Mitochondria As Sources and Targets of Methane
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This review summarizes the current knowledge on the role of mitochondria in the context of hypoxic cell biology, while providing evidence of how these mechanisms are modulated by methane (CH₄). Recent studies have unambiguously confirmed CH₄ bioactivity in various *in vitro* and *in vivo* experimental models and established the possibility that CH₄ can affect many aspects of mitochondrial physiology. To date, no specific binding of CH₄ to any enzymes or receptors have been reported, and it is probable that many of its effects are related to physico-chemical properties of the non-polar molecule. (i) Mitochondria themselves can be sources of endogenous CH₄ generation under oxido-reductive stress conditions; chemical inhibition of the mitochondrial electron transport chain with site-specific inhibitors leads to increased formation of CH₄ in eukaryote cells, in plants, and in animals. (ii) Conventionally believed as physiologically inert, studies cited in this review demonstrate that exogenous CH₄ modulates key events of inflammation. The anti-apoptotic effects of exogenously administered CH₄ are also recognized, and these properties also suggest that CH₄-mediated intracellular signaling is closely associated with mitochondria. (iii) Mitochondrial substrate oxidation is coupled with the reduction of molecular oxygen, thus providing energy for cellular metabolism. Interestingly, recent *in vivo* studies have shown improved basal respiration and modulated mitochondrial oxidative phosphorylation by exogenous CH₄. Overall, these data suggest that CH₄ liberation and effectiveness in eukaryotes are both linked to hypoxic events and redox regulation and support the notion that CH₄ has therapeutic roles in mammalian pathophysiology.

Keywords: methane, review, mitochondrion, apoptosis, gasotransmitter

INTRODUCTION

Methane (CH₄) is a small omnipresent molecule, the simplest alkane, and the most abundant organic gas in the atmosphere (1). It can help control the amount of hydroxyl radicals and neutralizes ozone in the troposphere (2) and also plays a role in global warming. More importantly, CH₄ can be synthesized biologically and several recent studies have revealed its bioactivity. In a pioneering study, radioactive ¹⁴C-labeled CH₄ was administered to the systemic circulation of sheep, and radioactive carbon dioxide (CO₂) was detected in the breath of animals. Since apart from burning, non-biological decomposition of CH₄ would need high temperature (above 1,000°C) or catalysts, recovery of ¹⁴CO₂ in the exhaled air suggest involvement of CH₄ in the cellular metabolism. Unfortunately, the underlying mechanisms were never resolved (3). In line with the data of Dougherty and coworkers, in a pilot study both ³H- and ¹⁴C-labeled CH₄ was administered to rats. The subsequent analyses revealed organ-dependent rates of retention and decomposition CH₄ in the cellular metabolism. Unfortunately, the underlying mechanisms were never resolved (3). In line with the data of Dougherty and coworkers, in a pilot study both ³H- and ¹⁴C-labeled CH₄ was administered to rats. The subsequent analyses revealed organ-dependent rates of retention and decomposition CH₄ in the cellular metabolism. Unfortunately, the underlying mechanisms were never resolved (3). In line with the data of Dougherty and coworkers, in a pilot study both ³H- and ¹⁴C-labeled CH₄ was administered to rats. The subsequent analyses revealed organ-dependent rates of retention and decomposition CH₄ in the cellular metabolism. Unfortunately, the underlying mechanisms were never resolved (3). In line with the data of Dougherty and coworkers, in a pilot study both ³H- and ¹⁴C-labeled CH₄ was administered to rats. The subsequent analyses revealed organ-dependent rates of retention and decomposition CH₄ in the cellular metabolism. Unfortunately, the underlying mechanisms were never resolved (3). In line with the data of Dougherty and coworkers, in a pilot study both ³H- and ¹⁴C-labeled CH₄ was administered to rats. The subsequent analyses revealed organ-dependent rates of retention and decomposition CH₄ in the cellular metabolism. Unfortunately, the underlying mechanisms were never resolved (3). In line with the data of Dougherty and coworkers, in a pilot study both ³H- and ¹⁴C-labeled CH₄ was administered to rats. The subsequent analyses revealed organ-dependent rates of retention and decomposition CH₄ in the cellular metabolism. Unfortunately, the underlying mechanisms were never resolved (3). In line with the data of Dougherty and coworkers, in a pilot study both ³H- and ¹⁴C-labeled CH₄ was administered to rats. The subsequent analyses revealed organ-dependent rates of retention and decomposition CH₄ in the cellular metabolism. Unfortunately, the underlying mechanisms were never resolved (3).
to its characteristics, availability and effectiveness, CH₄ became also a candidate gasotransmitter (6). The first reports about the protective effect of CH₄ against oxidative stress and inflammation caused by ischemia and reperfusion (IR) (7, 8) was followed by several studies (9–12). Because of its wide-ranging protective effects in many diverse disease models, it was proposed that CH₄ could be a new medical gas (13). Indeed, CH₄ is intrinsically non-toxic, without any known side effects. However, before the use in human clinical settings, the specific mechanism of action needs to be elucidated.

The mitochondrion is feasible target. Mitochondria are specialized subcellular structures that power various physiological roles, such as energy production, reactive oxygen species (ROS) formation, calcium homeostasis, and intrinsic apoptosis, all of which may be targets of CH₄ administration (3, 4). A previous review summarized the available findings on the biological role of CH₄, and it was proposed that CH₄ liberation is related to hypoxic events resulting in, or associated with mitochondrial dysfunction (6). Indeed, several studies have demonstrated that the effects of exogenously administered CH₄ in IR injuries can be grouped around a typical triad, namely anti-inflammatory, anti-oxidative, and anti-apoptotic properties. Notably, all these changes are also associated with mitochondrial functions, probably via non-specific physico-chemical alterations of membranes.

Importantly, concentrations of exogenously applied CH₄ are orders of magnitude higher than those reported for endogenous production (reviewed in Section "Endogenous CH₄ Formation Is Associated with Mitochondrial Dysfunction"). Due to the low solubility of CH₄ in the watery phase, the majority of the gas is exhaled while CH₄ is enriched at biological membrane interfaces, leading to higher local concentrations. Therefore, no direct conclusion can be made about the role of CH₄ as a messenger only based on studies with CH₄ treatment.

Overall, this review summarizes the effects of CH₄ on mitochondria along with the current knowledge and the best available evidences on the possible mode of action. First, mitochondria are discussed as sources of endogenous CH₄ generation under oxido-reductive stress conditions. Next, the consequences of exogenous CH₄ supplementation are outlined: how it modulates key events of inflammation that are associated with mitochondrial functions. Thereafter, CH₄-mediated intracellular signaling events are overviewed that are likely involved in cellular protection. Finally, the impact on the mitochondrial substrate oxidation in relationship with the anti-apoptotic effects is discussed.

ENDOGENOUS CH₄ FORMATION IS ASSOCIATED WITH MITOCHONDRIAL DYSFUNCTION

Mammalian methanogenesis has been considered an exclusive attribute of methanogenic Archaea, a group well distinguished from bacteria and eukaryotes. Nevertheless, to date, a number of studies have demonstrated the generation of non-bacterial CH₄ also in aerobic living systems, and it has also been proposed that the CH₄-producing phenomenon can be linked to the loss of the redox homeostasis.

It was shown in 2003 that hypoxia could lead to the generation of measurable amounts of non-bacterial CH₄ in isolated liver mitochondria (14). Increasingly, high amounts of CH₄ (between 0 and 2.3 nmol/mg protein) were generated after the addition of ascorbic acid and 1–100 mM hydrogen peroxide (H₂O₂), and the formation of CH₄ was related linearly to the quantity of mitochondria incubated. A breakthrough came when Keppler and colleagues (15) provided direct evidence of CH₄ generation in multi-cellular organisms under aerobic conditions. This key paper was followed by many studies that either supported or disagreed with the initial findings (16–19) where the common denominator was likely to be mitochondrial dysfunction. More importantly, it has been shown that oxido-reductive stress elicits aerobic CH₄ emission in plants (20).

Interestingly, in 2008, a study by Ghyczy et al. (21) demonstrated aerobic CH₄ emission in cultured endothelial cells exposed to hypoxia and metabolic distress. Mitochondrial dysfunction in this setting led to significant CH₄ generation (~2–23 nmol/mg mitochondrial protein), depending on the nature and intensity of the metabolic distress, and a similarly high and dose-dependent CH₄ generation was detected after ROS attack in the Udenfriend reaction.

Furthermore, it has been shown that the CH₄-producing phenomenon can be mimicked by the administration of sodium azide (NaN₃), a compound known to disrupt mitochondrial electron transport flow by specifically binding to cytochrome c (Cyt c) oxidase. In this in vivo study, the whole-body CH₄ production profile was determined in unrestrained animals after chronic NaN₃ administration (22). In this scenario, the stress-related methanogenic capacity of the rats was revealed in animals treated with antibiotics to eradicate the CH₄-producing intestinal flora (22). In a model of transient mitochondrial distress, the CH₄ generation of rats and healthy human volunteers was evaluated before and after excessive ethanol intake, and significant CH₄ production was demonstrated in both species (23). The phenomenon was again independent from the activity of methanogenic prokaryotes (23).

The CH₄-generating capacity of NaN₃ administration may also be associated with the generation of ROS (24, 25). It has been hypothesized that electrophilic methyl groups of biomolecules such as the phosphatidylcholine molecule might be carbon precursors (14, 18) and a potential source of CH₄ liberation. Nevertheless, the underlying mechanism is complicated by the various oxido-reductive stress answers of the mitochondria, e.g., throughout DNA methylation patterns and therefore gene expression changes. Such conditions can both affect gene expressions and activity of S-adenosylhomocysteine hydrolase (26) and may change the methionine metabolism, where CH₄ may be liberated as an intermediate compound. Recently, Althoff et al. (27) presented a novel chemical reaction that readily forms CH₄ from organosulphur compounds such as methionine, under highly oxidative conditions, ambient atmospheric pressure, and temperature. In this reaction, methyl sulfides are oxidized to the corresponding sulfoxides by a ferryl species, then, in the next phase, demethylation of the sulfoxide via homolytic bond cleavage leads to CH₄ formation (27).
NOVEL SIGNALING PATHWAYS INVOLVED IN CH₄-MEDIATED NUCLEAR AND MITOCOCHONDRIAL EFFECTS

CH₄ might alter the pattern of the activation of various signal transduction pathways and vice versa, the well-described triple effect (i.e., anti-inflammatory, antioxidant, and anti-apoptotic) of CH₄ may influence the upregulation and downregulation of cellular signaling cascades. A direct link between exogenously administered CH₄ and signaling targets have been reported recently (28, 29); however, a crosstalk with other bioactive gases and pathways cannot be excluded. Furthermore, no specific binding of CH₄ to any enzymes or receptors have been reported to date, and it is highly probable that many (if not all) of its effects are related to physico-chemical properties of the non-polar molecule.

In a recent study by Wang et al. (29, 30), a supersaturated (~1.5 mmol/l) CH₄-enriched saline solution was administered in a rat model of IR injury. High tissue concentrations of CH₄ (between 90 and 145 µmol/g) were achieved, which lead to increased expression of Nrf2 (also known as nuclear factor erythroid 2-related factor 2). Nrf2 undoubtedly plays a central role in the activation of antioxidant defense system in most living organisms. It is now well characterized that Nrf2 translocates and binds to the antioxidant response element (ARE) forming a complex in the nucleus and induces the expression genes antioxidant and detoxifying enzymes (Figure 1) (31, 32). A negative regulator of Nrf2–ARE pathway is the kelch-like ECH associating protein 1 (Keap1), which forms a cytoplasmic complex with Nrf2 and inhibits its translocation to the nucleus under basal conditions (33).

Wang and coworkers (29, 30) have found that CH₄ administration enhances Nrf2 expression at both the mRNA and protein levels. In this study, marked elevations in Nrf2 mRNA and protein levels were found while the regulatory Keap1 protein was degraded in a time dependent manner. Besides, activation of phosphatidylinositol 3-kinase–Akt pathway, an indirect mechanism involved in the synergistic activation of Nrf2–ARE oxidative stress response, may also play a role in CH₄ action (28, 34). As a result of CH₄-upregulated Nrf2/ARE signaling, activated downstream enzymes [e.g., hem oxygenase-1, superoxide dismutase (SOD), catalase, and γ-glutamyl cysteine ligase] attenuate the excessive production of ROS and result in preserved mitochondrial function (35) as well as anti-inflammatory (36–38) and anti-apoptotic effects (39, 40).

Among several transduction pathways, the forkhead box transcription factor class O (FoxO)-related antioxidant enzyme induction seems to be a novel candidate in the protective effects of CH₄ (41). Although FoxO in cooperation with tumor suppressor p53 (42) regulates cell cycle arrest, it is also responsible for ROS elimination by promoting the expression of numerous antioxidant genes and detoxifying enzymes (43); such as SOD, catalase, glutathione peroxidase 2, glutathione-S-transferase, and a sulfiredoxin (42). Moreover, it may influence mitochondrial homeostasis (44) through the modulation a serine/threonine-protein kinase, PTEN-induced putative kinase, thereby contributing to

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![Figure 1](https://example.com/image1.png)

**FIGURE 1** | Possible signaling pathways involved in the antioxidant, anti-apoptotic, and anti-inflammatory effect of methane. CH₄ may induce Nrf2/ARE-mediated activation of antioxidant and detoxifying enzymes. These attenuate the excessive production of reactive oxygen species (ROS) resulting preserved mitochondrial function as well as anti-inflammatory and anti-apoptotic effects. Second, complementary antioxidant pathways (e.g., FoxO) are also hypothesized to be activated. The non-polar nature of CH₄ may influence cell membrane permeability and ion channel function-related signal transduction as well. Nrf2, nuclear factor erythroid 2-related factor 2; ARE, antioxidant response element; HO-1, hem oxygenase-1; SOD, superoxide dismutase; CAT, catalase; γ-GCL, γ-glutamyl cysteine ligase; siRNA, small-interfering RNA; FoxO, forkhead box transcription factor class O; CH₄, methane; Keap1, kelch-like ECH associating protein 1; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; p65, transcription factor p65.
cell survival. Nevertheless, in the absence of data confirming that CH₄ acts on FoxO pathway (45) in animal disease models, this remains only a hypothesis.

**CH₄-MEDIATED ACTIONS ON THE MITOCHONDRIAL ELECTRON TRANSPORT SYSTEM (ETS)**

CH₄ has favorable distribution characteristics by penetrating membranes and diffusing into organelles including mitochondria (46); therefore, a potential effect of CH₄ on the mitochondrial respiration has also been emerged. Upon exogenous administration, CH₄ gets into the bloodstream through the alveoli of the lung and dissolves in the plasma. The cytoplasm/plasma solubility is near uniform for CH₄; however, this ratio is much higher in hydrophobic substances such as the phospholipid biomembranes of mitochondria (47, 48). Of interest, the protein complexes of the mitochondrial respiratory chain are partially embedded in the inner mitochondrial membrane, exposing parts of them to the hydrophobic lipid bilayer, which makes them potential targets of the CH₄.

The modulator effects of small gaseous molecules on mitochondrial respiration have been demonstrated in animal models of IR injury both in vivo and in hypoxic assays in vitro (11, 30, 49–51). Specifically, NO exerted protection through the activation of the mitochondrial K<sub>ATP</sub> channel opening (50) and induced a sustained mitochondrial depolarization (49). H₂S preserved mitochondrial membrane integrity and the complex I- and II-linked oxygen consumption rate (51). CH₄ restored the ADP-dependent mitochondrial respiration, i.e., the oxidative phosphorylation (11, 30). During oxygen deprivation, the mitochondrial ETS is manifested lower rates of non-phosphorylating basal respiration. In addition, the ADP-dependent oxygen consumption, or in other words oxidative phosphorylation, is significantly depressed. In contrast, reperfusion conditions induce leakage of electrons from the ETS into the intermembranous space (52) that leads to increased ROS formation. These results suggest the sensitivity of both the resting state of ETS and the mitochondrial bioenergetic function to IR injury.

General effects of CH₄ on IR-related mitochondrial dysfunction involve the restoration of the electron transport machinery of the inner mitochondrial membrane when oxygen concentration rises. In a study by Strifler et al. (11), mitochondria incubated in a medium with a gas phase containing 2.2% CH₄–air mixture displayed a significantly improved leak respiration and increased recovery of oxidative phosphorylation. These effects of CH₄ on the inner mitochondrial membrane can be explained with a hypothesis which presumes that CH₄ dissolves in biological membranes thereby changing its oxidative stress-related rigidity (7).

In parallel with the general effects on the mitochondrial ETS, CH₄ seems to exert site-specific action on protein complexes. Among the protein complexes of the mitochondrial ETS, complex IV (cytochrome c oxidase), which catalyzes the reduction of oxygen by ferricytochrome to H<sub>2</sub>O, is target of the CH₄ action. Indeed, endogenous CH₄ generation occurs in plant mitochondria (53) and in mammalian cells after inhibition of complex IV by NaN₃ (22). Meanwhile, exogenous CH₄ administration in IR injury conditions resulted in reduced Cyt c release from the inner mitochondrial membrane and lower Cyt c oxidase activity in liver mitochondria (11).

Nonetheless, the above observations are all related to the in vivo effects of exogenous CH₄ supplementation on mitochondria in oxido-reductive stress conditions. Interestingly, direct mitochondrial effects could not be shown when a 2.2% CH₄–air mixture was administered isolated mitochondria in vitro (11). In other words, direct effect of CH₄ on oxidative phosphorylation capacity and leak respiration in intact liver mitochondria cannot be shown in vitro. CH₄ has relatively low solubility in watery phase, ranging from 37.2 to 19.1 mg/l between 0 and 35°C at 1 standard atmosphere. Therefore, high concentrations should be applied exogenously to overcome the limitation of low gas solubility. This means that approximately 1 mmol/l/min CH₄ was usually applied as gas therapy (7) and 1.6 mmol/l in CH₄-enriched saline (29). Consequently, CH₄ concentrations in tissues upon CH₄ treatment are certainly higher than the average endogenous levels. Still, due to the high affinity of Cyt c oxidase for oxygen (54), CH₄ does not disproportionate oxygen and thus does not limit mitochondrial respiration.

**CH₄-MEDIATED ACTIONS ON APOPTOSIS**

The anti-apoptotic effect is one of the most studied properties of CH₄. Several studies demonstrated that CH₄ modulates the intrinsic pathway of apoptosis (9–11, 29, 30, 55–58). The intrinsic pathway of apoptosis, also called the mitochondrial pathway owing to the essential involvement of mitochondria (59), which are not only the site where anti- and pro-apoptotic proteins interact but also the origins of high range of signal pathways that initiate the activation of caspases through various mechanisms (60). The large family of Bcl-2 homologs involves key proteins of intrinsic apoptosis described to organize the process (61). They can be divided into two classes: anti-apoptotic Bcl-2 family proteins (such as Bcl-XL, Bcl-w, Mcl-1, A1, Bcl-Rambo, Bcl-L10, and Bcl-G) and pro-apoptotic proteins (such as Bcl-2 associated X protein (Bax), Bak, and Bok) (62). The primary anti-apoptotic function of Bcl-2 is to block the release of Cyt c. In contrast, upon stress, pro-apoptotic members of the Bcl-2 family are activated (Bak or Bax) which leads to the mitochondrial outer membrane permeabilization and subsequent release of intermembrane space proteins such as Cyt c. Cyt c is attached to the inner mitochondrial membrane and shuttles electrons between complex III and complex IV. In response to membrane damage, it releases to the cytosol and activate the initiator procaspase-9 within the apoptotic protease-activating factor-1 (Apaf-1) apoptosome complex (63). Once activated, caspase-9 activates effector procaspase-3 which, in turn, can cleave various protein substrates, leading to the morphological and biochemical features of apoptosis (64).

In IR, CH₄ supplementation may improve the level of key regulators of apoptosis, such as Bcl-2, Apaf-1, and caspases indirectly through an unknown pathway (10). Two recent studies of experimental retinal IR provided evidence for the effectiveness of CH₄ treatment on upregulation of anti-apoptotic Bcl-2 proteins, while reduced cleaved caspase 3- and 9 quantities (10, 57). Likewise, in an experimental spinal cord IR scenario, CH₄ was proven to significantly reduce the number of apoptotic cells...
The anti-apoptotic effect of CH₄. The release of cytochrome c and other inner mitochondrial membrane proteins are regulated by Bcl-2 family proteins through interplay between pro-apoptotic and anti-apoptotic proteins, which converge to Bax/Bak activation, thereby inducing mitochondrial outer membrane permeability. CH₄, methane; CLDN6, claudin 6; RIP 1, receptor-interacting kinase 1; ASK-1, apoptosis signal-regulating kinase 1; p-38, mitogen-activated protein kinase; Casp-3, caspase 3; Casp-9, caspase 9; Apaf-1, apoptotic protease-activating factor 1; Hrk/DP5, harakiri gene; Bcl-2, B-cell lymphoma 2 regulation protein; JNK, c-Jun N-terminal kinase; BAX, Bcl-2 associated X protein; BAK, Bcl-2 homologous antagonist/killer.

(confirmed by TUNEL staining) and also the amount of cleaved caspase-3 and caspase-9 proteins and to attenuate cytosolic Cyt c release (29). In addition, CH₄-rich fluids significantly reduced the expression of cleaved caspase-3 and also decreased the apoptotic cell number in rodents exposed to LPS-induced acute lung injury (58). In a hepatic IR model, CH₄ inhalation could effectively attenuate the apoptosis-linked morphological changes in the liver and the TUNEL positivity of hepatocytes (11). Similarly, caspase-3 expression was attenuated with CH₄-rich saline treatment which substantiated this finding (56). Moreover, CH₄-rich saline has pronounced neuroprotective effect in diabetic retinopathy in streptozotocin-induced diabetes, possibly by upregulating those cell cycle related microRNAs, which contribute to post-transcriptional regulation (65). A possible indirect way how CH₄ supplementation modulates apoptosis is to reduce the level of mitochondrial ROS formation. ROS and their by-products can oxidize the reduced thioredoxin-apoptosis signal-regulating kinase 1 complex, then activate apoptosis signal-regulating kinase and its downstream stress signaling targets, such as c-Jun NH(2)-terminal kinase (66). Another plausible explanation for the anti-apoptotic effect of CH₄ is reducing Cyt c release from the inner membrane, which has already been demonstrated in hepatic, myocardial, and spinal cord IR as well (9, 11, 29) (Figure 2).

PERSPECTIVES AND CONCLUDING STATEMENTS

In the human body, many gases are biologically active. Signaling roles were demonstrated for NO, CO, and H₂S, 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. CH₄ is a small, less reactive gas molecule, having a close symbiosis with bioactive gases in the intracellular spaces. The effects of exogenous CH₄ were clearly illustrated in detail in various tissues under different conditions. Of particular interest is that the recognized biological effects of CH₄ are not cell- or tissue specific, and an increased input may result in anti-inflammatory changes in cells and tissues. In this regard, it is tempting to speculate on a much broader, controller role for CH₄ in acute and chronic oxido-reductive stress conditions.

AUTHOR CONTRIBUTIONS

AM and PH designed and developed the concept of the manuscript. AS, LJ, ET, DÉ, and GV wrote the manuscript. ÁS prepared Figure 2. LJ prepared Figure 1. PH supervised and edited the manuscript. All authors discussed and commented on the manuscript at all stages.

FUNDING

This work was supported by Hungarian National Research, Development and Innovation Office (NKFIH) grant K120232 and K116861, EFOP-3.6.2-16-2017-00006, GINOP 2.3.2-15-2016-00015 and New National Excellence Program of the Ministry of Human Capacities UNKP-17-4. Nemzeti Kutatási és Technológiai Hivatal 10.13039/501100003827.
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Frontiers in Medicine | www.frontiersin.org November 2017 | Volume 4 | Article 195

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