Hypoxia-induced methane generation *in vivo* and *in vitro*: mechanism and significance

Ph.D. Thesis

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2008

Szeged, Hungary

CONTENTS

List of full papers related to the subject of the thesis

I. Ghyczy M, **Torday C**, Boros M: Simultaneous generation of methane, carbon dioxide, and carbon monoxide from choline and ascorbic acid: a defensive mechanism against reductive stress? FASEB J. 17(9):1124-6, 2003. **IF: 7.162**

II. Ghyczy M*, **Torday C***, Kaszaki J, Szabó A, Czóbel M, Boros M: Hypoxia-induced generation of methane in mitochondria and eukaryotic cells: an alternative approach to methanogenesis. Cell Physiol Biochem. 21(1-3):251-8, 2008.

IF: 3.557 (* = EQUAL CONTRIBUTION)

III. Ghyczy M*, **Torday C***, Kaszaki J, Szabó A, Czóbel M, Boros M: Oral phosphatidylcholine pretreatment decreases ischemia-reperfusion-induced methane generation and the inflammatory response in the small intestine. Shock 5:596-602, 2008**. IF: 3.325 (*= EQUAL CONTRIBUTION)**

List of abstracts related to the subject of the thesis

1. **Torday C**, Fónagy A, Wolfárd A, Ghyczy M, Boros M. Reduktív stressz által kiváltott metán képzõdés in vitro körülmények között. Magyar Sebészet, Supplementum, 43, 2001.
2. **Torday C**, Ghyczy M, Boros M. Biomolecules with electrophilic methyl groups may ameliorate reductive stress. Acta Physiol Hung 89(1-3), 287, 2002.
3. **Torday C**, Ghyczy M, Boros M. In vitro methane formation during reductive stress conditions. Shock 18(S1), 26, 2002.
4. Szabó A, Csipszer B, Czóbel M, **Torday C**, Kaszaki J, Ghyczy M, Boros M. The effects of systemic phosphatidylcholine treatment in hyper and hypodynamic endotoxemia. Eur Surg Res 36(S1), 117, 2004.
5. **Torday C**, Ghyczy M, Boros M. Methane production in endothelial cells - a consequence of free radical attack? Eur Surg Res 37(S1), 108-109, 2005.
6. Boros M, Ghyczy M, **Torday C**, Kaszaki J, Czóbel M. A gyulladás füstje: metán képződés hipoxia-reoxigenizáció alatt. Magyar Sebészet, Supplementum, 2007.
7. Boros M, Ghyczy M, **Torday C**, Kaszaki J, Czóbel M: Hypoxia-induced methane generation - mechanism and function. Inflammation Research 56(S2): S172, 2007.

INTRODUCTION

Methane (CH_4), the second most important anthropogenic greenhouse gas after CO_2 (Forster *et al.* 2007), is the most abundant reduced organic compound in the atmosphere. According to established knowledge, it is produced primarily by anaerobic bacterial activity in wetlands, rice fields, landfills and the gastrointestinal tract of ruminants, with non-bacterial emissions occurring from fossil fuel usage and biomass burning. The main tropospheric sink of CH_4 is chemical removal by the hydroxyl radical.

Aerobic life depends critically on redox homeostasis, an integrating network of oxidative and reductive processes. An abnormal increase in intracellular reducing power may occur through an interruption in the flow of electrons down the mitochondrial electron transport chain (Williamson *et al.* 1999, Niknahad *et al.* 1995). This occurs during hypoxia, when a deficiency of electron acceptor oxygen leads to decreasing ATP generation and progressive functional and structural cell damage (Chance, 1957).

Evidences suggest that an abnormal increase in reducing power (or "*reductive stress*") is associated with abnormal clinical states that may shorten the life span of cells (Ido *et al.* 1997). The clinical/pathophysiological equivalent to biochemical reductive stress is ischemia, when blood supply is inadequate to meet the demands of the tissues. Ischemia triggers a complex cascade of reactions and molecular events including altered Ca^{2+} homeostasis, mitochondrial dysfunction, and inflammatory activation.

Reperfusion, or reestablishment of the oxygen supply to the previously ischemic tissues is a precarious process, however, as the disturbed intracellular biochemistry leads to the generation of reactive oxygen species (ROS). Superoxide ($^{\bullet}O_2^-$), a key ROS produced during the incomplete reduction of oxygen forms the basis of "*oxidative stress*" (Zorov *et al.* 2006). This term is applied to *in vivo* situations in which damage is caused, either directly or indirectly, by an elevated level of ROS or the generation of other free radicals. Superoxide anion can release redox-active iron ions from iron-sulfur proteins and thus can lead to the generation of more aggressive species including the highly-damaging hydroxyl radical (Valko *et al.* 2005). The Haber-Weiss or iron-catalyzed Fenton-type reaction between hydrogen peroxide and a transition metal can be driven by other reducing radicals like ascorbate (Udenfriend reaction) as well.

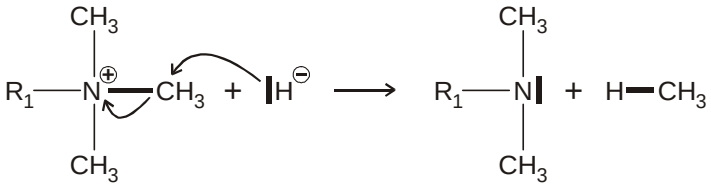
Hydroxyl radicals are short-lived (the *in vivo* half-life is approx. 10^{-9} sec) but their reactivity towards cellular constituents is several orders of magnitude higher than that of superoxide radicals and hydrogen peroxide (Halliwell and Gutteridge, 1992). Hydroxyl radical can damage virtually all types of macromolecules, carbohydrates, nucleic acids, lipids and amino acids: it is considered the most damaging oxygen species within the body. Its in vivo measurement is however, very difficult and specific markers (amino acid hydroxylation, protein, DNA adducts, and aromatic probes) are currently under intense investigation.

Ischemia and reperfusion (I-R) syndrome is a severe, multifactorial clinical condition and a good experimental model that has been widely employed to study oxido-reductive stress in vivo (Carden *et al*. 2000, Stallion *et al.* 2005). Although the etiology may vary, local ischemia always results in a change from an aerobic to an anaerobic metabolism, which profoundly affects both early and long-term tissue reactions. Today it is recognized that I-R is leading to antigen-independent inflammation, where several signals, including the release of constitutive cellular proteins act as activators of the immune system (Carden *et al.* 2000). In the resulting response, local expression of adhesion molecules, proinflammatory cytokines and systemic activation of the polymorphonuclear neutrophils (PMNs) are central inducers of tissue injury (Carden *et al.* 2000, Arumugam *et al.* 2004). This proinflammatory state increases tissue vulnerability to further injury, finally resulting in the amplification of the inflammatory response (Carden *et al*. 2000). In the gastrointestinal tract, a transient splanchnic flow reduction predisposes to the influx of luminal foreign material, and mucosal I-R therefore often progresses to the development of secondary septic complications.

A number of compounds have been demonstrated to protect against oxidative damage by inhibiting or quenching radical formation. Phosphatidylcholines (PCs) are a class of phospholipids which incorporate choline as headgroup. PC is ubiquitous membrane-forming entity, but experiments and clinical experience indicated that it may function as an active substance as well. In particular, various lines of evidence suggested that PC may decrease the magnitude of the inflammatory response, increases tolerance in experimental ischemia and hypoxia and inhibits mucosal damage caused by acids and other noxious agents in the gastrointestinal tract (Treede *et al.* 2007, Erős *et al.* 2006, Ghyczy *et al.* 2002). The therapeutical effect of parenteral PC and lyso-PC has been demonstrated in experimental sepsis models also (Drobnik *et al.* 2003, Yan *et al*. 2004, Ilcol *et al.* 2005).

Phospholipase D (PLD) is one of the key enzymes in the lipid metabolism which catalyzes the hydrolysis of PC to form phosphatidic acid (PA) and releasing the soluble choline into the cytosol. Choline is a natural amine, classified as a water-soluble essential nutrient (Blusztajn *et al.* 1983, 1998). Choline metabolites are needed for main physiological purposes including structural integrity, signaling roles for cell membranes, neurotransmission (acetylcholine synthesis). It is source for methyl groups (CH_3) required for methylation reactions via trimethylglycine (betaine) that participates in the S-adenosylmethionine (SAM) synthesis pathways (de Long *et al.* 2002, Ghosal *et al.* 1995). Humans can synthesize choline de novo in small amounts by converting phosphatidylethanolamine (PE) to PC in the liver by three methylation reactions, each using SAM as a CH_3 donor.

It has been widely believed that the biological efficacy of PC depends on the fatty acid moiety of the compound. In contrast**,** some other studies have suggested us that the protective role of PC is independent of the fatty acids and may be linked to electrophilic methyl groups (EMGs) of the headgroup, which may act as electron acceptors under certain in vivo conditions (Ghyczy *et al.* 2001). PC and acetylcholine react *in vitro* with the electron donor sodium benzothiolate in an irreversible redox reaction by the transfer of a pair of electrons to the electron-deficient methyl group, thus splitting this group from the positive N moiety (Stoffel *et al.* 1971). This electron-pair transfer between the biological electron acceptors and an artificial electron donor led us to speculate that a similar reaction may also take place in cells. In this sequence the nucleophilic hydride-ion from NADH is transferred to the electrophilic methyl group. This is followed by the splitting off of this methyl group with the formation of methane and the oxidation of NADH to NAD^+.



AIMS

Our main goal was to provide evidence that a methane-generating reaction is induced by transient hypoxia in aerobic systems. Further aims were to outline the mechanism of methane production and the possible components of the methanogenic pathway in mammalian cells

and organelles. Additionally, we aimed at determining the methanogenic potential of PC metabolites in vivo, and the effects and the possible role of PC in I-R-induced oxido-reductive stress response in the gastrointestinal tract.

METHODS

In vitro chemical experiments

Methyl group-containing PC metabolites including choline chloride (CC), N,N-dimethylethanolamine (DMEA), N-methylethanolamine (MEA), ethanolamine (EA), betaine, dimethylglycine (DMG), sarcosine (N-methylglycine) and glycine were tested in the presence of the hydroxyl free radical generating Udenfriend components (ASC, H_2O_2 and catalytic iron). The experiments were carried out in 5-ml gastight Supelco vials connected to 5-ml syringes through the septum of the cap. Methane concentration was analyzed by gas chromatography with flame-ionization detection (Carlo Erba Instruments HRGC 5300 Megaseries, Chrompack capillary column). Methane (CH_4), carbon dioxide (CO_2), and carbon monoxide (CO), nitrogen (N_2), and (O_2) concentrations were measured simultaneously by gas chromatography with TCD and column switching system, and the main column was filled with Hayesep Q 80/100 mesh.

The lucigenin-enhanced chemiluminescence assay

The 1 ml reaction mixture containing the components of the Udenfriend reaction and the examined PC metabolites in buffer were given to minivials containing lucigenin in 100 μM concentration. Chemiluminescence was measured in the tritium channel (set in the out-of-coincidence mode) of a Packard Tri-Carb 2100 Model liquid scintillation counter by the method of Ferdinandy et al. (2000).

Isolation of rat-liver mitochondria and mitochondrial subfractions

The isolation of mitochondria and mitochondrial fractions was performed according to the standard method of Schneider modified by Kristal and Yu (1998). Mitochondrial protein was determined according to Lowry *et al.* (1951). Mitochondrial integrity and oxygen consumption were analyzed by polarography at 25°C with an oxygraph equipped with a Clark oxygen electrode (Aqueous Phase Respiration System OXYGRAPH/Hansatech Instruments Ltd.).

Endothelial cell cultures

Endothelial cells were isolated and cultured in Dulbecco's phosphate-buffered saline according to a modified method of Grygliewski *et al.* (1986) and Rosen *et al*. (1981). The experiments were performed on 10-day-old endothelial cells (18-20x10^6 cells/flask).

Granulocyte studies

PMNs were separated from canine venous blood by the method of Guarnieri *et al*. (1987). Cell viability was tested by the trypan blue exclusion method. The superoxide radical production, the phorbol 12-myristate 13-acetate (PMA)-stimulated activity of the PMNs (1.5x10^6 cells/ml) were determined with a UV-1601 spectrophotometer (Shimadzu, Japan) at 550 nm.

***In vivo* large animal studies**

Three separate groups of experiments were performed on a total of 17 inbred dogs (average weight 12.7±2 kg) under sodium pentobarbital anesthesia (30 mg kg^{-1} i.v.).The right femoral artery and vein were cannulated for the measurement of mean arterial pressure (MAP) and for fluid or drug administration, respectively. A Swan-Ganz thermodilution catheter (Corodyn TD-E-N, 5011-110-7Fr; Braun Melsungen AG, Melsungen, Germany) was positioned into the pulmonary artery via the right femoral vein to measure the cardiac output (CO) by thermodilution, using a Cardiostar CO-100 computer (Experimetria Ltd., Budapest, Hungary).

An ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY, U.S.A.) was placed around the exposed superior mesenteric artery (SMA) to measure the mesenteric blood flow. A branch of a tributary of the ileal vein supplying the terminal part of the ileum was cannulated to measure mesenteric venous pressure. In all protocols, a period of 30 min was allowed for recovery from surgery. Ischemia was maintained for 60 min, followed by a 180-min reperfusion period.

Experimental protocol

The animals were randomly allocated into one or other of 3 experimental groups according to the feeding protocols. Groups 1 and 2 were fed with normal laboratory chow for 1 week before the experiments. In group 3, the animals were fed with a special diet containing 1% PC (Ssniff Spezialdiäten GmbH, 59494 Soest, Germany) in a dose of 50 g kg^{-1} day^{-1} for 6 days before the experiment. Group 1 (n=5) served as sham-operated control, while in groups 2 (n=6) and 3 (n=6), complete small intestinal ischemia was induced by occluding the superior mesenteric artery (SMA). Small intestinal tissue biopsy samples, peripheral blood samples

and gas samples from the expired air were taken before the occlusion of the SMA, and thereafter at the beginning and end of the reperfusion period.

Hemodynamic measurements

Pressure and blood flow signals were monitored continuously and registered with a computerized data-acquisition system (SPELL Haemosys; Experimetria Ltd., Budapest, Hungary).

Intramucosal pH measurements

A silastic balloon catheter (TGS Tonomitor, Tonometrics Inc., Worcester, Massachusetts, U.S.A.) was introduced through a small enterotomy into the intestinal lumen. Arterial blood gases and intramucosal pCO_2 were measured with a blood-gas analyzer (AVL, Graz, Austria). Intramucosal pH (pHi) was calculated by using the modified Henderson-Hasselbach formula with a correction factor for 30-min equilibration (Fiddian-Green, 1989).

Intestinal superoxide production

Superoxide production in freshly minced intestinal biopsy samples was assessed by a lucigenin-enhanced chemiluminescence assay (Ferdinandy *et al.* 2000). Chemiluminescence was measured at room temperature in a liquid scintillation counter by using a single active photomultiplier positioned in out-of-coincidence mode.

Tissue myeloperoxidase activity

The activity of myeloperoxidase (MPO), was measured on ileal biopsy samples by the method of Kuebler *et al.* (1996). The MPO activities of the samples were measured at 450 nm (UV-1601 spectrophotometer, Shimadzu, Japan), and the data were referred to the protein content.

Exhaled methane measurement

The trachea was intubated with a cuffed endotracheal tube (Portex Tracheal Tube) and the animals breathed spontaneously throughout the experiment. 2500 ml of expired air was collected. 1-ml gas samples were taken from the bag to determine methane concentrations.

The rate of methane formation was determined by gas chromatography and a Chrompack capillary column.

Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). *In vitro* data were analyzed by two-way ANOVA tests

followed by the Bonferroni test. Data are expressed as means ± standard deviation (SD). For the *in vivo* data, Friedman repeated measures analysis of variance on ranks was applied within the groups. Time-dependent differences from the baseline were assessed by Dunn's method. Differences between groups were analyzed with Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison. In this context, median values and 75^{th} and 25^{th} percentiles are given; p values < 0.05 were considered significant.

RESULTS AND DISCUSSION

In vitro studies without biological structures

We determined the relative effectiveness of potential methyl group donor compounds (CC > S- methylmethionine > PC > S-adenosylmethionine > betaine > carnitine > acylcarnitine) and reducing agents (ASC > NADH >NADPH > glutathione > dithiotreitol > NAC) in terms of methane generation, and the most potent ASC and choline chloride were used in further experiments. We have investigated the formation of methane in a chemical model reaction from choline in the presence of H_2O_2, catalytic iron, and ASC. Each step of the reaction pathway was studied systematically, methane formation as well as the possible evolution of other gases was monitored in several concentration ranges and in different ratios of the components. No reactions occurred in the absence either of Fe^{3+} or H_2O_2. In all experimental series, the reactions started immediately with visible gas formation, color changes, and an elevation of temperature. Methane generation *in vitro* increased linearly with the amount of H_2O_2 and choline and the concentration of catalytically active iron. The optimal ratio of oxidant to reducing agent was 1:0.5 in this setting. The reactions as judged by gas evolution came to an end after 20-30 min, and the completed reaction can be restarted only by the addition of H_2O_2. In this system CO_2 and CO are formed parallel to methane generation. The results indicate that the reduced carbon moiety derives from choline, while ASC generates CO and CO_2.

In the second series of *in vitro* chemical experiments the methane-generating capacity of PC metabolites was examined in a hydroxyl radical-generating chemical reaction. When CC and the choline metabolites were tested, significant methane liberation was detected in the gas phase. For CC, DMEA, MEA and EA, the amount increased linearly with the number of

methyl groups in the molecules; whereas for the other choline products (i.e. EA, betaine, DMG, sarcosine and glycine) significant methane liberation was not observed.

The methane-producing choline metabolites with alcoholic moiety in the molecule (i.e. CC, DMEA, MEA and EA) effectively inhibited hydroxyl free radical generation in the Udenfriend reaction ($p < 0.05$), while the non- methane producers betaine, DMG, sarcosine and glycine with carboxyl moiety did not display free radical-scavenging capacity determined by the lucigenin-enhanced chemiluminescence assay. The effectiveness was proportional to the amount of methane generated and the number of methyl groups in the compounds.

CC in the range 1-100 mM dose-dependently decreased the hydroxyl radical concentration generated by the Udenfriend reaction (chemical model of hydroxyl radical generation) and exhibited a similar effectiveness as the standard hydroxyl free radical scavengers DMSO, mannitol and DMTU. These results suggest that an elevated reducing power could potentially not only reduce oxygen, but also electron acceptor biomolecules thus leading to the formation of methane.

Experiments with mitochondria

The mitochondria are a major source of intracellular ROS production and all components of our chemical model reaction (other than free iron) are ubiquitous constituents in comparatively high concentrations in the matrix. Reductive stress in this system was generated by hypoxia and by adding several reducing agents (including ASC, NADH, NADPH, dithiothreitol, reduced glutathione, NAC). An increasingly high amount of methane was reproducibly generated after the addition of ASC and 1-100 mM H_2O_2. Methane formation was linearly related to the amount of mitochondria incubated (between 0 and 10 mg protein/2 ml), the amount of H_2O_2 added (between 0 and 20 mM), and the pH of the reaction mixture. Acidic pH increased methane formation, but there was a significant methane evolution even at pH 7.0. Catalase (300 U/ml) completely abolished the increase in methane production, and this indicates that mitochondrial H_2O_2 is required for the hypoxic activation of the methane generating reaction. DMTU (10 mM), pyruvate (10 mM), and mannitol (10 mM) were less effective (~80% inhibition was observed), while the addition of superoxide dismutase (100 U/ml) did not affect methane generation. It is important to note that the rate of H_2O_2 formation in rat liver tissue is very high (380 nmol/min/g tissue, *i.e.*, ~10% of total oxygen consumption) and catalase is absent from the mitochondrial matrix (Halliwell *et al.*

2000, Chance *et al.* 1979, Sohal *et al.* 1990, Nulton-Persson *et al.* 2001). The Fe^{3+} ion represents a further important component, as reductive stress (the accumulation of electrons in NADH) can liberate the Fe^{3+} ion from ferritin and reduce it to Fe^{2+}. This in turn may catalyze the formation of hydroxyl radicals through the iron-catalyzed Haber-Weiss reaction (Thomas *et al.* 1985, Liochev *et al.* 2002).

Secondly, rat liver mitochondrial subfractions were prepared to examine methane production of each under the effect of the hydroxyl free radical-generating Udenfriend components (H_2O_2, ASC and catalytically active iron). The highest methane production was observed in the matrix (2.96 nmol/mg protein, ($p < 0.05$), much less methane was generated in the intermembrane space (0.197 nmol/mg protein), and only very low amounts were detected in the inner membrane (0.08 nmol/mg protein) and outer membrane (0.042 nmol/mg protein) fractions.

Endothelial cell culture studies

In a further step, we have examined the consequences of modulation of the mitochondrial electron transport in confluent cultures of porcine aortic endothelial cells. In these studies, chemical hypoxia was evoked by different ways. The treatments included inhibition of glucose uptake and anaerobic glycolysis, application of site-specific inhibitors of the mitochondrial electron transport chain alone or in combination with glycolysis inhibitors, application of an uncoupling agent, and treatment of the cells with increasing concentrations of the hydroxyl radical generating Udenfriend system. Methane generation was always observed when compounds with such different modes of primary action were used.

The level of methane production increased significantly ($p < 0.05$) over the 2 nmol/mg baseline, reaching 8-10 nmol/mg when intact endothelial cells were deprived of glucose (when glucose was omitted from the reaction mixture) and after the inhibition of glycolysis with NaF, 2-DDG and IAA. Metabolic inhibition of complex IV by NaCN and NaN_3 increased the methane production further ($p < 0.05$) and resulted in ~15 nmol/mg. Simultaneous glucose deprivation or glycolysis inhibition did not enhance this response further. Higher amounts of liberated methane (20-23 nmol/mg) were observed after the inhibition of oxidative phosphorylation by the application of uncoupling agent 2,4-dinitrophenol (2,4-DNP), and similarly high and dose-dependent methane generation was measured after the hydroxyl radical-generating Udenfriend reaction. The results revealed that

disturbance of the normal mitochondrial function leads to significant, dose-dependent methane generation in endothelial cells, the extent depending on the type and intensity of the metabolic distress.

Granulocytes

The PMA-activated PMN granulocyte assay (based on the reduction of cytochrome c) was used to investigate the effects of CC and its metabolites on leukocyte activation. In this system ROS were generated upon activation of the NADPH oxidase complex of the PMNs. The methane producing PC metabolites (i.e. CC, DMEA and MEA) and EA proved to be efficient ($p < 0.05$), while the nonproducers, betaine, DMG, sarcosine and glycine did not inhibit the activity of isolated PMN granulocytes. These series of experiments using freshly isolated and PMA-activated PMN leukocytes furnished evidence that methane-producing PC metabolites inhibit the formation of ROS and may therefore be defined as 'antioxidants'.

In vivo experiments

The aims were to establish whether the dietary administration of PC can protect the reperfused small bowel mucosa by its acting as an anti-inflammatory agent and to investigate this possibility in association with *in vivo* methane generation. Thus, in a further step, we examined the consequences of modulation of the PC input on endogenous methane formation, and we have reported a series of experimental results that support the hypothesis that a PC-enriched diet may be protective during intestinal I-R injury. The examined parameters were: hemodynamics, superoxide production, tissue myeloperoxidase changes, intramucosal pHi, PCO_2 gap and *in vivo* methane production.

The results revealed that the postischemic period was characterized by general signs of reperfusion damage, significant tissue acidosis with ROS generation, and leukocyte accumulation. These local responses were accompanied by increased methane production in the exhaled air during the early phase of reoxygenation.

The results also indicated that a PC-enriched diet may indeed counteract mucosal superoxide generation, efficiently decrease intestinal PMN accumulation and prevent the decrease in mucosal pHi. Furthermore, our results demonstrate that PC or its metabolites can suppress the methane generation after transient ischemia. Although the delivery of more PC prevented I-R-induced mucosal inflammation, this protocol actually decreased the postischemic production of methane as well. This parallel change in the oxidative and

reductive arms of the process seems to be controversial. A possible explanation for the PC-induced negative feedback may involve a dual functionality of PC metabolites. Theoretically, choline can be oxidized to betaine, dimethylglycine, sarcosine (N-methylglycine), and glycine by donating electrons to an electron acceptor, for example, to ROS, and this pathway can then negatively influence ROS generation as a whole. In this case, methanogenic metabolites will generate methane by accepting an electron, whereas non-producers will react with a radical to form water.

These molecules may react differentially to the various changes in the redox milieu in accordance with an abnormal shift toward reducing or oxidizing conditions in the cell. Indeed, the results of our *in vitro* experiments indicated that a concerted reaction of choline and ascorbate can lead to the simultaneous generation of oxidized and reduced carbon-containing gas molecules: methane, CO_2, and CO. Nevertheless, further investigations are needed to determine the effects of individual PC metabolites on oxidoreductive stress markers.

Here it should be added that Keppler *et al.* have shown that plants can also produce methane in response to photo energy without the need for a protein to catalyze the reaction (Keppler *et al.* 2006). These laboratory experiments proved that living plants, plant litter and the structural plant component pectin emit CH_4 to the atmosphere under aerobic conditions. Later further studies (Wang *et al.* 2008, Vigano *et al.* 2008, McLeod *et al.* 2008) have confirmed Keppler's work. More importantly, these data strongly support our previous *in vitro* and *in vivo* findings as well.

In conclusion, a steady state of reducing power or redox balance may be as important for the normal functioning of aerobic cells as is a constant pH. Conversely, redox imbalance may be as common and important a feature of abnormal clinical states as is acid-base imbalance. Attention has been focused in the past on "*oxidative stress*". Oxidative stress has long been assumed to be the main cause of oxygen free radical activity and its damaging consequences in biological systems. This assumption may be mistaken and may account for the disappointing performance of antioxidants in clinical practice (Gutteridge 1999). The reverse imbalance, "*reductive stress*", is far more common and potentially life-threatening. The underlying cause for the pathological conditions which are misleadingly named today oxidative stress, is reductive stress or elevated/displaced reducing equivalent. This can be normalized only by electron acceptors. The defense mechanism which may operate in

biological systems against such reductive stress may be the capture of electrons by EMGs and the consequent irreversible evolution of methane gas.

SUMMARY OF NEW FINDINGS

1. The formation of methane from choline and from its metabolites in the presence of the hydroxyl radical producing Udenfriend reaction (hydrogen peroxide, catalytic iron, and ascorbic acid) was reported. In this system, carbon monoxide and carbon dioxide are formed from the ascorbate molecule in parallel with methane generation.
2. PC metabolites with alcoholic moiety in the molecule displayed an effectiveness to counteract oxygen radical production, proportional to the amount of methane generated and the number of methyl groups in the compounds. The efficacy of CC to eliminate hydroxyl free radical is in the same order as that of the known hydroxyl radical scavenger molecules mannitol, DMSO and DMTU.
3. The formation of methane by rat liver mitochondria was reported for the first time.
4. The mechanism by which methan can be generated *in vitro* and *in vivo* has been elaborated; methane generation is a consequence of transient oxygen deprivation in aerobic cells. The methane-generating reaction is a defensive response to reductive stress which provides protection against redox imbalance in living systems.
5. The formation of methane by an aerobic organism was reported for the first time. The generation of methane may be linked to a tissue response to ischemia and associated with abnormal ROS generation in the gastrointestinal tract.
6. PC metabolites containing both electron donor and acceptor groups may have a function to counteract intracellular oxygen radical production.
7. Increased dietary uptake of PC exerts an anti-inflammatory influence in the gastrointestinal tract.

ACKNOWLEDGMENT

Here I would like to commemorate to Professor Gábor Petri, who invited me to the Institute of Experimental Surgery (today Institute of Surgical Research), supported my career and showed me the way to go in my scientific research.

I am grateful to Profs. Sándor Nagy and Mihály Boros, former and present directors of the Institute of Surgical Research, who helped my research work from the beginnings. I am deeply indebted to Professor Boros for giving me all kinds of professional and human help in my research work, and for his stimulating support to write my thesis.

I am especially indebted to Dr. Miklós Ghyczy (Cologne, Germany) for his constructive and valuable scientific advices.

Also, I thank all my colleagues and the technical staff of my Institute for their contributions to my experimental work. I would like to thank especially Gabriella Patyik and Andrea Fónagy for the skillful help, for their precise and scrupulous work. I would like to express my gratitude to all those who had contributed in some way toward the completion of the thesis.