequence of oxidative stress by oxidant species such as peroxynitrite, leads to the decreased fluidity of lipid membranes and impaired deformability and pathological aggregability of erythrocytes, severely affecting the hemorheological properties of blood. The peroxidation of membrane lipids of red blood cells decreases the fluidity of the affected cellular membranes, contributing to reduced capillary blood flow and, in severe cases, capillary stasis may develop.[141,142] Of interest, in a recent study, the *in vitro* incubation of oxidized whole blood samples with CH_4 significantly improved both the deformation and aggregation parameters of red blood cells, suggesting that there is an additional level of protection being exerted by the molecule.[109]

These experimental findings all suggest an influence of CH_4 on membrane sparing or recovery in oxido-reductive environments. In order to avoid the potentially fatal outcome of an increased oxido-reductive potential, molecular participants of a living system should be quickly brought into use to save or regenerate membranes, which are responsible not only for separation, but also for the maintenance of a steady state *via* channels, pores, and membrane proteins. Compared to NO, CH_4 may reach higher concentrations when dissolved in water or colloid solutions, and ROS generation can lead to a higher level of fixation of CH_4 in a lipid environment, such as the mitochondrial membrane.

Apolar CH_4 may enter the cytoplasm and mitochondrial matrix and dissolve in the hydrophobic non-polar lipid tails of the phospholipid biomembranes, in theory influencing its physicochemical condition, which is essential for the normal functioning of embedded proteins and ion channels. When compressed hydrocarbon gases were investigated quantitatively by microcalorimetry on the growth of *Saccharomyces cerevisiae*, the inhibitory action increased in the order $CH_4 \ll$ ethane $<$ propane $<$ i-butane $<$ *n*-butane, which correlated with the hydrophobicity, suggesting that hydrocarbon gases interact with some hydrophobic regions of the cell membrane. In support of this, the authors found that UV-absorbing materials at 260 nm were released from the yeast cells and, what is more, scanning electron microscopy demonstrated morphological changes in these cells.[143]

Membrane rigidity relates to the degree of lipid peroxidation; this means that when the amount of oxidised lipids is increased, the fluidity of membranes is reduced. CH_4 dissolved in biological membranes may affect this process, thereby influencing the stereo figure of membrane proteins that

determines their accessibility and morphology. The peroxidation of polyunsaturated fatty acids and direct triggering of cytochrome c release from mitochondria are a well-known consequence of IR injury. The tissue nitrotyrosine level is an indicator of protein nitration, this being an irreversible process associated with elevated peroxynitrite levels. Peroxynitrite, among its other damaging effects, is a potent initiator of membrane lipid peroxidation.[144] Earlier, it was reported that IR-associated lipid peroxidation decreases the membrane fluidity in various tissues and in erythrocytes.[141,145] Red blood cells are rich in iron, which catalyzes the ROS formation through the Fenton reaction, making them highly susceptible to IR damage.[146,147] The membrane and cytoskeleton are together responsible for changing the shape of erythrocytes.[148] Lipid peroxidation severs the connection between the two components and, consequently, both the deformability and aggregation of the erythrocyte is influenced in a negative way.[149,150] Along these lines, we evaluated the red blood cell velocity as a measure of perfusion in intestinal serosal microvessels, which was significantly reduced after mesenteric IR. In contrast, normoxic CH_4 treatment maintained a satisfactory microcirculation throughout the reperfusion period and upon normoxic CH_4 treatment for 10 min; the red blood cell deformability improved at low-to-moderate shear stress rates (1.5–7 Pa), suggesting a direct effect of CH_4 in the partial restoration of membrane fluidity and/or membrane–cytoskeleton junctions. Furthermore, the tissue nitrotyrosine levels in the CH_4-treated group did not differ from those in the controls.[109]

8.10.2 CH_4 Accumulation May Indirectly Influence the Intracellular Signaling Reactions that Lead to Anti-inflammatory Effects

A recent study by Zhang *et al.* substantially expanded our scope of knowledge on the anti-inflammatory mechanism of action of CH_4 and demonstrated that MRS treatment can protect mice from LPS-induced endotoxin shock, and DSS-induced colitis by suppressing the TNF-α and IL-6 production.[118] Furthermore, MRS-treated mice had a lower bacterial load in blood after intraabdominal *E. coli* (1×10^7 ip) infection. CH_4 treatment attenuated the phosphorylation of NF-κB, JNK, ERK, and P38MAPK in LPS-stimulated macrophages in an IL-10-dependent manner *via* enhanced activation of PI3K/AKT signaling, leading to GSK-3β phosphorylation both *in vitro* and *in vivo*.

In another model, spinal cord IR was accompanied by the activation of caspase-9 and caspase-3 and significantly increased the cytochrome c release from the mitochondria into the cytoplasm. Increased mRNA and amounts of TNF-α, IL-1β, CXCL1, and ICAM-1 were observed in IR; however, the increases and the apoptotic effects were blocked upon CH_4 administration.[4]

These findings suggest that higher concentrations of CH_4 lead to anti-inflammatory responses *via* master switches such as Nrf2/Keap1 or NF-κB.

Nrf2 has also been shown to have a key role in signaling the antioxidant response element (ARE)-mediated regulation of gene expression. As it happens, CH_4 induces the time-dependent nuclear translocation of Nrf2 protein and, in addition, the increased nuclear Nrf2 is accompanied by the down-regulation of the Nrf2 inhibitor Keap1 in the cytoplasmic fraction.[4] This occurs in association with the phosphorylation and nuclear translocation of the NF-κB p65 subunit. The nucleoplasmic ratio of phospho-NF-κB p65 was increased at 72 h post injury relative to sham-operated rats, but this increase was inhibited by CH_4 treatment. Furthermore, after Nrf2 knockdown by intrathecal siRNA pre-treatment, the nuclear accumulation of phospho-NF-κB p65 occurred, unlike that in CH_4-treated rats. To sum up, lots of data point to an indirect anti-cytokine effect of CH_4 by influencing the NF-κB and Nrf2 activation.

8.11 Conclusions

In the human body, many gases are biologically active. Signaling roles have been demonstrated for NO, CO, and H_2S, and it has become clear that gaseous mediators form complex intracellular pathways and regulate numerous physiological processes, separately, or more often, in antagonistic or synergistic ways. Research on gases and derivatives has become a topic of great interest, and recently four characteristics were listed to define gasotransmitters (namely simplicity, availability, volatility, and effectiveness), and six criteria were proposed that make a gas physiologically important. Gasotransmitters are (1) small molecules of gas, (2) freely permeable to membranes, (3) endogenously generated in mammalian cells with specific substrates and enzymes, (4) have well-defined specific functions at physiologically relevant concentrations, (5) their functions can be replicated by their exogenously applied counterparts, and (6) have specific cellular and molecular targets.[151] If we discuss the available findings on the generation and biological effects of CH_4 from such aspects, current evidence does not wholly support the gasotransmitter concept, but it does support the notion that CH_4 is bioactive and its liberation and effectiveness are both linked to hypoxic events. Today, the overall evidence based on research findings suggests that excretion of CH_4 in the breath in mammals reflects not only the intestinal microbial fermentation, but also its unidentified generation from target cells. It could be possible that the formation and constant build-up of ROS 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, and may be an integral feature of cellular responses to changes in the oxidative status in all eukaryotes. In this respect, the available information suggests that hypoxia-induced non-archaeal CH_4 generation may be a necessary phenomenon of aerobic life.

The significance of endogenous CH_4 production in the gastrointestinal system is also an open question. The mucosa embraces complex systems,

where a balance should be kept between inward and outward transport, growth, and differentiation, where the preservation or restoration of the epithelial and endothelial barriers is of vital importance. In contrast with other organs, the gut wall is persistently exposed to potentially invading microorganisms, bacterial toxins, products of phagocytic cells, and non-bacterial antigens that can cross the mucosal epithelium, and this presupposes the action of a system which tunes or modulates the constant pro-inflammatory activity in the gastrointestinal tract. Again, it is tempting to speculate that a low, but stable level of CH_4 is required to maintain the inflammatory signals in their resting condition.

CH_4 is a small, omnipresent, low-reactivity gas molecule, having a close symbiosis with bioactive gases in the intracellular spaces. It is lighter than air and readily expelled in a concentration gradient to achieve equilibrium. Of particular interest is that the recognized biological effects of CH_4 are not cell- or tissue specific, and an increased input may result in acute and chronic changes in cells and tissues. Is CH_4 really a gasotransmitter? Here, it should be noted that the term 'gasotransmitter' does not essentially cover 'biological activity', but that there is significant overlap between the groups. In this regard, it is tempting to speculate on a much broader, controller role for CH_4, like in an internal combustion engine, where it is necessary to regulate the air–fuel ratio in order to burn the fuel completely with the available oxygen.

Whereas the results indicate a bioactive role for higher concentrations of exogenous CH_4, this is not obvious for endogenous sources; and there is still no clear-cut evidence that CH_4 in the endogenously produced concentration range (1–10 ppmv) has a role in cellular physiology. The effects of exogenous CH_4 have been illustrated in detail in various tissues under different conditions. Nevertheless, in a discussion of these aspects of CH_4 biology, it should be borne in mind that there is a conceptual difference between the baseline levels or the physiological generation of a gas (*e.g.*, NO, CO, and H_2S) after *de novo* induction or discordant alteration by inducer factors and that, after exogenous pharmacological doses, the affected processes or evolving responses may therefore be dependent on the number of molecules and/or the reactivity of the microenvironment. The effectiveness of CH_4 surely reflects a multifaceted response of complex biological systems, consistent with a situation whereby biological adaptations occur in response to low continuous exposure of a compound that would be visible at higher or larger doses. The endogenous nature of CH_4 combined with the ability to activate simultaneously multiple cytoprotective pathways in higher amounts has made this compound attractive for the development of new concepts – and also therapies linked to inflammation and oxido-reductive stress.

Is CH_4 a therapeutic gas? After our paper describing the anti-inflammatory properties of CH_4, the accompanying editorial commentaries asked this question and today the answer is that it is most likely the case.[3,152,153] Numerous *in vitro* and *in vivo* approaches have shown that CH_4 is bioactive, several studies have reported that increased CH_4 input ameliorates the signs

and sequelae of inflammatory activation, and common findings have shed light on many details of the mechanism of action. Yet, none of the mechanistic hypotheses presented here can so far fully explain the anti-inflammatory efficacy of CH_4. Needless to say, understanding the molecular pathways that account for the inflammatory consequences of acute and chronic oxido-reductive stress should help clarify this issue.

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References

1. R. L. Beaver and W. E. Field, *J. Agromedicine*, 2007, **12**(2), 3.
2. H. Ragins, H. Shinya and W. I. Wolff, *Surg., Gynecol. Obstet.*, 1974, **138**(4), 554.
3. M. Boros, M. Ghyczy, D. Érces, G. Varga, T. Tőkes, K. Kupai, C. Torday and J. Kaszaki, *Crit. Care Med.*, 2012, **40**, 1269.
4. L. Wang, Y. Yao, R. He, Y. Meng, N. Li, D. Zhang, J. Xu, O. Chen, J. Cui, J. Bian, Y. Zhang, G. Chen and X. Deng, *Free Radical Biol. Med.*, 2017, **103**, 69.
5. G. Strifler, E. Tuboly, E. Szél, E. Kaszonyi, C. Cao, J. Kaszaki, A. Mészáros, M. Boros and P. Hartmann, *PLoS One*, 2016, **11**(1), e0146363.
6. S. Baldursdottir, K. Sigvaldason, S. Karason, F. Valsson and G. H. Sigurdsson, *Acta Anaesthesiol. Scand.*, 2010, **54**(7), 821.
7. R. Conrad, *Environ. Microbiol. Rep.*, 2009, **1**, 285.
8. S. Kirschke, P. Bousquet, P. Ciais, M. Saunois, J. G. Canadell, E. J. Dlugokencky, P. Bergamaschi, D. Bergmann, D. R. Blake, L. Bruhwiler, P. Cameron-Smith, S. Castaldi, F. Chevallier, L. Feng, A. Fraser, M. Heimann, E. L. Hodson, S. Houweling, B. Josse, P. J. Fraser, P. B. Krummel, J. F. Lamarque, R. L. Langenfelds, C. Le Quere, V. Naik, S. O'Doherty, P. I. Palmer, I. Pison, D. Plummer, B. Poulter, R. G. Prinn, M. Rigby, B. Ringeval, M. Santini, M. Schmidt, D. T. Shindell, I. J. Simpson, R. Spahni, L. P. Steele, S. A. Strode, K. Sudo, S. Szopa, G. R. van der Werf, A. Voulgarakis, M. van Weele, R. F. Weiss, J. E. Williams and G. Zeng, *Nat. Geosci.*, 2013, **6**, 813.
9. G. Etiope and B. Sherwood Lollar, *Rev. Geophys.*, 2013, **51**(2), 276.
10. B. C. McBride and R. S. Wolfe, *Biochemistry*, 1971, **10**, 2317.
11. J. Ellermann, R. Hedderich, R. Bocher and R. K. Thauer, *Eur. J. Biochem.*, 1988, **172**, 669.
12. K. J. Purdy, *Adv. Appl. Microbiol.*, 2007, **62**, 63.
13. P. H. Janssen and M. Kirs, *Appl. Environ. Microbiol.*, 2008, **74**, 3619.

14. Y. Liu and W. B. Whitman, *Ann. N. Y. Acad. Sci.*, 2008, **1125**, 171.
15. A. Brune, in *Handbook of Hydrocarbon and Lipid Microbiology*, ed. K. N. Timmis, Springer, Heidelberg, 2010, p. 707–728.
16. F. Keppler, J. T. Hamilton, M. Brass and T. Rockmann, *Nature*, 2006, **439**, 187.
17. F. Keppler, M. Boros, C. Frankenberg, J. Lelieveld, A. McLeod, A. M. Pirttila, T. Rockmann and J. P. Schnitzler, *Environ. Chem.*, 2009, **6**, 459.
18. D. Bruhn, I. M. Moller, T. N. Mikkelsen and P. Ambus, *Physiol. Plant.*, 2012, **144**, 201.
19. K. Lenhart, F. Althoff, M. Greule and F. Keppler, *Biogeosciences*, 2015, **12**, 1907.
20. B. Wang, H. Longyu, L. Wei and Z. Wang, *Atmos. Environ.*, 2013, **80**, 290.
21. F. Althoff, K. Benzing, P. Comba, C. McRoberts, D. R. Boyd, S. Greiner and F. Keppler, *Nat. Commun.*, 2014, **5**, 4205.
22. K. Lenhart, M. Bunge, S. Ratering, T. R. Neu, I. Schüttmann, M. Greule, C. Kammann, S. Schnell, C. Müller, H. Zorn and F. Keppler, *Nat. Commun.*, 2012, **3**, 1046.
23. K. Lenhart, B. Weber, W. Elbert, J. Steinkamp, T. Clough, P. Crutzen, U. Pöschl and F. Keppler, *Glob. Change Biol.*, 2015, **21**, 3889.
24. K. Lenhart, T. Klintzsch, G. Langer, G. Nehrke, M. Bunge, S. Schnell and F. Keppler, *Biogeosciences*, 2016, **13**, 3163.
25. M. Ghyczy, C. Torday, J. Kaszaki, A. Szabó, M. Czóbel and M. Boros, *Cell. Physiol. Biochem.*, 2008, **21**(1–3), 251.
26. E. Tuboly, A. Szabó, G. Erős, A. Mohácsi, G. Szabó, R. Tengölics, G. Rákhely and M. Boros, *J. Breath Res.*, 2013, **7**(4), 9.
27. F. Keppler, J. T. G. Hamilton, W. C. McRoberts, I. Vigano, M. Brass and T. Rockmann, *New Phytol.*, 2008, **178**, 808.
28. I. Vigano, H. van Weelden, R. Holzinger, F. Keppler, A. McLeod and T. Rockmann, *Biogeosciences*, 2008, **5**, 937.
29. A. R. McLeod, S. C. Fry, G. J. Loake, D. J. Messenger, D. S. Reay, K. A. Smith and B. W. Yun, *New Phytol.*, 2008, **180**, 124.
30. Z. P. Wang, Z. Q. Xie, B. C. Zhang, L. Y. Hou, Y. H. Zhou, L. H. Li and X. G. Han, *Environ. Sci. Technol.*, 2011, **45**, 9531.
31. M. M. Qaderi and D. M. Reid, *Physiol. Plant.*, 2009, **137**, 139.
32. D. J. Messenger, A. R. McLeod and S. C. Fry, *Plant, Cell Environ.*, 2009, **32**, 1.
33. A. Wishkerman, S. Greiner, M. Ghyczy, M. Boros, T. Rausch, K. Lenhart and F. Keppler, *Plant, Cell Environ.*, 2011, **34**, 457.
34. E. Tuboly, A. Szabó, D. Garab, G. Bartha, Á. Janovszky, G. Erős, A. Szabó, Á. Mohácsi, G. Szabó, J. Kaszaki, M. Ghyczy and M. Boros, *Am. J. Physiol.: Cell Physiol.*, 2013, **304**(2), C207.
35. M. Boros, E. Tuboly, A. Mészáros and A. Amann, *J. Breath Res.*, 2015, **9**(1), 014001.
36. R. K. Thauer, *Microbiology*, 1998, **44**, 2377.
37. M. Ghyczy, C. Torday and M. Boros, *FASEB J.*, 2003, **17**(9), 1124.

38. M. Ghyczy and M. Boros, *Br. J. Nutr.*, 2001, **85**(4), 409.
39. I. M. Møller, in *Annual Plant Reviews, Plant Mitochondria*, ed. D. C. Logan, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2007, vol. 31, p. 185.
40. J. H. Bond Jr., R. R. Engel and M. D. Levitt, *J. Exp. Med.*, 1971, **133**, 572.
41. Y. Peled, T. Gilat, E. Liberman and Y. Bujanover, *J. Pediatr.*, 1994, **153**, 560.
42. M. D. Levitt, J. K. Furne, M. Kuskowski and J. Ruddy, *Clin. Gastroenterol. Hepatol.*, 2006, **4**, 123.
43. B. P. de Lacy Costello, M. Ledochowski and N. M. Ratcliffe, *J. Breath Res.*, 2013, 7(2), 024001.
44. G. Kim, F. Deepinder, W. Morales, L. Hwang, S. Weitsman, C. Chang, R. Gunsalus and M. Pimentel, *Dig. Dis. Sci.*, 2012, **57**(12), 3213.
45. P. Pitt, K. M. de Bruijn, M. F. Beeching, E. Goldberg and L. M. Blendis, *Gut*, 1980, **21**(11), 951.
46. D. Polag, O. Leiß and F. Keppler, *Sci. Total Environ*, 2014, **481**, 582.
47. A. Minocha and S. Rashid, *Dig. Dis. Sci.*, 1997, **42**, 672.
48. A. Szabó, V. Ruzsanyi, K. Unterkofler, Á. Mohácsi, E. Tuboly, M. Boros, G. Szabó, H. Hinterhuber and A. Amann, *J. Breath Res.*, 2015, **9**(1), 016009.
49. D. Roccarina, E. C. Lauritano, M. Gabrielli, F. Franceschi, V. Ojetti and A. Gasbarrini, *Am. J. Gastroenterol.*, 2010, **105**(6), 1250.
50. A. B. Sahakian, S. R. Jee and M. Pimentel, *Dig. Dis. Sci.*, 2010, **55**(8), 2135.
51. D. Kunkel, R. J. Basseri, M. D. Makhani, K. Chong, C. Chang and M. Pimentel, *Dig. Dis. Sci.*, 2011, **56**(6), 1612.
52. G. A. Weaver, J. A. Krause, T. L. Miller and M. J. Wolin, *Gut*, 1986, **27**, 698.
53. F. Keppler, A. Schiller, R. Ehehalt, M. Greule, J. Hartmann and D. Polag, *J. Breath Res.*, 2016, **10**(1), 016003.
54. M. C. Bennett, G. W. Mlady, Y. H. Kwon and G. M. Rose, *J. Neurochem.*, 1996, **66**(6), 2606.
55. K. Nose, Y. Nunome, T. Kondo, S. Araki and T. Tsuda, *Anal. Sci.*, 2005, **21**, 625.
56. S. Kittelmann, C. S. Pinares-Patiño, H. Seedorf, M. R. Kirk, S. Ganesh, J. C. McEwan and P. H. Janssen, *PLoS One*, 2014, **9**(7), e103171.
57. J. R. Bratten, J. Spanier and M. P. Jones, *Am. J. Gastroenterol.*, 2008, **103**(4), 958.
58. E. A. Melcher, M. D. Levitt and J. L. Slavin, *Nutr. Cancer*, 1991, **16**, 85.
59. T. H. Florin, G. Zhu, K. M. Kirk and N. G. Martin, *Am. J. Gastroenterol.*, 2000, **95**, 2872.
60. P. Vernia, M. D. Camillo, V. Marinaro and R. Caprilli, *Eur. J. Clin. Nutr.*, 2003, **57**, 1116.
61. M. Di Stefano, C. Mengoli, M. Bergonzi, C. Klersy, W. Pagani, E. Micelia and G. R. Corazza, *Am. J. Gastroenterol.*, 2015, **110**(6), 891.

62. J. Serra, F. Azpiroz and J. R. Malagelada, *Gastroenterology*, 1998, **115**(3), 542.
63. S. R. McWilliams, P. D. Mc Laughlin, O. J. O'Connor, A. N. Desmond, A. Ní Laoíre, F. Shanahan, E. M. Quigley and M. M. Maher, *J. Neurogastroenterol. Motil.*, 012, **18**(4), 419.
64. R. B. Lasser, J. H. Bond and M. D. Levitt, *N. Engl. J. Med.*, 1975, **293**(11), 524.
65. E. P. Steffey, B. H. Johnson, E. I. Eger 2nd and D. Howland Jr., *Anesth. Analg.*, 1979, **58**(5), 405.
66. A. C. Soares, H. M. Lederman, U. Fagundes-Neto and M. B. de Morais, *J. Clin. Gastroenterol.*, 2005, **39**, 512.
67. K. Triantafyllou, C. Chang and M. Pimentel, *J. Neurogastroenterol Motil.*, 2014, **20**, 31.
68. M. Pimentel, A. G. Mayer, S. Park, E. J. Chow, A. Hasan and Y. Kong, *Dig. Dis. Sci.*, 2003, **48**, 86.
69. D. Cloarec, F. Bornet, S. Gouilloud, J. L. Barry, B. Salim and J. P. Galmiche, *Gut*, 1990, **31**, 300.
70. K. M. Lee, C. N. Paik, W. C. Chung, J. M. Yang and M. G. Choi, *Eur. J. Gastroenterol. Hepatol.*, 2013, **25**(6), 726.
71. M. Pozuelo, S. Panda, A. Santiago, S. Mendez, A. Accarino, J. Santos, F. Guarner, F. Azpiroz and C. Manichanh, *Sci. Rep.*, 2015, **5**, 12693.
72. K. Gottlieb, V. Wacher, J. Sliman and M. Pimentel, *Aliment. Pharmacol. Ther.*, 2016, **43**(2), 197.
73. M. Pimentel, H. C. Lin, P. Enayati, B. van den Burg, H. R. Lee, J. H. Chen, S. Park, Y. Kong and J. Conklin, *Am. J. Physiol.: Gastrointest. Liver Physiol.*, 2006, **290**, 1089.
74. Y. Liu, H. S. Luo, C. B. Liang, W. Tan, H. Xia and W. J. Xu, *Zhonghua Yixue Zazhi*, 2013, **93**(6), 459.
75. J. Jahng, I. S. Jung, E. J. Choi, J. L. Conklin and H. Park, *Neurogastroenterol. Motil.*, 2012, **24**, 185.
76. M. Pimentel, Y. Kong and S. Park, *Dig. Dis. Sci.*, 2004, **49**, 84.
77. A. R. Hede, L. Andersson and C. Post, *Acta Pharmacol. Toxicol.*, 1985, **57**(4), 291.
78. R. J. Basseri, B. Basseri, M. Pimentel, K. Chong, A. Youdim, K. Low, L. Hwang, E. Soffer, C. Chang and R. Mathur, *Gastroenterol. Hepatol.*, 2012, **8**, 22.
79. R. Mathur, M. Amichai, K. S. Chua, J. Mirocha, G. M. Barlow and M. Pimentel, *J. Clin. Endocrinol. Metab.*, 2013, **98**, E698.
80. R. Mathur, D. Goyal, G. Kim, G. M. Barlow, K. S. Chua and M. Pimentel, *Res. J. Endocrinol. Metab.*, 2014, **2**(2), 2053–3640.
81. H. Zhang, J. K. DiBaise, A. Zuccolo, D. Kudrna, M. Braidotti, Y. Yu, P. Parameswaran, M. D. Crowell, R. Wing, B. E. Rittmann and R. Krajmalnik-Brown, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 2365.
82. P. J. Turnbaugh, R. E. Ley, M. A. Mahowald, V. Magrini, E. R. Mardis and J. I. Gordon, *Nature*, 2006, **444**, 1027.

83. B. S. Samuel and J. I. Gordon, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 10011.
84. R. Mathur, G. Kim, W. Morales, J. Sung, E. Rooks, V. Pokkunuri, S. Weitsman, G. M. Barlow, C. Chang and M. Pimentel, *Obesity*, 2013, **21**(4), 748.
85. Y. Su, Y. H. Luo, L. L. Zhang, H. Smidt and W. Y. Zhu, *Microb. Biotechnol.*, 2015, **8**(3), 519.
86. C. Alarcón de la Lastra, S. Sánchez-Fidalgo, I. Villegas and V. Motilva, *Curr. Pharm. Des.*, 2004, **10**, 3505.
87. M. Zhou, R. Wu, W. Dong, A. Jacob and P. Wang, *Am. J. Physiol.: Regul., Integr. Comp. Physiol.*, 2008, **294**(1), R84.
88. P. Balakumar and S. Kathuria, *Br. J. Pharmacol.*, 2012, **166**(7), 1981.
89. L. Xin, X. Sun and S. Lou, *PLoS One*, 2016, **11**(3), e0150925.
90. T. Li, S. He, S. Liu, Z. Kong, J. Wang and Y. Zhang, *Free Radical Res.*, 2015, **49**(10), 1269.
91. A. Moustafa and Y. Habara, *Free Radical Biol. Med.*, 2016, **90**, 47.
92. M. Magierowski, K. Magierowska, M. Hubalewska-Mazgaj, J. Adamski, D. Bakalarz, Z. Sliwowski, R. Pajdo, S. Kwiecien and T. Brzozowski, *Pharmacol. Res.*, 2016, **114**, 235.
93. S. U. Christl, P. R. Murgatroyd, G. R. Gibson and J. H. Cummings, *Gastroenterology*, 1992, **102**(4 Pt 1), 1269.
94. M. D. Levitt and J. H. Bond, *N. Engl. J. Med.*, 1969, **69**, 122.
95. G. R. Gibson, J. H. Cummings and G. T. Macfarlane, *J. Appl. Bacteriol.*, 1988, **65**(3), 241.
96. G. R. Gibson, J. H. Cummings, G. T. Macfarlane, C. Allison, I. Segal, H. H. Vorster and A. R. Walker, *Gut*, 1990, **31**, 679.
97. T. Koeck, X. Fu, S. L. Hazen, J. W. Crabb, D. J. Stuehr and K. S. Aulak, *J. Biol. Chem.*, 2004, **279**(26), 27257.
98. M. Shen, D. Fan, Y. Zang, Y. Chen, K. Zhu, Z. Cai, Y. Liu, X. Sun, J. Liu and J. Gong, *J. Neurol. Sci.*, 2016, **369**, 361.
99. X. Liu, M. J. Miller, M. S. Joshi, D. D. Thomas and J. R. Lancaster, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**(5), 2175.
100. W. Cui, F. Qi, Y. Zhang, H. Cao, J. Zhang, R. Wang and W. Shen, *Plant Cell Rep.*, 2015, **34**, 435.
101. F. Qi, Z. Xiang, N. Kou, W. Cui, D. Xu, R. Wang, D. Zhu and W. Shen, *Physiol. Plant.*, 2017, **159**, 366.
102. K. Zhu, W. Cui, C. Dai, M. Wu, J. Zhang, Y. Zhang, Y. Xie and W. Shen, *Environ. Exp. Bot.*, 2016, **129**, 37.
103. M. K. Samma, H. Zhou, W. Cui, K. Zhu, J. Zhang and W. Shen, *Biometals*, 2017, **30**, 97.
104. G. Potters, T. P. Pasternak, Y. Guisez, K. J. Palme and M. A. K. Jansen, *Trends Plant Sci.*, 2007, **12**, 98.
105. G. C. Pagnussat, M. Simontacchi, S. Puntarulo and L. Lamattina, *Plant Physiol.*, 2002, **129**, 954.
106. Y.-T. Lin, M.-Y. Li, W.-T. Cui, W. Lu and W.-B. Shen, *J. Plant Growth Regul.*, 2012, **31**, 519.

107. Y. Lin, W. Zhang, F. Qi, W. Cui, Y. Xie and W. Shen, *J. Plant Physiol.*, 2014, **171**, 1.
108. Y. Zhu, W. Liao, M. Wang, L. Niu, Q. Xu and X. Jin, *J. Plant Physiol.*, 2016, **195**, 50.
109. A. T. Mészáros, T. Büki, B. Fazekas, E. Tuboly, K. Horváth, M. Z. Poles, Sz. Szűcs, G. Varga, J. Kaszaki and M. Boros, *Surgery*, 2017, pii: S0039-6060(17)30067-3, DOI: 10.1016/j.surg.2016.12.040.
110. V. G. Deinega, *Farmakol. Toksikol.*, 1968, **31**(4), 494.
111. B. Szczesny, K. Modis, K. Yanagi, C. Coletta, S. Le Trionnaire, A. Perry, M. E. Wood, M. Whiteman and C. Szabo, *Nitric Oxide*, 2014, **41**, 120.
112. D. Gero, R. Torregrossa, A. Perry, A. Waters, S. Le-Trionnaire, J. L. Whatmore, M. Wood and M. Whiteman, *Pharmacol. Res.*, 2016, **113**, 186.
113. O. Chen, Z. Ye, Z. Cao, A. Manaenko, K. Ning, X. Zhai, R. Zhang, T. Zhang, X. Chen, W. Liu and X. Sun, *Free Radical Biol. Med.*, 2016, **90**, 1.
114. L. Versichelen, G. Rolly and H. Vermeulen, *Br. J. Anaesth.*, 1996, **76**(5), 668.
115. R. W. Dougherty, J. J. O'Toole and M. J. Allison, *Proc. Soc. Exp. Biol. Med.*, 1967, **124**, 1155.
116. S. M. Carlisle, P. A. Burchart, C. McCauley and R. A. Surette, *Appl. Radiat. Isot.*, 2005, **62**, 847.
117. A. Szabó, K. Unterkofler, P. Mochalski, M. Jandacka, V. Ruzsanyi, G. Szabó, Á. Mohácsi, S. Teschl, G. Teschl and J. King, *J. Breath Res.*, 2016, **10**(1), 017105.
118. X. Zhang, N. Li, H. Shao, Y. Meng, L. Wang, Q. Wu, Y. Yao, J. Li, J. Bian, Y. Zhang and X. Deng, *Sci. Rep.*, 2016, **6**, 29359.
119. R. He, L. Wang, J. Zhu, M. Fei, S. Bao, Y. Meng, Y. Wang, J. Li and X. Deng, *Biochem. Biophys. Res. Commun.*, 2016, **470**(1), 22.
120. K. Song, M. Zhang, J. Hu, Y. Liu, Y. Liu, Y. Wang and X. Ma, *BMC Surg.*, 2015, **15**, 92.
121. A. Matsuzawa and H. Ichijo, *Biochim. Biophys. Acta*, 2008, **1780**(11), 1325.
122. Z. Ye, O. Chen, R. Zhang, A. Nakao, D. Fan, T. Zhang, Z. Gu, H. Tao and X. Sun, *Shock*, 2015, **44**, 181.
123. D. B. Zorov, C. R. Filburn, L.-O. Klotz, J. L. Zweier and S. J. Sollott, *J. Exp. Med.*, 2000, **192**, 1001.
124. S. S. Hayreh, M. B. Zimmerman, A. Kimura and A. Sanon, *Exp. Eye Res.*, 2004, **78**, 723.
125. L. Liu, Q. Sun, R. Wang, Z. Chen, J. Wu, F. Xia and X. Q. Fan, *Brain Res.*, 2016, **1646**, 327.
126. J. Wu, R. Wang, Z. Ye, X. Sun, Z. Chen, F. Xia, Q. Sun and L. Liu, *Biochem. Biophys. Res. Commun.*, 2015, **466**, 155.
127. W. Liu, D. Wang, K. Liu and X. Sun, *Med. Hypotheses*, 2012, **79**, 308.
128. S. K. Niture, R. Khatri and A. K. Jaiswal, *Free Radical Biol. Med.*, 2014, **66**, 36.

129. D. F. Fan, H. J. Hu, Q. Sun, Y. Lv, Z. H. Ye, X. J. Sun and S. Y. Pan, *Brain Res.*, 2016, **1633**, 62.
130. S. Srinivasan and N. G. Avadhani, *Free Radical Biol. Med.*, 2012, **53**(6), 1252.
131. G. Solaini, A. Baracca, G. Lenaz and G. Sgarbi, *Biochim. Biophys. Acta*, 2010, **1797**(6–7), 1171.
132. S. Dröse and U. Brandt, *Adv. Exp. Med. Biol.*, 2012, **748**, 145.
133. R. B. Hamanaka and N. S. Chandel, *Trends Biochem. Sci.*, 2010, **35**(9), 505.
134. A. Weidinger and A. V. Kozlov, *Biomolecules*, 2015, **5**(2), 472.
135. R. G. Hansford, B. A. Hogue and V. Mildaziene, *J. Bioenerg. Biomembr.*, 1997, **29**(1), 89.
136. E. B. Tahara, F. D. Navarete and A. J. Kowaltowski, *Free Radical Biol. Med.*, 2009, **9**, 1283.
137. G. C. Brown and V. Borutaite, *Mitochondrion*, 2012, **12**(1), 1.
138. C. E. Cooper and G. C. Brown, *J. Bioenerg. Biomembr.*, 2008, **40**(5), 533.
139. C. Wang and R. J. Youle, *Annu. Rev. Genet.*, 2009, **43**, 95.
140. E. Tuboly, A. Mészáros and M. Boros in *Methanogenesis: Biochemistry, Ecological Functions, Natural and Engineered Environments*, ed. G. G. Badalians, Nova Science Publishers, Inc., New York, 2014, ch. 2, p. 19.
141. G. E. Dobretsov, T. A. Borschevskaya, V. A. Petrov and Y. A. Vladimirov, *FEBS Lett.*, 1977, **84**(1), 125.
142. B. Vollmar and M. D. Menger, *Langenbecks Arch. Surg.*, 2011, **396**(1), 13.
143. S. Kawachi, Y. Hara, T. Arao, Y. Suzuki and K. Tamura, *Biosci., Biotechnol., Biochem.*, 2010, **74**(10), 1991.
144. N. Hogg and B. Kalyanaraman, *Biochim. Biophys. Acta*, 1999, **1411**(2–3), 378.
145. H. Watanabe, A. Kobayashi, T. Yamamoto, S. Suzuki, H. Hayashi and N. Yamazaki, *Free Radical Biol. Med.*, 1990, **8**(6), 507.
146. O. K. Baskurt, A. Temiz and H. J. Meiselman, *Free Radical Biol. Med.*, 1998, **24**(1), 102.
147. H. L. Reid, J. A. Dormandy, A. J. Barnes, P. J. Lock and T. L. Dormandy, *Lancet*, 1976, **1**(7961), 666.
148. W. H. Reinhart, *Biorheology*, 2001, **38**(2–3), 203.
149. N. Mohandas and J. A. Chasis, *Semin. Hematol.*, 1993, **30**(3), 171.
150. E. Kayar, F. Mat, H. J. Meiselman and O. K. Baskurt, *Biorheology*, 2001, **38**(5), 405.
151. R. Wang, *Trends Biochem. Sci.*, 2014, **39**(5), 227.
152. W. Liu, D. Wang, H. Tao and X. Sun, *Med. Gas Res.*, 2012, **2**, 25.
153. M. P. Fink, *Crit. Care Med.*, 2012, **40**(4), 1379.