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

Ph.D. Thesis

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CONTENTS

List of papers related to the subject of the thesis **4**

List of abbreviations **5**

1. INTRODUCTION **6**

1.1. Reductive stress and oxidative stress **6**

1.2. Free radicals, free radical scavengers and protective mechanisms **7**

1.3. Ischemia-reperfusion **7**

1.4. Phosphatidylcholine **8**

1.5. Choline and its metabolites **8**

1.6. Electrophilic methyl groups **9**

2. AIMS **9**

3. MATERIALS AND METHODS **10**

3.1. In vitro chemical experiments **10**

3.1.1. Methane-producing capacity of choline metabolites **10**

3.1.2. In vitro experiments with the hydroxyl radical generating Udenfriend reaction **10**

3.1.3. The lucigenin-enhanced chemiluminescence assay **11**

3.2. Experiments with mitochondria **11**

3.2.1. Isolation of rat-liver mitochondria **11**

3.2.2. Isolation of mitochondrial fractions **12**

3.2.3. Measurement of mitochondrial methane formation **12**

3.2.4. Measurement of methane formation in mitochondrial subfractions after free radical generation **13**

3.3. Tissue culture experiments **13**

3.3.1. Chemical hypoxia of endothelial cells **13**

3.4. Granulocyte studies **13**

3.5. In vivo large animal studies **14**

3.5.1. Hemodynamic measurements **15**

3.5.2. Intramucosal pH measurements **15**

3.5.3. Intestinal superoxide production **15**

3.5.4. Tissue myeloperoxidase activity **15**

3.5.5. Exhaled methane measurement **15**
3.5.6. Experimental protocol **16**
3.6. Methane measurement **16**
3.7. Statistical analysis **16**
4. RESULTS **17**
4.1. In vitro studies without biological structures **17**
4.2. Experiments with mitochondria **23**
4.3. Endothelial cell culture studies **25**
4.4. Granulocytes **28**
4.5. In vivo experiments **28**
4.5.1. Hemodynamics **29**
4.5.2. Superoxide production **29**
4.5.3. Tissue myeloperoxidase changes **30**
4.5.4. Intramucosal pH and PCO_2 gap **31**
4.5.5. In vivo methane production **31**
5. DISCUSSION **32**
6. SUMMARY OF NEW FINDINGS **37**
7. ACKNOWLEDGEMENTS **38**
8. REFERENCES **39**
9. ANNEX **47**

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)**

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1. **Torday C**, Fónagy A, Wolfárd A, Ghyczy M, Boros M. Reduktív stressz által kiváltott metán képzõdés in vitro körülmények között. Magyar Sebészet Suppl., 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 Suppl., 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.

List of abbreviations

Cardiac output	CO
Choline chloride	CC
2-deoxy-D-glucose	2-DDG
2,4-dinitrophenol	2,4-DNP
Dimethylglycine	DMG
Dimethylsulfoxide	DMSO
Dimethylthiourea	DMTU
Electrophilic methyl group	EMG
Ethanolamine	EA
Ethylene diamine tetraacetic acid	EDTA
N-2-Hydroxyethylpiperazone-n-2-Ethanesulfonic Acid	HEPES
Iodoacetate	IAA
Ischemia-reperfusion	I-R
L-ascorbic acid	ASC
Mean arterial pressure	MAP
Myeloperoxidase	MPO
N,N-dimethylethanolamine	DMEA
N-methylethanolamine	MEA
Nicotinamide adenine dinucleotide	NAD
Phenylmethanesulphonylfluoride	PMSF
Phorbol 12-myristate 13-acetate	PMA
Phosphatidylcholine	PC
Phosphatidylethanolamine	PE
Polymorphonuclear neutrophil	PMN
Sodium azide	NaN_3
Sodium cyanide	NaCN
Sodium fluoride	NaF
Soybean trypsin inhibitor	STI
Superior mesenteric artery	SMA

1. 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.

1.2. Reductive stress and oxidative stress

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, 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.

1.2. Free radicals, free radical scavengers and protective mechanisms

Today, oxido-reductive stress and the damage that results from it have been implicated in a wide number of disease processes including atherosclerosis, autoimmune disorders, neuronal degeneration and cancer. However, superoxide anion ($\bullet O_2^-$), the one-electron reduction state of O_2, is formed in many autoxidation reactions and by the electron transport chain during

normal cellular metabolism in all aerobic species. On the other hand, 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. The first step of this catalytic cycle involves reduction of ferric ion to ferrous:

$$Fe^{3+} + \bullet O_2^- \rightarrow Fe^{2+} + O_2$$

The second step is the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$$

The net Haber-Weiss reaction:

$${}^{\bullet}O_2^- + H_2O_2 \rightarrow {}^{\bullet}OH + HO^- + O_2$$

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. In vivo measurement of hydroxyl radicals is however, very difficult and specific markers (amino acid hydroxylation, protein, DNA adducts, and aromatic probes) are currently under intense investigation.

1.3. Ischemia-reperfusion

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.

1.4. Phosphatidylcholine

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.* 2008). 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).

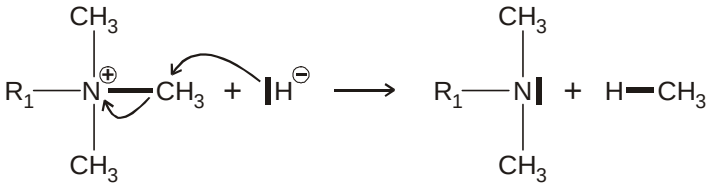
1.5. Choline and its metabolites

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 methylation. Three methylation reactions are required, each using SAM as a CH_3 donor. In rats, dietary choline deficiency is associated with an increased incidence of spontaneous liver cancer and increased sensitivity to carcinogenic chemicals. A number of mechanisms have been proposed to explain the cancer-promoting effects of choline deficiency: (a) decreased methylation of DNA, resulting in abnormal DNA repair; (b) increased oxidative stress, increasing the likelihood of DNA damage; (c) stimulation of apoptosis; and (d) activation of cell-signaling protein kinase C molecule (Zeisel *et al.* 1991)

The choline-containing phospholipids PC and sphingomyelin, are precursors for the intracellular messenger molecules, diacylglycerol and ceramide. Two other choline metabolites, platelet activating factor (PAF) and sphingophosphorylcholine, are also known to be cell-signaling molecules. Besides, choline is a precursor for acetylcholine, an important neurotransmitter.

1.6. Electrophilic methyl groups

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^+.



2. 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.

3. MATERIALS AND METHODS

3.1.1. In vitro chemical experiments

Methyl group-containing PC metabolites as test compounds included choline chloride (CC), N,N-dimethylethanolamine (DMEA), N-methylethanolamine (MEA), ethanolamine (EA), betaine, dimethylglycine (DMG), sarcosine (N-methylglycine) and glycine. Dimethylsulfoxide (DMSO) was applied as positive control. The experiments were carried out in 5-ml gastight Supelco vials connected to 5-ml syringes through the septum of the cap. The total volume of the reaction mixture was 1 ml and it contained the following components: 1 mM hydrogen peroxide (H_2O_2), 0.5 mM L-ascorbic acid (ASC), 10 μM $FeCl_3$ or $FeCl_2$ and 6 μM EDTA, 1 mM test compound or DMSO in 5 mM K-phosphate buffer (Ghyczy et al. 2003). All chemicals were purchased from Sigma Co. The reaction mixture was adjusted to pH 7.4, the tubes were then closed and air was removed by bubbling nitrogen through the mixture for 20 min. The reaction was started by the addition of H_2O_2 with a Hamilton syringe through the septum of the cap. Any gas mixture generated expanded spontaneously into a 5 ml volume syringe inserted through the septum of the cap. The methane concentration of the gas phase was analyzed in 0.5-ml gas samples by gas chromatography (Carlo Erba Instruments HRGC 5300 Megaseries, Chrompack capillary column) 60 min after initiation of the reaction.

3.1.2. In vitro experiments with the hydroxyl radical generating Udenfriend reaction

The experiments were performed in 22 ml gas-tight Supelco vials equipped with a gas-tight open cap with a septum. The vials were connected to a 100 ml Hamilton syringe through the septum of the cap; thus the generated gases could expand spontaneously into the syringe. The composition of the gas phase was analyzed by gas chromatography with TCD and column switching system, and the main column was filled with Hayesep Q 80/100 mesh. Methane (CH_4), carbon dioxide (CO_2), and carbon monoxide (CO), nitrogen (N_2), and (O_2) concentrations were measured simultaneously from 1 ml gas samples taken from the gas phase from the top of the reaction mixture. The 10 ml reaction mixture contained equimolar amounts (1 mol/l) of H_2O_2, L-ascorbic acid, 10 mmol/l $FeCl_3$ or $FeCl_2$ and CC in 500 mmol/l potassium-phosphate buffer. Parallel experiments were performed after hypoxia (20 min incubation in N_2 atmosphere). The reaction was always started by the addition of H_2O_2 with a Hamilton syringe through the gas-tight septum of the cap; the pH of the reaction mixture was

adjusted to pH 7.0 beforehand (the pH was always measured at the beginning and at the end of the experiments). Temperature changes within the Supelco vials were monitored and registered with an RPH 01 drop-temperature sensor connected to a 3.1 Haemosys Data Acquisition System (Experimetria Ltd.). The experiments were repeated with linearly decreasing the concentration of the individual components. The gas formation decreased linearly, and the detection limit was reached in the 100 micromolar range.

3.1.3. The lucigenin-enhanced chemiluminescence assay

The 1 ml reaction mixture containing the components of the Udenfriend reaction and the examined methyl-group containing PC metabolites in buffer were given to minivials containing lucigenin. The resulting chemiluminescence was measured in the tritium channel (set in the out-of-coincidence mode) of a Packard Tri-Carb 2100 Model liquid scintillation counter. The manipulations were performed without external light after 2 min dark adaptation. Changes in chemiluminescence were detected in the presence or absence of 1 mM methyl group-containing test compounds and the results were expressed as percentages of the control values.

3.2. Experiments with mitochondria

3.2.1. Isolation of rat-liver mitochondria

The isolation of mitochondria was performed according to the standard method of Schneider modified by Kristal and Yu 1998. Briefly, male Wistar rats (200-250 g) were housed in a controlled environment with free access to food and water. The rats were anesthetized with ether and killed by decapitation, and their livers were removed while they were under aseptic conditions. The livers of two rats were immediately placed in a glass Potter homogenizer and homogenized in ice-cold medium containing 250 mM mannitol, 75 mM saccharose, 0.1 mM EDTA (sodium salt), 10 mM HEPES, and protease inhibitors (10 μM/ml PMSF, 10 μM leupeptin, 10 μM STI, and 500 U/ml aprotinin), pH 7.4. The homogenate was centrifuged at 650 g for 6 min, and the resulting supernatant was centrifuged at 7800 g for 15 min. The pellet was resuspended in the initial medium containing protease inhibitors (10 μM /ml PMSF and 250 U/ml aprotinin) and centrifuged at 7800 g for 15 min. Centrifugation was repeated, and the final pellet (without PMSF) containing the mitochondria was used for the experiments within 2 h. Each experiment was repeated with mitochondria stored at -20°C for 24 h. Solutions were prepared with bidistilled methane-free water, and all

the procedures were performed at 4°C. 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.). Briefly, mitochondria (0.5 mg/ml protein) were placed in a 2 ml cell, and the respiratory parameters were determined using 5 mM succinate and 0.45 μmol ADP as substrates. Each experiment was repeated with at least five different mitochondrial preparations.

3.2.2. Isolation of mitochondrial fractions

Mitochondria isolated from rat livers were washed and centrifuged twice with phosphate buffer at 8000 g, suspended in hypotonic 20 mM phosphate buffer (pH 7.4) and centrifuged at 35000 g for 20 min. The supernatant containing the intermembrane space fraction (IMS) was removed. The sediment was then suspended in 10 mM HEPES buffer containing a protease inhibitor cocktail (10 μg/ ml STI, 10 μM leupeptin and 500 U/ml aprotinin) and centrifuged at 1900 g for 25 min. The supernatant was centrifuged at 35000 g for 20 min at 4 ^{o}C to obtain the outer mitochondria membrane fraction (OM), while the sediment was resuspended in distilled water (mitoplast fraction) and freeze-thawed 4 times. After a 25 min centrifugation at 35000 g the supernatant was separated as matrix (M) fraction. The sediment was resuspended in distilled water and washed twice with centrifugation at 35000 g for 15 min to obtain the inner membrane (IM) fraction.

3.2.3. Measurement of mitochondrial methane formation

2 ml of mitochondrium suspension (0.25 to 5 mg/ml protein) was incubated in 5-ml gas-tight Supelco vials at 37°C in a reaction medium containing 20 mM potassium phosphate buffer (154 mM KCl, 3 mM $MgCl_2$, 5 mM succinic acid), 0.1 mM $FeCl_3$, 0.06 mM EDTA and 5 mM ASC. The reaction mixture was adjusted to pH 7.4. A 20-min exposure to N gas was used to induce hypoxia and the reaction was started by the addition of 10 mM H_2O_2. The samples were kept in a water bath at 37°C for 60 min, the reaction was stopped by immersion in ice-cold water and the samples were subsequently kept on ice until analysis. 200-ml aliquots of the headspace gas were obtained with a Hamilton gas-tight syringe and the rate of methane formation was determined by gas chromatography.

3.2.4. Measurement of methane formation in mitochondria subfractions after free radical generation.

These experiments were performed in 5-ml gas-tight Supelco vials under continuous stirring. The reaction mixtures contained 2 ml of mitochondrial fraction (1.5 mg protein/vial) in 20 mM potassium phosphate buffer, 5 mM ASC, 0.1 mM Fe^{3+}/0.06 mM EDTA and 10 mM H_2O_2 (Udenfriend reaction components) in a total volume of 2.5 ml. The reaction was identical to described above, 200-ml aliquots of the headspace gas were obtained and methane formation was determined by gas chromatography.

3.3. Tissue culture experiments

The cells were isolated and cultured according to a modified method of Grygliewski *et al.* 1986 and Rosen *et al*. 1981. Endothelial cells were verified by light microscopy via the non-overlapping cobblestone morphology and the positive staining for the presence of Factor VIII-related antigen by routine indirect immuno-fluorescence assay (Grigliewski *et al.* 1986). The experiments were performed on 10-day-old endothelial cells (18-20x10^6 cells/flask). Before the start of the studies, the culture medium was changed for Dulbecco's phosphate-buffered saline with or without glucose (5 ml/flask) and the Nunclon caps were changed for Supelco gas-tight caps.

3.3.1. Chemical hypoxia of endothelial cells

In these studies, a confluent layer of endothelial cells was exposed to site-specific inhibitors of complex IV of the mitochondrial electron transport chain (NaCN and NaN_3); inhibitors of glycolysis (NaF, 2-DDG and IAA), and the uncoupler of the electron transport from phosphorylation (2,4-DNP) in the concentration range of 1-100 nmol/ml. The oxygen free radical-generating Udenfriend system was used at concentrations of 1-1000 nmol/ml concentration range. Other approaches included the removal of glucose from the reaction medium or exposure to inhibitors of glycolysis, together with metabolic poisons. The total gas volume above the cell layer was 36 ml, and 500-µl gas samples were analyzed in all cases. After measurement of the methane concentration, the contents of the flask were dissolved in 1 M NaOH, and the total protein content was determined by the method of Lowry *et al*.

3.4. Granulocyte studies

PMNs were separated from canine venous blood by the method of Guarnieri *et al*. 1987. Following dextran sedimentation and hypotonic lysis (to remove contaminating erythrocytes),

Ficoll-Hipaque gradient centrifugation was performed. Cell viability was tested by the trypan blue exclusion method. The PMNs ($1.5x10^6$ cells/ml) were incubated at 37 ^oC in the presence of 40 μM ferricytochrome c in phosphate buffer, and the generation of superoxide anion was detected with a UV-1601 spectrophotometer (Shimadzu, Japan) at 550 nm. The spontaneous radical production and the phorbol 12-myristate 13-acetate (PMA)-stimulated activity of the PMNs were determined. The extent of release of superoxide into the reaction mixture was calculated from the linear portion of the cytochrome c reduction plot, using a molar absorption coefficient of $19.1x10^{-3}$ M^{-1} cm^{-1}.

3.5. *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 experiments were carried out in adherence to the NIH guidelines for the use of experimental animals and the study was approved by the Ethical Committee for the Protection of Animals in Scientific Research at the University of Szeged. The animals were deprived of food but not water for 18 h before the start of the experiments.

The right femoral artery and vein were cannulated for the measurement of mean arterial pressure (MAP) and for fluid or drug administration, respectively. The animals were placed in a supine position on a heating pad for maintenance of the body temperature between 36 and 37 ^oC, and received an infusion of Ringer's lactate at a rate of 10 ml kg^{-1} h^{-1} during the experiments. 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).

After a midline abdominal incision, the root of the superior mesenteric artery (SMA) was dissected free. An ultrasonic flow probe **(**Transonic Systems Inc., Ithaca, NY, U.S.A.) was placed around the exposed 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 with a 2-F polyethylene catheter 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.

3.5.1. Hemodynamic measurements

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

3.5.2. 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 RG. 1989).

3.5.3. Intestinal superoxide production

Superoxide production in freshly minced intestinal biopsy samples was assessed by a lucigenin-enhanced chemiluminescence assay (Ferdinandy *et al.* 2000). Approximately 25 mg of intestinal tissue was placed in 1 ml of Dulbecco's solution (pH 7.4) containing 5 μM lucigenin. Chemiluminescence was measured at room temperature in a liquid scintillation counter by using a single active photomultiplier positioned in out-of-coincidence mode, in the presence or absence of the superoxide scavenger nitroblue tetrazolium (NBT; 20 μl). NBT-inhibited chemiluminescence was considered an index of intestinal superoxide generation.

3.5.4. Tissue myeloperoxidase activity

The activity of myeloperoxidase (MPO), a marker of tissue leukocyte infiltration, was measured on ileal biopsy samples by the method of Kuebler *et al.* (1996). Briefly, the tissue samples were homogenized with Tris-HCl buffer (0.1 M, pH 7.4) containing 0.1 mM PMSF to block tissue proteases, and then centrifuged at 4 ^{o}C for 20 min at 24000 g. 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.

3.5.5. Exhaled methane measurement

The trachea was intubated with a cuffed endotracheal tube (Portex Tracheal Tube) and the animals breathed spontaneously throughout the experiment. The sufficiency of normoxic ventilation was checked by repeated analysis of blood gases, including the partial pressure of oxygen in arterial blood samples (AVL, Graz, Austria). The endotracheal tube was connected to a non-rebreathing one-way rectifier valve system (Ambu GmbH, Germany). During breath

sampling, the expiratory outlet of the system was attached to a gastight flexible aluminum bag (Plastigas, Linde Gas, Cologne, Germany) and 2500 ml of expired air was collected. 1-ml gas samples were taken from the bag to determine methane concentrations by gas chromatography.

3.5.6. 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.

3.6. Methane measurement

The rate of methane formation was determined by gas chromatography with flame-ionization detection (Carlo Erba Instruments HRGC 5300 Megaseries) and a Chrompack capillary column. The samples were measured in triplicate: the coefficient of variation of the measurements was less than 7%. Possible atmospheric contamination of the samples was investigated; the baseline methane (room air) concentration was determined in separate measurements. For calibration, a standard methane gas mixture was used. The methane contents of the experimental samples were determined from the linear part after the background methane concentration had been subtracted.

3.7. 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); p values < 0.05 were considered significant.

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.

4. RESULTS

4.1. In vitro studies without biological structures

The findings are set out in Figures 1-4. No reactions occurred in the absence either of Fe^{3+} or H_2O_2 (data not shown.). In all experimental series, the reactions started immediately with visible gas formation, color changes, and an elevation of temperature. First, 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. The results show that methane generation in vitro increased linearly with the amount of H_2O_2 (Figure 1) and choline (between 0 and 2 mol/l) and the concentration of catalytically active iron (between 0 and 20 mmol/l). With increasing ASC concentration, methane generation reaches maximum (50 µmol/total gas volume) at 0.5 M and then declines (Figure 1 c); in other words, 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.

CO and CO_2 were also present in the gas phase (Figure 2b and c). CO_2 evolved in a measurable amount within 30 s after the start of the reaction, while CO appeared parallel to methane formation. Methane formation was significantly enhanced at acidic pH, and similarly, the pH decreased in parallel to methane generation. The reaction is exothermic; 45°C, 60°C, 74°C, and 95°C peak was measured after 0.25 M, 0.5 M, 1 M, and 2 M H_2O_2, respectively. In parallel to heat generation, the gas volume increased: the expansion moved the piston of the syringe attached to the reaction tube in proportion to the amount of the generated CO_2. The alteration of choline concentration affected exclusively the methane generation and did not influence CO or CO_2 production. The same amount of CO (3.5 µM/total gas volume) and CO_2 (1300 µM/total gas volume) was formed with or without choline, while methane formation was exclusively related to the presence of choline (or other

EMGs). When the amount of the individual components was decreased, methane formation decreased linearly and the detection limit was reached in the 0.1 mM range.

In the second series the methane-generating capacity of PC metabolites was tested in a hydroxyl radical-generating chemical reaction (Figure 3). Approximately 120 000 nmol methane was generated from DMSO. When CC and the choline metabolites were tested, the order of magnitude was lower, but 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; 3750 nmol methane was generated from CC, 2960 nmol from DMEA, and 1820 nmol from MEA ($p \leq 0.05$), whereas for the other test compounds (i.e. EA, betaine, DMG, sarcosine and glycine) significant methane liberation was not observed (Figure 3).

The lucigenin-enhanced chemiluminescence assay was used to determine the reactive oxygen species-scavenging capacity of the choline metabolites; the findings are depicted in Figure 4A and 4B. In these experiments, the extent of hydroxyl free radical generation in the Udenfriend reaction was quantified by means of the luminol-enhanced chemiluminescence assay in the presence or absence of test compounds, while DMSO, dimethylthiourea (DMTU) and mannitol were used as positive controls.

The methane-producing choline metabolites with alcoholic moiety in the molecule (i.e. CC, DMEA, MEA and EA) effectively inhibited ROS generation ($p < 0.05$), while the non-methane producers betaine, DMG, sarcosine and glycine did not display free radical-scavenging capacity. The effectiveness was proportional to the amount of methane generated and the number of methyl groups in the compounds (Figure 4A).

CC in the range 1-100 mM dose-dependently decreased the hydroxyl radical concentration generated by the Udenfriend reaction, and exhibited a similar effectiveness as the standard hydroxyl free radical scavengers DMSO, mannitol and DMTU (Figure 4B).

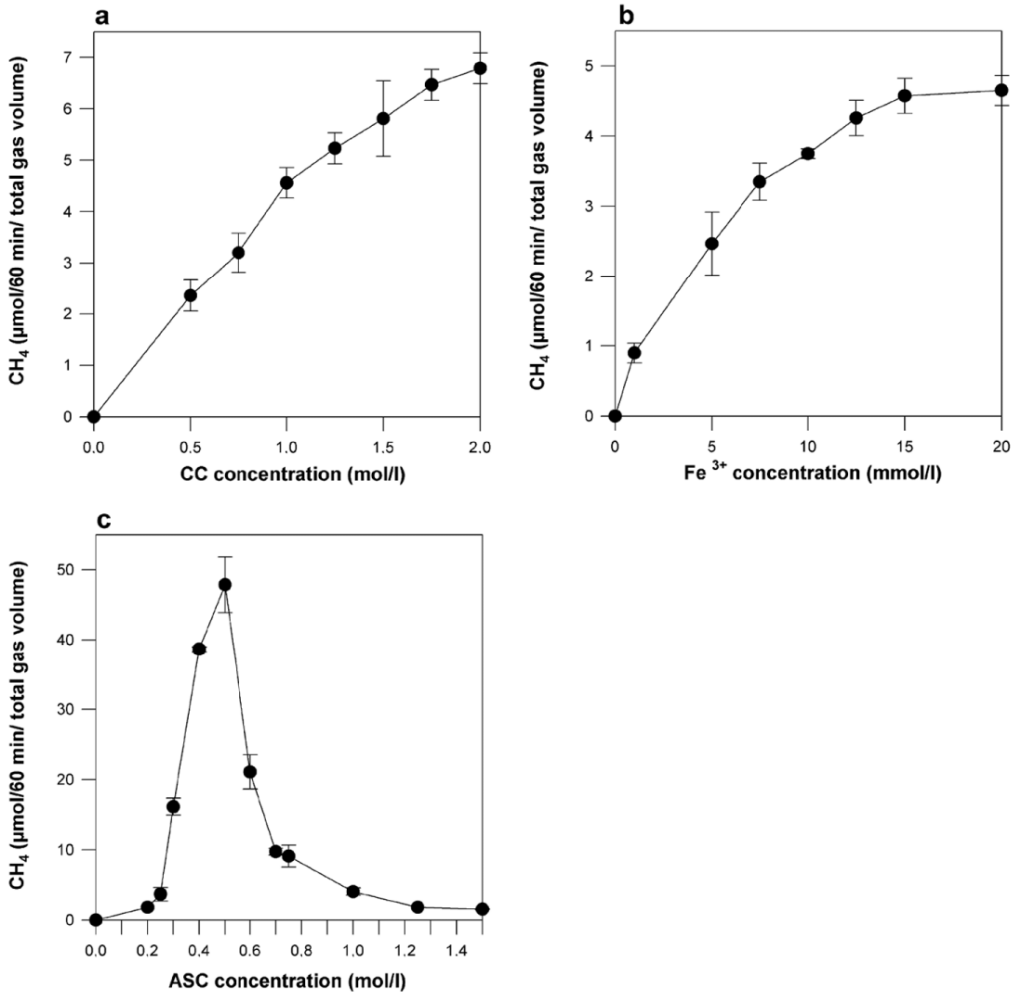


Figure 1. In vitro methane generation during a chemical model of reductive stress. Graphs show choline dependence (**a**), Fe^{3+} dependence (**b**), and ascorbic acid (ASC) dependence of the reaction (**c**).

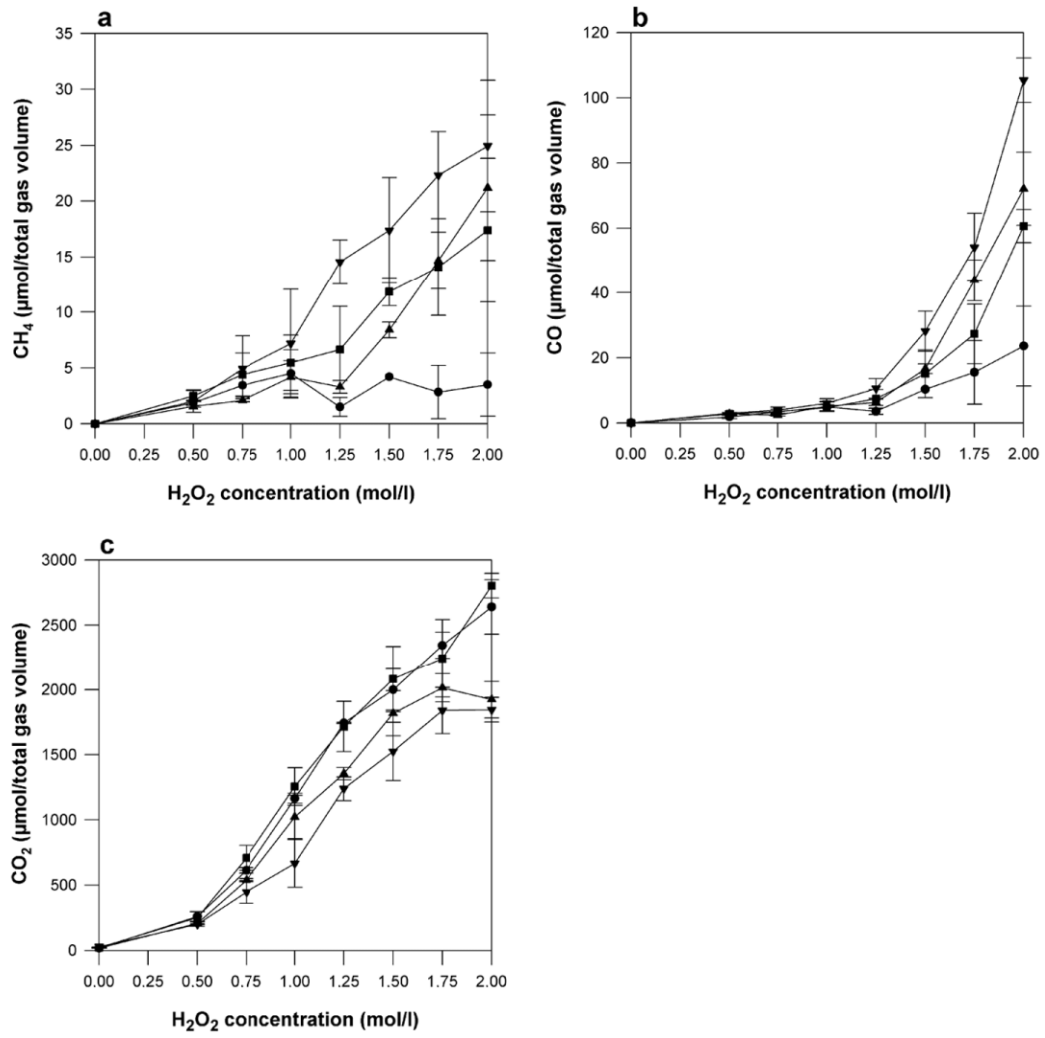


Figure 2. In vitro generation of methane, carbon monoxide (CO), and carbon dioxide (CO2) in a reaction mixture containing 500 mM K-phosphate buffer, 0.5 M ascorbate, and 10 mM $FeCl_3$. The reaction was started by adding H_2O_2 in increasing concentration. The gas production was measured 8 min (●), 60 min (▲), 120 min (■), and 24 h (▼) after initiating the reaction, respectively. **a)** Methane production; **b)** CO concentration changes; **c)** CO_2 production.

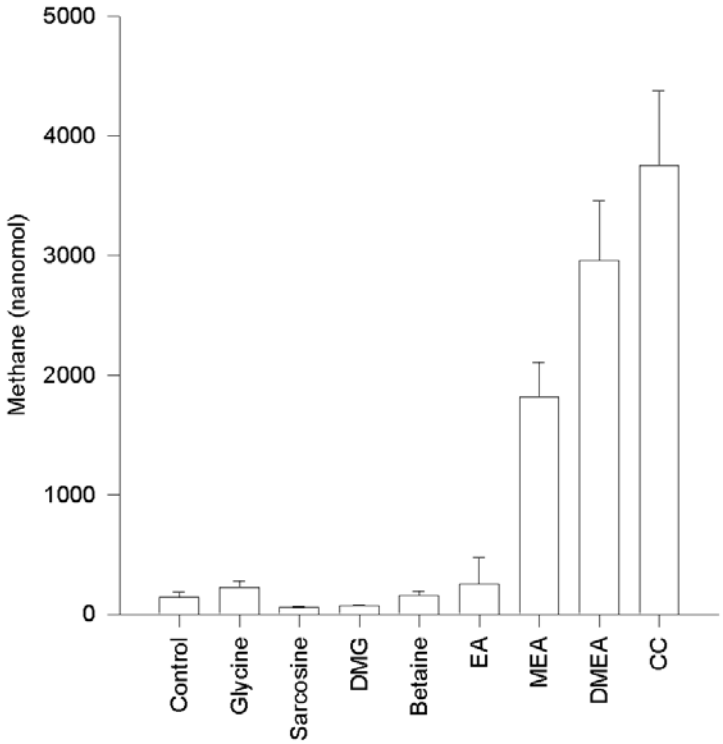


Figure 3. In vitro methane-producing capacity of choline and choline metabolites, using a chemical model of hydroxyl radical generation. The bars indicate mean ± SD.

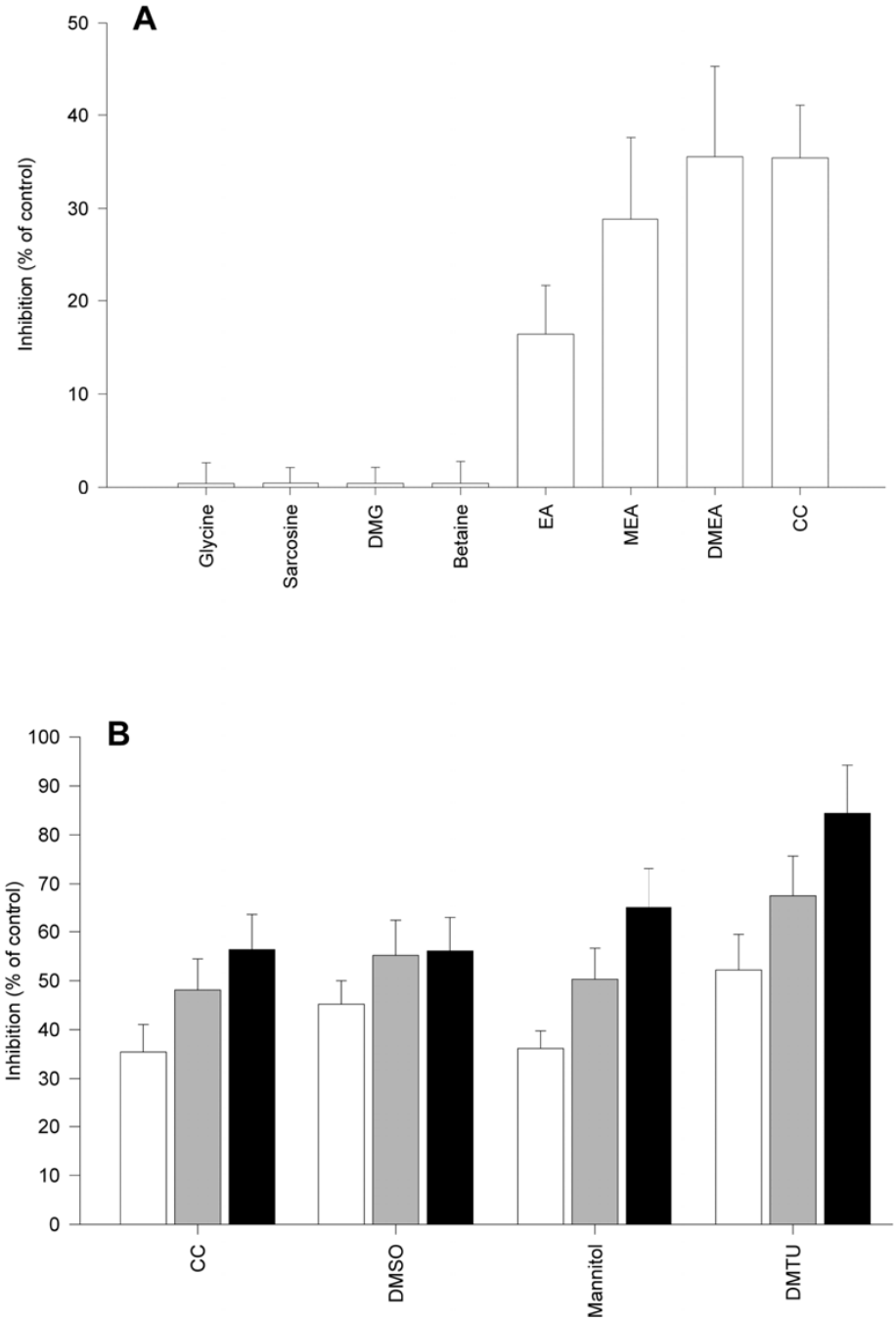


Figure 4A, 4B. Changes in chemiluminescence detected in the presence of 1 mM test compounds (choline metabolites) after induction of the Udenfriend reaction (upper panel). The degree of inhibition of chemiluminescence was measured with increasing choline (CC), DMSO, mannitol or DMTU concentrations (empty bars: 1 mM, grey bars: 10 mM, black bars: 100 mM) in identical chemical reactions (lower panel). The bars indicate mean ± SD.

4.2. Experiments with mitochondria

The possible formation of methane was explored in experiments with isolated rat liver mitochondria. 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 (as shown in Figure 5). 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 (see inside graph in Figure 5). 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 (data not shown).

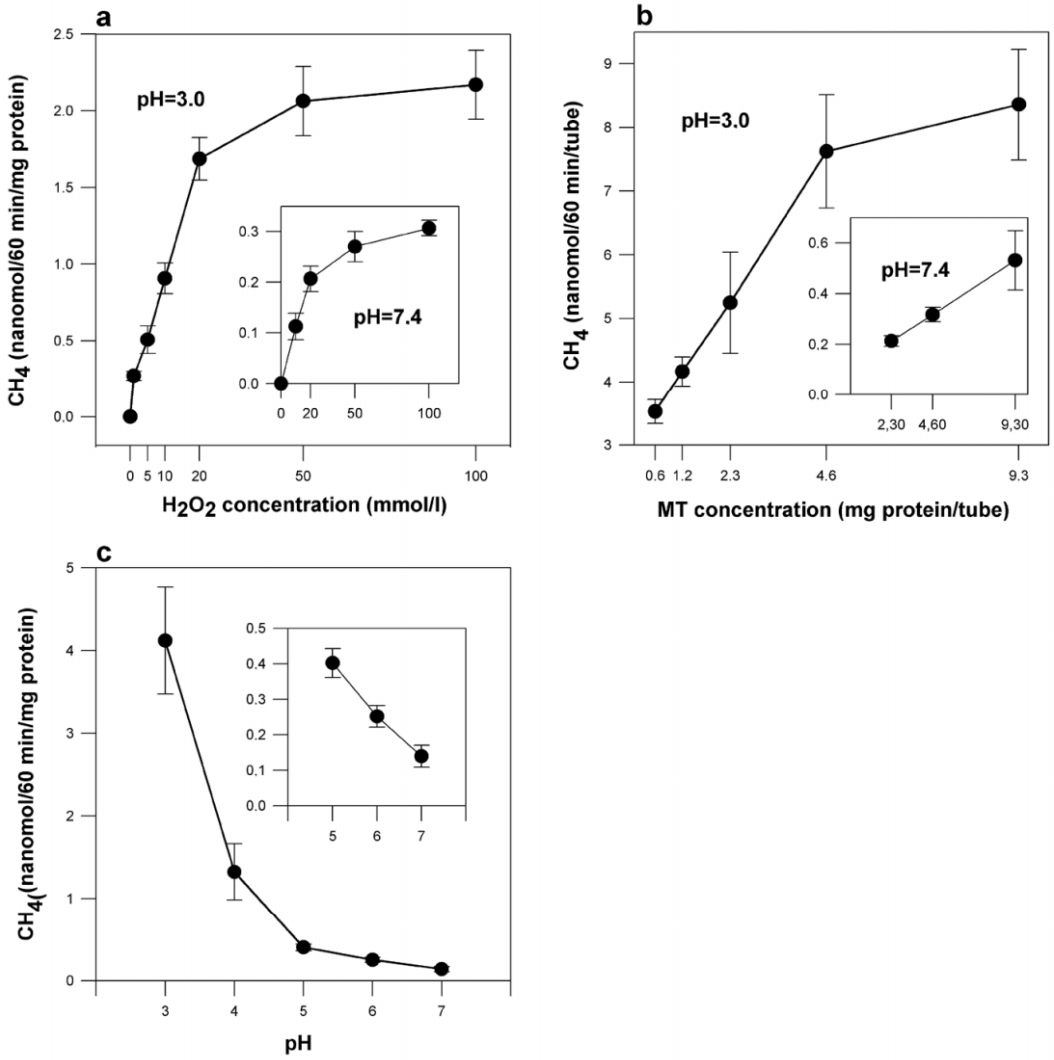


Figure 5. Methane production of rat liver mitochondria. Results obtained with increasing peroxide (H_2O_2) concentration (**a**), increasing mitochondrial (MT) protein concentration (**b**), or as a function of pH changes (**c**).

Secondly, rat liver mitochondrial subfractions were incubated in gastight vials with hydroxyl free radical-generating Udenfriend components (H_2O_2, ASC and catalytically active iron) to examine methane production of each. The highest methane production was observed in the matrix (2.96 nmol/mg protein, $p \leq 0.05$), 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 (Figure 6)

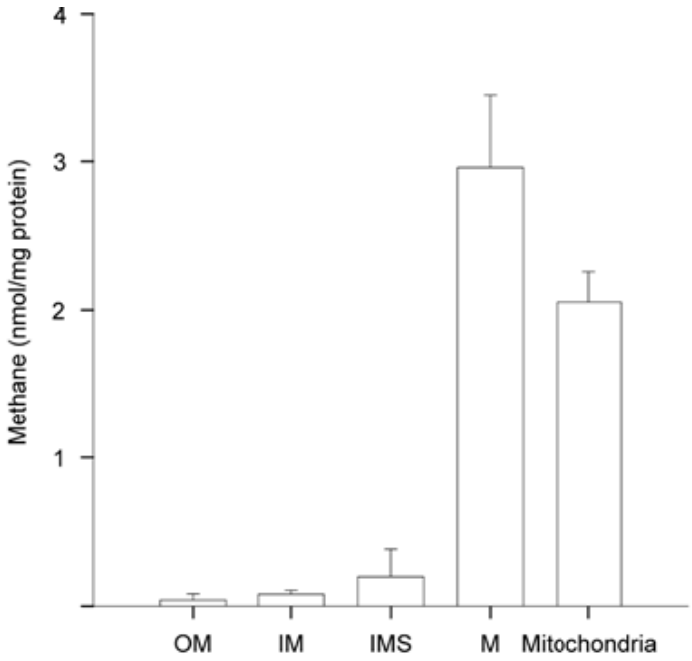


Figure 6. Methane production of rat liver mitochondria and mitochondrial subfractions. The results were obtained with intact mitochondria or matrix (M), intermembrane space (IMS), inner membrane (IM) and outer membrane (OM) fractions in a reaction mixture containing K-phosphate buffer, ascorbate and $FeCl_3$. The reaction was started by adding hydrogen peroxide and the gas production was measured 60 min after initiation of the reaction. The bars indicate mean ± SD.

4.3. Endothelial cell culture studies

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 (Figure 7). Metabolic inhibition of complex IV by NaCN and NaN_3 increased the methane production further ($p \leq 0.05$) and resulted in ~15 nmol/mg. Simultaneous glucose deprivation or glycolysis inhibition did not enhance this response further (Figure 8). Higher amounts of liberated methane (20-23 nmol/mg) were observed after the inhibition of oxidative phosphorylation with 2,4-DNP (Figure 9), and similarly high and dose-dependent methane generation was measured after the Udenfriend reaction (Figure 10).

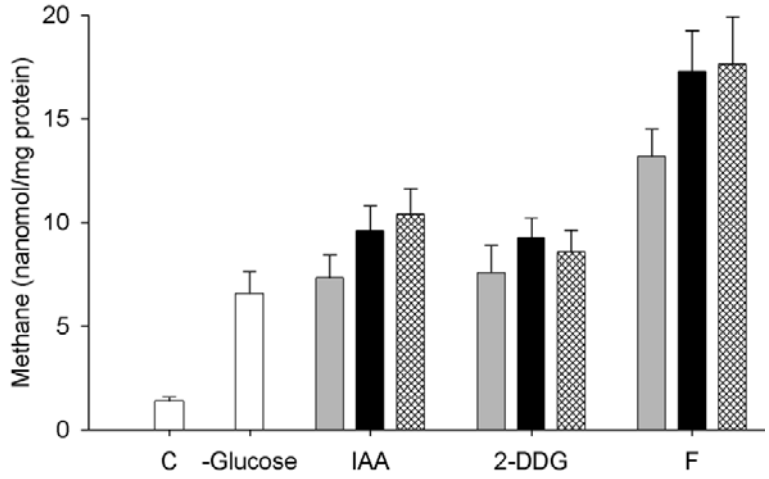


Figure 7. Effects of chemical hypoxia alone or hypoxia with glucose deprivation on methane generation of control (C) or treated primary aortic endothelial cells. The treatments included glucose deprivation (-Glucose), inhibition of glucose uptake and anaerobic glycolysis using 2-deoxy-D-glucose (2-DDG), iodoacetate (IAA) or sodium fluoride (F) (grey bars indicate 10 μM, black bars 100 μM, hatched bars 1 mM)

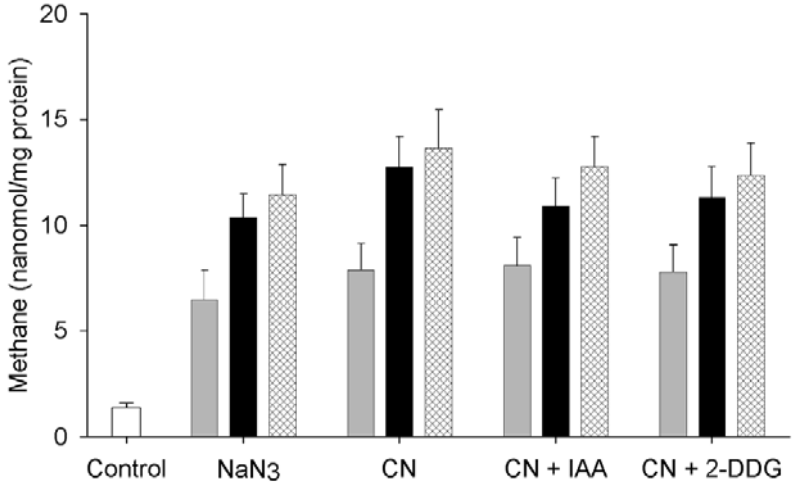


Figure 8. Effects of site-specific inhibitors of the mitochondrial electron transport chain (inhibitors of complex IV) sodium cyanide (NaCN) and sodium azide (NaN_3) alone or in combination with glycolysis inhibitors on methane generation of primary aortic endothelial cells (grey bars indicate 1 μM, black bars 10 μM, hatched bars 100 μM). Control (C) = mitochondria without inhibitors.

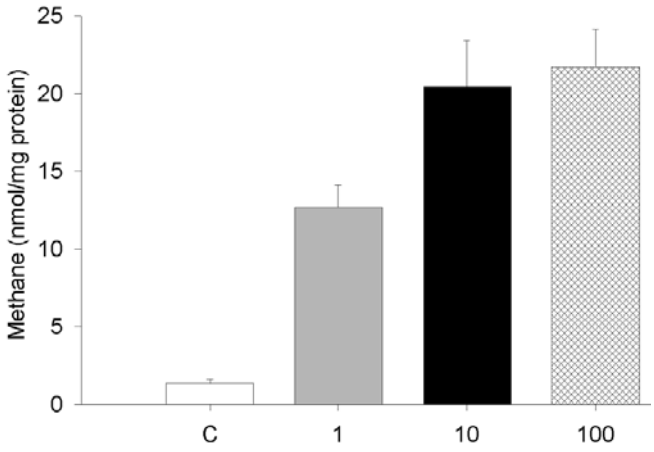


Figure 9. Methane generation of control or treated primary aortic endothelial cells. Application of the uncoupling agent 2,4- dinitrophenol (2,4-DNP) in 1-100 μM range (grey bars indicate 1 μM, black bars 10 μM, hatched bars 100 μM

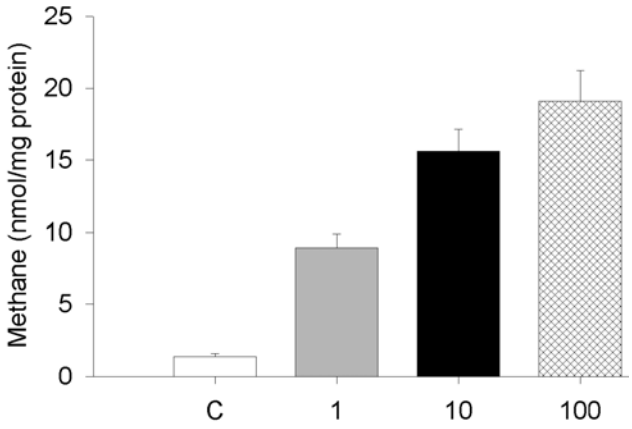


Figure 10. Treatment of the cells with increasing concentrations (1-100 μM) of the hydroxyl radical-generating Udenfriend system. The bars indicate mean ± SD.

4.4. Granulocytes

The PMA-activated PMN granulocyte assay 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 (Figure 11).

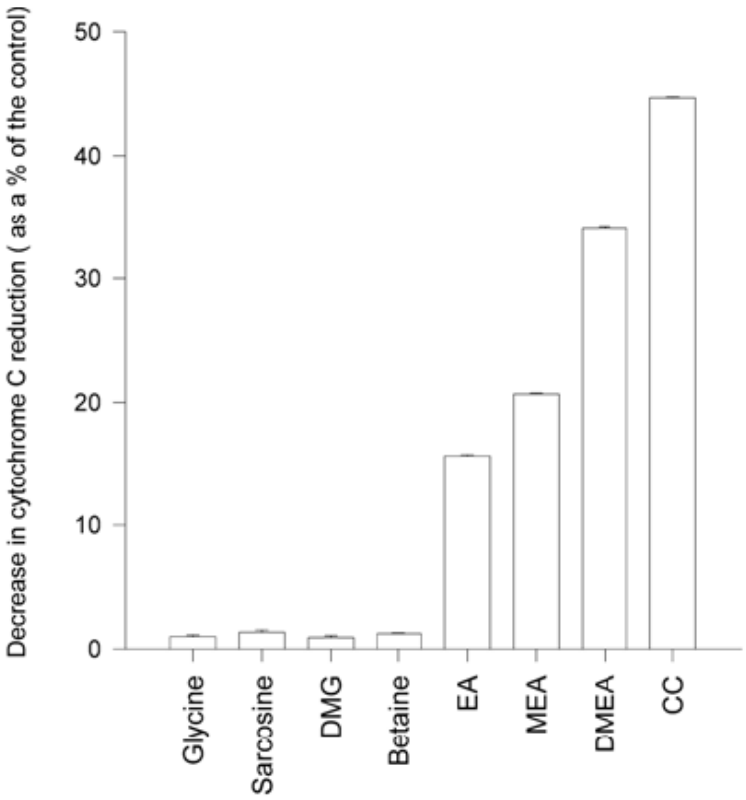


Figure 11. The reduction of cytochrome c by PMA-activated leukocytes (PMNs). PMA-activated PMNs were treated with 1mM chlorine chloride (CC) or the choline metabolites N-methylethanolamine (MEA), N,N-methylethanolamine (DMEA), ethanolamine (EA), betaine, dimethylglycine (DMG), sarcosine and glycine. Each column represents the mean of 3 to 5 determinations with PMNs isolated from the peripheral blood of healthy dogs. The bars indicate mean ± SD.

4.5. In vivo experiments

4.5.1. Hemodynamics

The baseline values of the recorded macrohemodynamic variables did not differ significantly in the different groups, and in the sham-operated group, there were no significant hemodynamic changes as compared with the control valuesduring the experimental period (data not shown).The baseline for cardiac output and MAP in the I-R group was not significantly different ($p < 0.05$) from those for the PC treated I-R group 3. In groups 2 and 3, the 60-min arterial occlusion and 180-min reperfusion-induced changes in total vascular resistance were rather similar; no significant differences were observed between the groups.

4.5.2. Superoxide production

The ROS-producing capacity of the small intestinal biopsy samples did not change in the sham-operated animals. The superoxide production of the mucosa was gradually and significantly increased during reperfusion after the release of the SMA occlusion. The oral PC treatment stabilized this parameter at the control level (Figure 12).

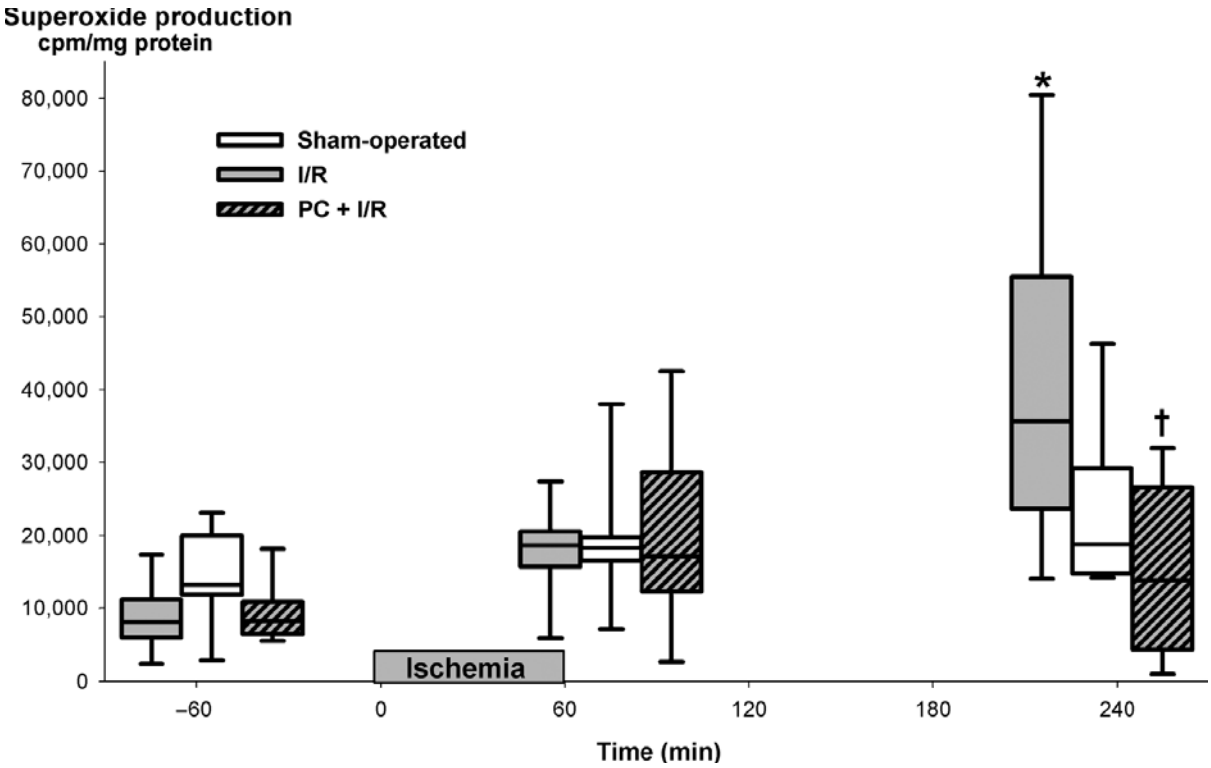


Figure 12. Changes in mucosal superoxide production (counts per minute per milligram protein) in the sham-operated (open boxes), intestinal I-R (gray boxes), and PC-treated I-R

(hatched boxes) groups. The box plots demonstrate the median values and the 25th and 75th percentiles (lower and upper part of the box) and the lowest and the highest values of the range (lower and upper whiskers). *P < 0.05 within groups versus baseline values, †P < 0.05 for PC-treated I-R group versus I-R group values.

4.5.3. Tissue myeloperoxidase changes

The MPO activity was significantly increased in the mucosa in the nontreated I-R group as compared with the baseline or the sham-operated group (Figure 13). In PC-treated I-R group 3, a significantly lower MPO activity was found after the 60-min intestinal ischemia. The PC-enriched diet also led to a significantly decreased ischemia-induced MPO activity 180 min after reperfusion.

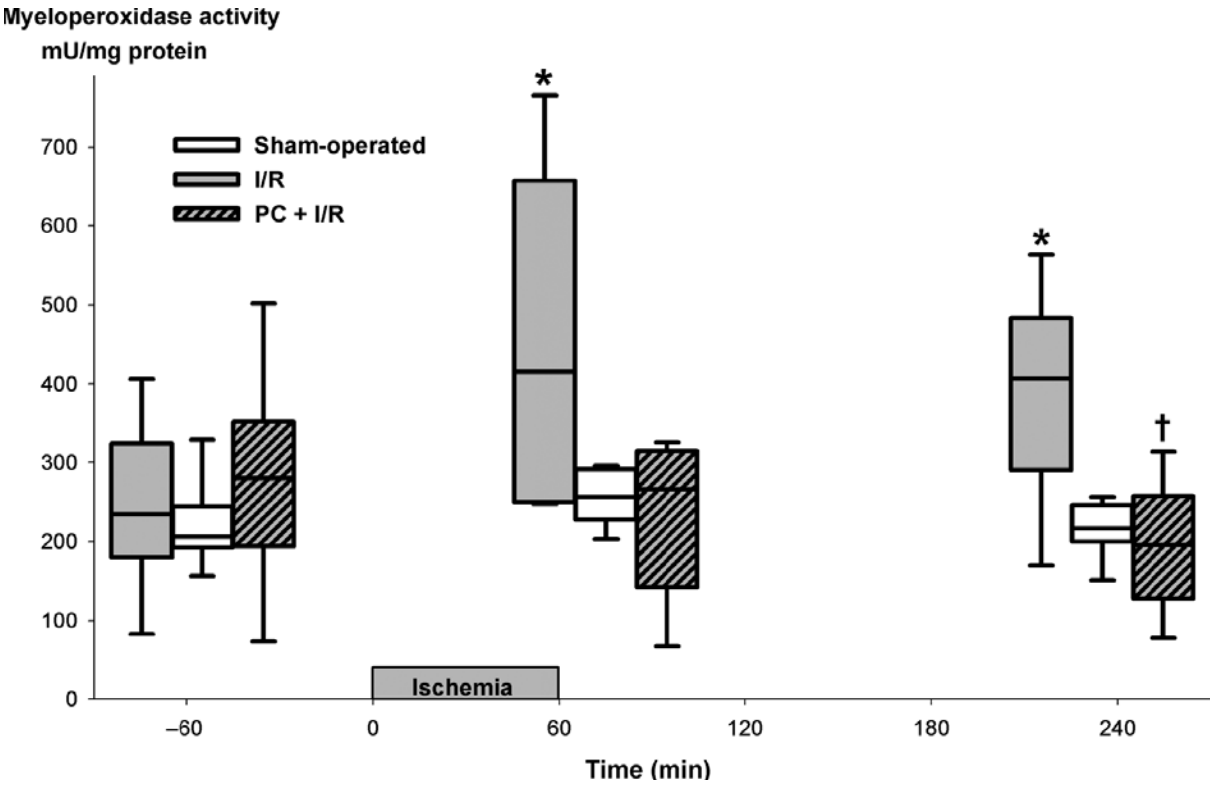


Figure 14. Changes in intestinal MPO activity (milliunits per milligram protein) in ileal biopsy samples before and after 60-min complete arterial occlusion and after 180-min reperfusion in the sham-operated (open boxes), intestinal I-R (gray boxes), and oral PC-treated I-R (hatched boxes) groups. The box plots demonstrate the median values and the 25th and 75th percentiles (lower and upper part of the box) and the lowest and the highest values

of the range (lower and upper whiskers). *p < 0.05 within groups versus baseline values; †p < 0.05 for treated groups versus sham-operated group values.

4.5.4. Intramucosal pH and PCO_2 gap

The pHi of the intestine is a reliable index of local tissue perfusion (Fiddium-Green 1989, Boros *et al.* 1994). In the sham-operated group, the pH of the arterial blood and the pHi remained essentially constant throughout the experiment (data not shown). After arterial occlusion, the pHi in the ileum diminished dramatically from the baseline 7.3 to approximately 6.8. In this group, after release of the occlusion of the SMA, the intestinal pHi remained significantly lower than the corresponding level for the sham-operated control group and for PC-treated I-R group 3 throughout the reperfusion period. In group 3, restitution of themesenteric blood flow resulted in a slow increase in intestinal pHi to 7.09 at the end of the observation period, a median value not significantly different from the baseline.

Another sensitive parameter of tissue perfusion is PCO_2 gap, a difference of local tissue and arterial PCO_2. At the end of ischemia, the PCO_2 gap increased significantly and remained significantly higher than the corresponding level for the sham-operated control group and for the PC-treated I-R group throughout the reperfusion period.

4.5.5. In vivo methane production

The exhaled methane concentration was increased markedly after the 60-min ischemia and remained significantly elevated for 1 h during reperfusion. The methane level decreased up to the end of the experiments but remained higher than the baseline values. In PC-treated I-R group 3, the breath methane concentrations were not increased significantly after the start of the reperfusion, and the values were significantly lower than those in the nontreated I-R group 2 (Figure 15).

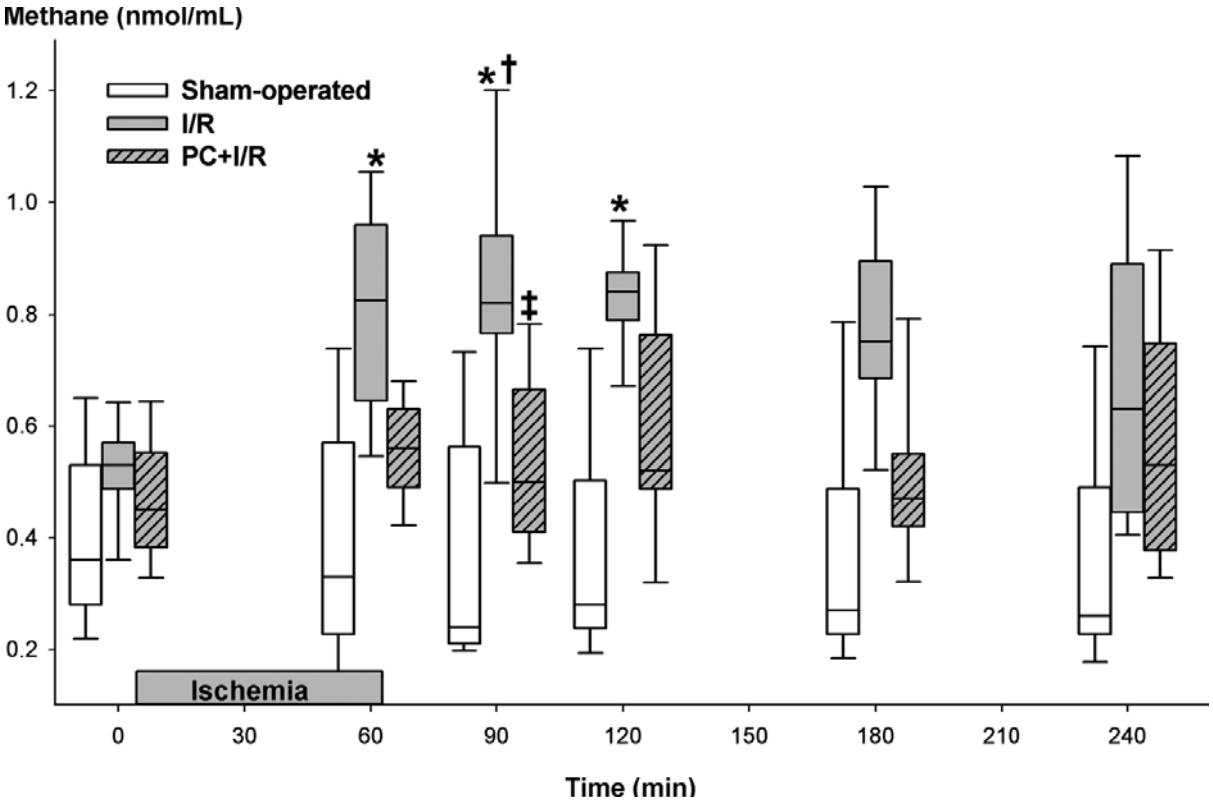


Figure 15. Changes in exhaled methane level in breath samples in the sham-operated (open boxes), intestinal I-R (gray boxes), and PC-treated I-R (hatched boxes) groups. The box plots demonstrate the median values and the 25th and 75th percentiles (lower and upper part of the box) and the lowest and the highest values of the range (lower and upper whiskers). *P < 0.05 for treated groups versus baseline values; †P < 0.05 between groups versus shamoperated group values; ‡P G 0.05 for PC-treated I-R group versus I-R group values.

5. DISCUSSION

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. Although the consequences of Fenton reaction have been extensively studied, the interactions described by us, which may provide a model of reductive stress generated in biological systems (and to the protective response to it), have not been previously reported. 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.

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. H_2O_2 is an intermediate species of oxygen metabolism ubiquitously present in the mammalian organism, and mitochondria produce H_2O_2 at elevated rates by a high degree of reduction of the respiratory carriers. Catalase, which decomposes H_2O_2, abolished the increase in methane production. This indicates that mitochondrial H_2O_2 is required for the hypoxic activation of the methane-generating reaction. 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).

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. The mechanistic details of this pathway suggest a new concept to explain the loss of methyl groups in pathologies with an abnormally elevated mitochondrial NADH/NAD^+ ratio. In the past, the formation of methane was thought to be an exclusive attribute of methanogenic archaea, the only organisms known to produce methane from decomposing organic matter in anaerobic freshwater environment and in the gastrointestinal tract of mammals (Thauer 1998). The bacterial fermentation takes place under strict anaerobic conditions, as the formation of methane by an aerobic organism has hitherto not been previously reported.

The results also indicated that methane-generating biomolecules in this pathway may actually counteract ROS generation and exhibit a strong inhibitory potential for the production of hydroxyl radicals. The efficacy of CC was proved to be of the same order as that of the established hydroxyl radical scavenger mannitol, DMSO and DMTU, and PC metabolites with alcoholic moiety in the molecule (i.e., choline, N,N- dimethylethanolamine and N-methylethanolamine) displayed an effectiveness proportional to the amount of methane generated and the number of methyl groups in the compounds (see Figures 5, 6 and 17). A

similar series of experiments using isolated activated PMN leukocytes furnished evidence that methane-producing PC metabolites inhibit the formation of ROS and may therefore be defined as ‘antioxidants’ (Figure 11).

In mitochondria substrate oxidation by the electron transport chain creates a proton gradient across the inner membrane and fuels ATP synthesis by F0F1-ATP synthase. The electron transport chain through which the electrons shuttle must be in a steady state as regards the input of electrons and the availability of the electron acceptor oxygen. A continuing lack of oxygen will cause an abnormally elevated mitochondrial NADH/NAD^+ ratio and the collapse of ionic homeostasis, leading to dissipation of the transmembrane potential. However, ROS are also produced at significant rates by reactions intrinsic to the normal aerobic metabolism, thus the “redox homeostasis” appropriately includes an intracellular balance between ROS generation and scavenging (Droge 2006). A number of conditions lead to impairment of the redox homeostasis, thereby inducing the formation of ROS by transferring the excess electrons to oxygen outside the transport chain, to a site where PC or free choline is present. It must be emphasized that choline may be liberated from membrane-bound PC through the redox-sensitive activation of phospholipase D (PLD). PLD hydrolyzes PC to phosphatidic acid and choline, and it has been implicated as a signal-activated key enzyme in a wide range of physiological responses (Tappia *et al.* 2006).

In a further step, we have examined the consequences of modulation of the mitochondrial electron transport in in confluent cultures of porcine aortic endothelial cells, 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 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.

Our understanding of the pivotal position of a redox imbalance and inflammation in the pathogenesis of I-R raises questions concerning the opportunities to prevent or treat this syndrome. 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. There is firm evidence that leukocyte adherence is of critical importance during I-R, with the potential to amplify the production of ROS and other proinflammatory mediators (Boros *et al*. 2003, Massberg *et al.* 1998). Leukocyte activation evokes a profound vasoconstrictor effect, leading to hypoperfusion or even a no-reflow phenomenon, with complete cessation of the tissue microcirculation (Menger *et al*. 1997). The tissue pHi and the PCO_2 gap are well-established, indirect parameters of hypoxic changes, and balloon tonometry is an appropriate tool with which to evaluate the effectiveness of therapies aimed at counteracting a microcirculatory dysfunction in the gastrointestinal tract (Fiddian-Green 1989, Creteur *et al*. 2006). The results of our in vivo study 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 (Figure 16). 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 (Figure 16).

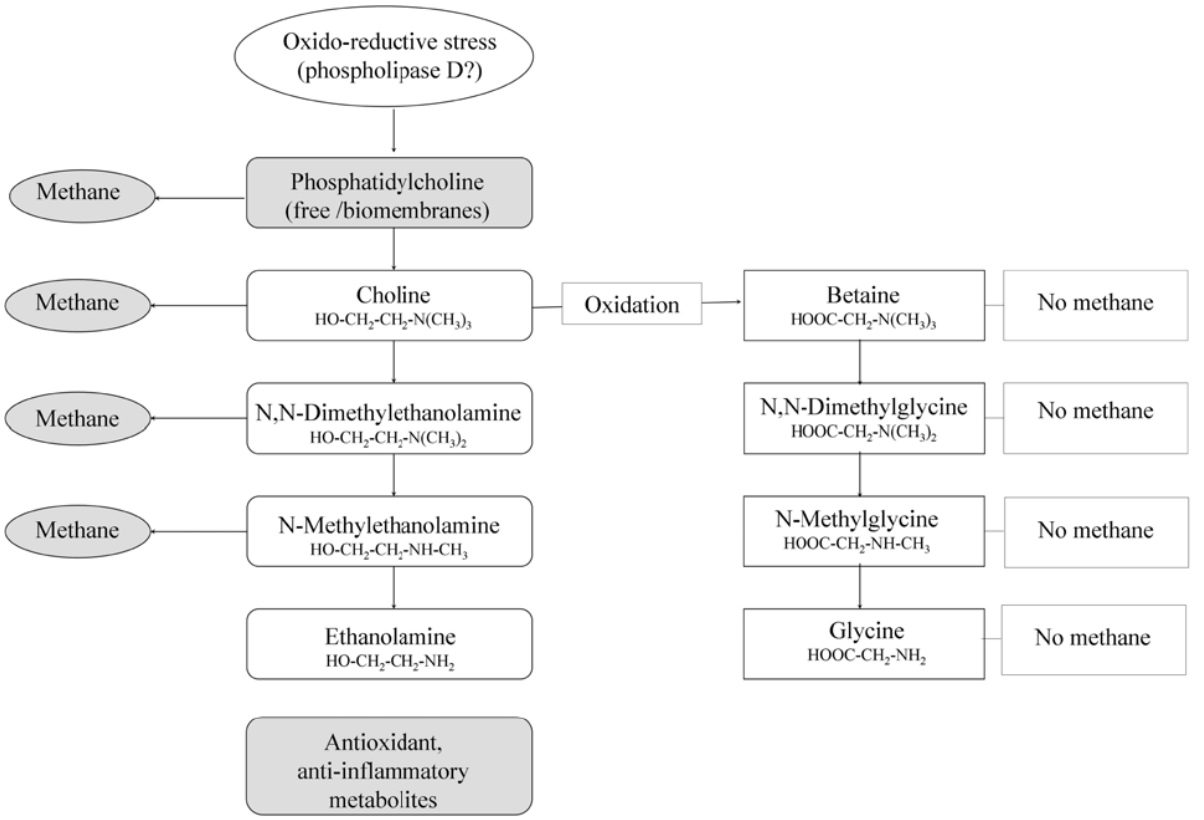


Figure 16. Schematic diagram of a proposed mechanism for the oxido-reductive stress-induced in vivo methanogenic response.

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.

6. 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. 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. 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.
4. The formation of methane by rat liver mitochondria was reported for the first time.
5. 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.
6. Phosphatidylcholine metabolites containing both electron donor and acceptor groups may have a function to counteract intracellular oxygen radical production.

7. The formation of methane by an aerobic organism has hitherto not been previously reported. The generation of methane may be linked to a tissue response to ischemia and associated with abnormal ROS generation in the gastrointestinal tract.
8. The methane-generating reaction is a novel defensive response to reductive stress which provides protection against redox imbalance in living systems.
9. Increased dietary uptake of PC exerts an anti-inflammatory influence in the gastrointestinal tract.

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9. ANNEX