Methane bioactivity and interactions with other biological gases

András T. Mészáros M.D.

University of Szeged,
Institute of Surgical Research, Szeged, Hungary

Ph.D. Thesis

University of Szeged, Faculty of Medicine,
Doctoral School of Multidisciplinary Medicine
Szeged, Hungary

Supervisors:

Mihály Boros M.D., Ph.D., D.Sc.
University of Szeged, Institute of Surgical Research
Szeged, Hungary

Andrey V. Kozlov M.D., Ph.D.
Ludwig Boltzmann Institute for Experimental and Clinical Traumatology
Vienna, Austria

2017

List of full papers related to the subject of the thesis:

I. **Mészáros AT,** Büki T, Fazekas B, Tuboly E, Horváth K, Poles MZ, Szűcs Sz, Varga G, Kaszaki J, Boros M: Methane inhalation preserves the epithelial barrier during ischemia and reperfusion in the rat small intestine. *Surgery*, 2017. (accepted for publication**)** **IF: 3.309**

II. Tuboly E*, **Mészáros A***, Boros M: Nonbacterial biotic methanogenesis, possible mechanisms and significance. In: *Methanogenesis: Biochemistry, Ecological Functions, Natural and Engineered Environments. Badalians G.G. (ed.). Nova Science Publishers, Inc. NY, USA 2014,* Chapter 2, pp. 19-49. ISBN 978-1-63321-567-2. ***Equal contribution**

III. Dumitrescu SD*, **Meszaros AT***, Puchner S, Weidinger A, Boros M, Redl H, Kozlov AV: EPR analysis of extra- and intracellular nitric oxide in liver biopsies. *Magnetic Resonance in Medicine*, 2016. (Epub ahead of print) *** Equal contribution IF: 3.782**

IV. Boros M, Tuboly E, **Mészáros A**, Amann A: The role of methane in mammalian physiology – is it a gasotransmitter? *Journal of Breath Research*, 2015; 9: 014001 **IF: 4.177**

V. Kaszaki J, **Mészáros A**, Büki T, Varga G, Érces D, Boros M: Pathophysiology of intestinal ischemia and reperfusion – novel therapeutic possibilities. *Magyar Belorvosi Archívum*, 2013; 66: 6-12.

VI. Kozlov AV, Bernardi P, Lancaster J, **Meszaros AT**, Weidinger A: Mitochondrial pathways linking acute inflammation and liver failure, a hypothesis about key role of mitochondrial ROS. **Submitted for publication**

List of full papers related to the field of the thesis:

I. Tuboly E, Futakuchi M, Varga G, Érces D, Tőkés T, **Mészáros A**, Kaszaki J, Suzui M, Imai M, Okada A, Okada N, Boros M, Okada H. C5a inhibitor protects against ischemia/reperfusion injury in rat small intestine. *Microbiology and Immunology*, 2016; 60(1): 35–46. **IF: 1.428**

II. Strifler G, Tuboly E, Szél E, Kaszonyi E, Cao C, Kaszaki J, **Mészáros A**, Boros M, Hartmann P Inhaled methane limits the mitochondrial electron transport chain dysfunction during experimental liver ischemia - reperfusion injury. *PLoS One*, 2016;11(1):e0146363 **IF: 3.057**

III. Érces D, Nógrády M, Varga G, Szűcs S, **Mészáros AT,** Fischer-Szatmári T, Cao C, Okada N, Okada H, Boros M, Kaszaki J. Complement C5a inhibition improves late hemodynamic and inflammatory changes in a rat model *of non-o*cclusive mesenteric ischemia. *Surgery*, 2016;159(3):960-71 **IF: 3.309**

Cumulative IF: 19.062

I. Introduction

1. Oxygen and aerobic life

In most vertebrate tissues the majority of oxygen (O_2) is consumed by mitochondria, the powerhouses of cells. Mitochondria are spatially separated intracellular sites hosting a series of oxido-reductive reactions coupled to proton pumping. Electrons are carried by respiratory complexes to cytochrome c oxidase, where O_2, the final electron acceptor, is reduced to H_2O. This process contributes to more than 90% of the total O_2 consumption of the human body. Protons are pumped through the inner mitochondrial membrane with the energy produced by oxidation-reduction steps, and this electro-chemical gradient is the driving force of the ATP synthesis.

During the electron transfer partially reduced superoxide ($O_2^{\bullet -}$) is produced as well, which is the primary reactive oxygen species (ROS) in the eukaryotic cell (Chance *et al.*, 1961). There are at least 10 known enzymes in mammalian mitochondria which are able to produce ROS (Andreyev *et al.*, 2005; Quinlan *et al.*, 2012). ROS production is tightly regulated and secured by several lines of antioxidant defense systems. Today it is commonly accepted that physiological levels of ROS regulate a variety of signaling steps (e.g. NF-κB, Nrf-2, STAT3 pathways) directly and indirectly (Hamanaka *et al.*, 2010; Niture *et al.*, 2014; Weidinger *et al.*, 2015).

2. Hypoxia, reoxygenation and inflammation

The impairment of arterial blood flow (ischemia) leads to tissue hypoxia, limiting mitochondrial oxidative phosphorylation. The dysfunction of mitochondria is central to hypoxic tissue injuries, resulting in impaired ATP production, an increase in ATP hydrolysis to maintain the mitochondrial membrane potential ($\Delta\psi_m$), dysregulated mitochondrial Ca^{2+} homeostasis, elevated ROS production and mitochondrial permeability transition (Di Lisa *et al.*, 2006; Bernardi *et al.*, 2015).

The duration of ischemia determines the survival of the affected tissue, thus timely restoration of the nutritive blood flow is essential to recover organ function. Reperfusion, however, paradoxically worsens the initial damage done by the ischemia itself. This is mainly due to the reintroduction of molecular O_2 to previously ischemic tissue, leading to an imbalance between ROS production and detoxification (Granger *et al.*, 1981). Pathological ROS production in the reperfusion period is fueled by mitochondrial, cytosolic and extracellular sources; the most important ROS source in the postischemic tissue is probably xanthine oxidoreductase (XOR) (Harrison, 2002; Khambata *et al.*, 2015). Hypoxic conditions are inducing inflammation, and inflammatory disease states are frequently accompanied by tissue hypoxia. Ischemia-reperfusion (IR) injury is also characterized by an inflammatory response and an important contributing factor to the oxidative tissue damage is the activation of the innate immune system. The umbrella term “inflammatory mediator” covers molecules and cells contributing to the initiation, propagation and controlled cessation of the inflammation cascade. Most

of them are “soluble” compounds such as cytokines and small proteins, but ROS and gaseous compounds, such as nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H_2S) represent special classes of inflammatory mediators (Liu *et al.*, 2012) as well.

An important feature of reperfusion injury is the no-reflow phenomenon. The peroxidation of membrane lipids of erythrocytes by oxidant species, such as peroxynitrite (ONOO), decreases the deformability of the affected cellular membranes, contributing to reduced capillary blood flow and in severe cases, capillary stasis develops (Dobretsov *et al.*, 1977, Vollmar *et al.*, 2011).

3. Intestinal ischemia-reperfusion (IR) injury and the epithelial barrier

Occlusive or non-occlusive mesenteric ischemia with subsequent reperfusion results in oxidative injury to the mucosa, which leads to irreversible structural damage to biological membranes impairing their permeability and facilitating the leakage of toxins and bacteria from the intestinal lumen to the blood. The gut mucosa is metabolically very active, but O_2 levels are rather low in the lumen of the bowel (Levitt, 1971; Glover *et al.*, 2016). During inflammation the intestinal tissue can become profoundly hypoxic, or even anoxic despite the non-vascular origin of the disease. To maintain the function of the inter-epithelial tight junctions (TJs) of the selectively permeable mucosal barrier, a continuous energy supply is needed. ATP depletion (mesenteric ischemia) leads to the dissolution of TJs, but existing TJs can be “reused” if ATP levels are restored within a short period of time (Tsukamoto *et al.*, 1997; Bush *et al.*, 2000).

Being a component of the cell wall of Gram-negative bacteria, which are known to occur in abundance in the intestine, bacterial lipopolysaccharide (LPS, endotoxin) is thought to play an important role in the development of systemic inflammation, sepsis and multiple organ dysfunction syndrome originating from the gut after low flow states and IR conditions (Olofsson *et al.*, 1985). The LPS-linked reactions are mediated by Toll-like receptors (TLRs) and as part of the cellular stress response, the generation of both $O_2^{\bullet -}$ and NO is one of the major consequences of LPS exposure in various TLR4-expressing cell types (Mittal *et al.*, 2014; Vaure *et al.*, 2014).

4. Biologically active gases

Gasotransmitters are defined by four characteristics (simplicity, availability, volatility and effectiveness, respectively), and six additional criteria were recently listed by Wang: they are (1) small molecules of gas; (2) freely permeable to membranes; (3) endogenously generated in mammalian cells with specific substrates and enzymes; (4) have well-defined specific functions at physiologically relevant concentrations; (5) the functions can be mimicked by their exogenously applied counterparts; and (6) have specific cellular and molecular targets. Today the most well-known gasotransmitter compounds are NO, H_2S and CO (Wang, 2014).

4.1. NO

NO possesses an unpaired electron and is therefore regarded as a free radical. Being uncharged, NO readily crosses free biological membranes with passive diffusion in all directions. Under normoxic conditions, NO is mainly produced by various NO synthase isoforms (iNOS, nNOS, eNOS). Apart from vasodilation, NO has several physiological functions in cellular signaling (Hirst *et al.*, 2011) and the majority of the physiological effects are based on the activation of soluble guanylate cyclase, resulting in increased cGMP levels (Toledo *et al.*, 2012).

4.2. Methane (CH_4)

CH_4 is the most reduced form of carbon, the smallest organic compound and the simplest alkane. Because of its non-polar properties, the solubility of CH_4 is two orders of magnitude higher in membrane lipids than in the aqueous phase (Miller *et al.*, 1977; Meyer *et al.*, 1980). In physiologically relevant concentrations CH_4 has no toxic effects, unless O_2 delivery is heavily compromised. Under ambient conditions CH_4 is accepted to be not reactive, but in the troposphere CH_4 is oxidized to CO_2 by hydroxyl radicals (Cantrell *et al.*, 1990; Hurkuck *et al.*, 2012).

In the gastrointestinal system of most mammals carbohydrates are fermented and the carbon dioxide (CO_2) released during this process is subsequently reduced to CH_4 by the obligate anaerobe prokaryotes, the methanogenic Archaea (Conrad *et al.*, 1999). The gas produced is then excreted with the flatus, leaves through the skin or can be measured in roughly one third of humans in the exhaled breath (Nose *et al.*, 2005; de Lacy Costello *et al.*, 2013). The non-microbial formation of CH_4 in eukaryotic mitochondria was first demonstrated *in vitro* by Ghyczy in 2003. It was later shown that during oxido-reductive stress conditions in animal and plant cell cultures and also *in vivo,* measurable quantities of CH_4 are formed (Ghyczy *et al.*, 2008; Wishkerman *et al.*, 2011; Keppler *et al.*, 2016).

Much attention has recently been paid to the bioactivity of CH_4 in eukaryotes and to its possible therapeutic projections. The biological effect of CH_4 in the mammalian organism was first shown by Pimentel and colleagues (Pimentel *et al.*, 2006). They reported that CH_4 slows the small intestinal propulsive motility and augments contractile activity. Correlations of detectable CH_4 in the exhaled air of humans and peristalsis changes during irritable bowel syndrome have been repeatedly shown as well (Lee *et al.*, 2013; Pozuelo *et al.*, 2015). An anti-inflammatory potential for CH_4 was first reported by Boros and colleagues in experimental mesenteric IR (Boros *et al.*, 2012). Over the past few years many other papers have been published on the anti-inflammatory effects of CH_4 in various animal models of IR, hypoxia and sterile inflammation. Most publications addressed four aspects of CH_4 activity, namely (1) the modulation of pro-inflammatory cytokine release; (2) anti-apoptotic effects; (3) the suppressed generation of oxidative stress biomarkers with concurrent potentiating of endogenous antioxidant systems and (4) improved organ functions (Song *et al.* 2015; Chen *et al.* 2016; Liu *et al.* 2016).

4.3. Relationship between O_2 and other gases: NO-linked effects in hypoxia and reperfusion

Disturbances in macro- and microcirculation either by primarily vascular origin or during secondary inflammatory disorders can profoundly alter the oxygenation profile of tissues. Many of the endogenous enzymes that utilize O_2 are able to bind other gases as well. The complex interplay of gaseous compounds is of particular importance in hypoxic pathologies, since in the lack of O_2 new reactions can emerge, highlighting those processes which are usually in the background.

NO production by the NOS isoforms is an O_2-dependent mechanism. Under hypoxic and anoxic conditions NO can be formed from nitrite (NO_2^-) and from nitrate (NO_3^-), with further reduction through NO_2^-. One of the main NO_2^- reducing pathways in humans is XOR. The NO formation from NO_2^- under hypoxia is viewed as a salvage mechanism (Dalsgaard *et al.*, 2007). During reperfusion, formation of peroxynitrite (ONOO) and the deleterious effects of NO are mostly connected. ONOO and ONOO-derived radicals (e.g. lipid hydroperoxides) can readily oxidize and/or nitrate biomolecules including tyrosine residues, thiols, DNA and unsaturated fatty-acid-containing phospholipids.

The interplay of CH_4 with NO in mammals has not yet been investigated systematically. On the one hand, it was demonstrated that normoxic CH_4 ventilation decreases tyrosine nitrosylation after an IR injury (Boros *et al.*, 2012), a process which involves NO. On the other hand, it has been shown that the inhibition of the mitochondrial cytochrome c oxidase (complex IV), an important target of NO under hypoxia, leads to CH_4 generation (Wishkerman *et al.*, 2011; Tuboly *et al.*, 2013). Moreover, some of the effects exerted by CH_4 in model systems of inflammation can be explained by the indirect modulation of functions of NO. It is likely that the two gases are able to modulate the effect of each other at membrane interfaces, where their concentration is at its peak.

II. Aims

1. Our first general aim was to explore the consequences and effects of normoxic CH_4 administration in IR-induced inflammation. The following specific goals have been addressed.

- The first specific goal was to characterize the changes in epithelial and endothelial permeabilities induced by IR challenge in the rat small intestine. With this aim, we monitored the early and later biochemical consequences, the structural and hemodynamic changes in the intestinal mucosa;
- The second goal was to detect specific biochemical markers of both oxidative and nitrosative stress to investigate the possible mechanism of action of exogenous CH_4-based treatments;
- The third goal is based on our hypothesis that CH_4 has direct influence on cell membrane rigidity. Therefore an additional goal was to investigate the influence of CH_4 on changes in erythrocyte deformability provoked by oxidative stress *in vitro*.

2. Our second general aim was to better understand the mechanisms of CH_4 action in models of tissue hypoxia with elevated ROS and reactive nitrogen species (RNS) levels, with special emphasis on the possible relationship of CH_4 with other biological gases, such as NO; the following specific goals have been addressed in this part of the study.

- The first goal was to establish a bacterial endotoxin-based *in vivo* experimental model of inflammation to follow the changes in locally generated NO levels and to develop a specific and sensitive method to detect NO generation in the model established before;
- The second goal was to examine the potential interplay of CH_4 with NO directly investigating *in vivo* and *ex vivo* effects of CH_4 administrations on the NO generation by means of electron paramagnetic resonance (EPR) technique and on-line chemiluminescence analyses.

III. Materials and methods

1. An *in vivo* study to investigate the effects of CH_4 treatments in mesenteric IR

1.1. Animals, surgical procedure and study design

Male Sprague-Dawley rats were used in accordance with the EU Directive 2010/63 for the protection of animals used for scientific purposes, and the study was approved by the National Scientific Ethical Committee on Animal Experimentation, with the license number V/148/2013. In study 1 (the "early reperfusion" study), the animals were killed 60 min after the re-establishment of the mesenteric blood flow, while in the second set (the "late reperfusion" study), the reperfusion period lasted for 180 min. Group 1 served as a sham-operated control, while in Group 2 (IR) the superior mesenteric artery (SMA) was occluded for 45 min. In CH_4-treated Group 3 (IR+CH_4) an artificial gas mixture containing 2.2% CH_4, 21% O_2 and 76.8% N_2 was administered for 5 min before the end of the 45-min ischemia and for 10 min at the beginning of the reperfusion.

1.2. Mucosal permeability measurements

The epithelial permeability (EP) was determined with the 4 kDa fluorescein isothiocyanate-dextran (FD4) method as described previously (Cuzzocrea *et al.*, 1997). The EP index was defined as the percentage of the ratio of the lumen and plasma concentrations of FD4. The vascular permeability (VP) index was determined using the azo dye Evans blue method as described previously (Szentpali *et al.*, 2001). The VP index was defined as the percentage of the ratio of the tissue and plasma concentrations of Evans blue.

1.3. Morphological analysis

Full-thickness ileum biopsies were taken after 30 min of reperfusion. After staining with hematoxylin and eosin, photomicrographs were recorded with a 40x objective connected to a digital camera. The extent of superficial epithelial damage of the terminal ileum was evaluated by means of

fluorescence confocal laser scanning endomicroscopy (CLSEM). The mucosal surface of the terminal ileum was exposed surgically and after topical application of the fluorescent dye acriflavin non-overlapping fields were evaluated by a modified semiquantitative scoring system (Érces *et al.*, 2016).

1.4. Hemodynamics

The SMA flow signals were measured continuously and recorded with a computerized data acquisition system. An intravital orthogonal polarization spectral imaging technique was used for the visualization of the serosal microcirculation of the ileum. Changes in red blood cell (RBC) velocity in the postcapillary venules were determined off-line by a computer-assisted image analysis system.

1.5. Biochemical measurements

The activity of myeloperoxidase (MPO) as a marker of tissue leukocyte infiltration was measured in the pellet of ileum biopsy homogenates (Kuebler *et al.*, 1996). The plasma ET-1 concentration at the end of the "late reperfusion" experiments was determined in by means of an ELISA kit and expressed as fmol/mL. The rate of $O_2^{\bullet -}$ production in freshly minced intestinal biopsy samples was assessed by using the lucigenin-enhanced chemiluminescence assay (Ferdinandy *et al.*, 2000). Free nitrotyrosine (NTyr), as a marker of ONOO generation, was measured in intestinal tissue samples by ELISA. The NTyr content was normalized to the protein content of the small intestinal homogenate and expressed in ng/mg.

1.7. CH_4 concentration measurement

In a separate set of experiments, tissue CH_4 concentration was measured multiple times in anesthetized rats after the inhalation of room air or artificial air containing 2.2% exogenous CH_4 by means of photoacoustic spectroscopy (PAS) (Tuboly *et al.*, 2013). Each time, a 200 mg ileum sample or 1 mL of blood was taken and the tissue specimen was placed in a glass vial connected to the chamber of the PAS device. The CH_4 values were corrected for background levels and expressed in parts-per-million (ppm).

2. *In vitro* and *ex vivo* studies for the detection of NO generation

2.1. LPS treatment and study design

LPS from *Escherichia coli* serotype 026:B6 (activity ≥500,000 EU/mg) was used for the dose dependence experiments for electron paramagnetic resonance (EPR) spectroscopy analysis. Six groups of rats were injected with various doses (0.2, 1.3, 2.5, 4.7, 6.3, and 8.5 mg/kg body weight) of LPS dissolved in saline. Control animals were injected with saline only. Samples were then collected 16 h after the LPS injection.

2.2. EPR spectroscopy

EPR spectra were recorded at liquid nitrogen temperature at short- and long-scale ranges. The general settings for a short range were as follows: modulation frequency, 100 kHz; microwave

frequency, 9.425 GHz; microwave power, 8.3 mW; modulation amplitude, 5 G; and gain, 200. NO–Hb complexes were recorded at 3300±200 G. The general settings for the long range care were as follows: modulation frequency, 100 kHz; microwave frequency, 9.429 GHz; microwave power, 30 mW; and modulation amplitude, 6 G. Liver spectra were recorded at 3200±500 G. Signals were quantified by the determination of magnitudes and by double integration of components of spectra.

2.3. NO trapping

Iron-bound NO in the intestinal tissue of rats with CH_4 treatment was measured using the method described earlier (Kozlov *et al.*, 2001). Stated briefly, rats were injected subcutaneously with sodium diethyldithiocarbamate (Na-DETC) and $FeSO_4$ before SMA occlusion. DETC forms a water-insoluble DETC-Fe complex with iron, which is able to bind and stabilize NO as a DETC-Fe-NO complex, permitting the measurement of NO in the natural compartment where it was formed.

2.4. An *in vitro* study to analyze anoxic NO release

Shock-frozen rat liver tissue was homogenized in a 1:10 ratio with an incubation buffer containing 106 mM KCl, 5 mM KH_2PO_4, 20 mM Tris-HCl and 0.5 mM EDTA. NO release from liver homogenate was measured on-line by means of chemiluminescence under anoxic conditions and 37°C. The pH of the solution was set to 6.0 to mimic ischemic tissue with severe acidosis. The samples were equilibrated with nitrogen or nitrogen containing 2.2% CH_4 under continuous gas flow for 10 minutes. Afterwards, 4.4 mM $NaNO_2$ was added and the NO released from the homogenate was measured in real-time for a 15 minute period.

3. An *in vitro* microrheological study

Venous blood from healthy volunteers was placed into three groups, and incubated for 120 minutes at 37°C on a rollerbed before RBC aggregation and deformability measurements. A non-treated sample served as the negative control. Oxidative stress was induced with the addition of phenazine methosulfate (PMS) and incubation for 120 minutes (Rabai *et al.*, 2010). In the third, CH_4-treated group, the headspace of the sample was continuously perfused with a gas mixture containing 2.2 % CH_4 in normoxic air for 10 minutes after at the end of the PMS incubation protocol. RBC deformability and the aggregation of samples were determined by means of ektacytometry and light-transmission aggregometry, respectively. Elongation index (EI) of RBCs was calculated as the (length-width)/(length+width) of the pattern for 9 different shear stress values ranging from 0.5 Pa to 50 Pa (Hardeman *et al.*, 1994). The degree of aggregation was characterized by the aggregation index, which is calculated using the area below the light intensity curve in a 10 second period.

4. Statistical analysis

For a statistical evaluation of the data, GraphPad Prism 5.01 for Windows was used. The statistical analysis was performed by a two-way analysis of variance of repeated measures followed

by Bonferroni post-hoc test in normally distributed data and Kruskal-Wallis one-way analysis of variance on ranks combined with Dunn's method for pairwise multiple comparisons in groups showing a non-Gaussian distribution. Pearson's test was used to assess the statistical significance ($p<0.0001$) of correlations. Median values and 75^{th} and 25^{th} percentiles are given and in each case, p values <0.05 were considered significant.

IV. Results

1. The kinetics of CH_4 transport

The CH_4 concentration in the baseline samples of non-CH_4-producer animals remained below the background levels in the arterial blood and in the ileum tissue prior to the beginning of the experiment. After 5 minutes of normoxic CH_4 inhalation with a flow rate of 300 mL/min, at the end of the SMA ischemia substantially increased CH_4 concentrations were detected in the systemic arterial blood and there was a slight increase in the ileum as well. 10 minutes into the reperfusion phase, at the end of the 15 min inhalation of normoxic CH_4-air mixture, CH_4 concentration in the ileal tissue also increased. In samples taken at the 60^{th} minute of the reperfusion, 50 minutes after the end of CH_4 treatment, no significant amounts of CH_4 were found in intestinal or blood samples.

2. The small intestinal permeability

Small intestinal epithelial barrier function

The epithelial permeability (EP) index was determined to assess the barrier function of the intestinal mucosa during the re-establishment of the blood flow to the previously ischemic tissues. The EP did not change *in the early reperfusion* in sham-operated, control animals, while the plasma levels of FD4 rose steeply in the IR group, which indicates a rapid loss of the epithelial barrier function. Normoxic CH_4 treatment resulted in significantly lower EP levels, implying there were preserved interepithelial junctions. *Later in the reperfusion*, the EP index in non-treated animals decreased, suggesting an improved barrier function as compared to that in the early phase; while in the CH_4-treated group, the change was similar to those observed in the early phase of reperfusion. The EP index remained at the baseline level in the control animals.

Small intestinal microvascular barrier function

The vascular permeability (VP) assessed by Evans blue extravasation increased in the early reperfusion relative to sham-operated control animals, but statistically significant differences between the experimental groups were not detected either in the early or the later phases of reperfusion.

Micro- and macrocirculatory changes

Prior to the induction of SMA occlusion, the RBC velocity in the microvessels of the ileal serosa was similar in all groups. In the 15^{th} min of reperfusion, the intestinal microcirculation of the IR

group was significantly impaired. In the IR+CH_4-treated groups the RBC velocity did not differ from the sham-operated groups, implying improved microcirculation. By the 120 min of the reperfusion, no differences could be seen among the groups.

The SMA blood flow was assessed continuously during the experiments. In the IR and IR+CH_4 groups, the complete cessation of blood flow was followed by different reactions during reperfusion. The SMA flow in IR groups remained significantly low as compared to that for the sham-operated and IR+CH_4-treated groups; while in CH_4-treated animals, the SMA flow was significantly higher.

ROS and RNS levels

Tissue NTyr concentration is an indicator of protein nitration produced by a chemical reaction associated with ONOO generation. The NTyr levels were elevated by the 180 min of reperfusion in the IR group as compared to those in the sham-operated controls, while the levels did not differ from the controls in the CH_4-treated group. $O_2^{\bullet -}$, was detected in ileal biopsies at the beginning of experiments, and no differences were observed among groups. At 15 min after the reestablishment of blood flow, the samples from the IR group contained significantly higher levels of $O_2^{\bullet -}$ than those from the control and CH_4-treated animals.

Tissue ET-1 levels

The ET-1 concentration was measured from plasma samples at the end of the reperfusion period. In the IR group, there was a significant elevation at 180 min after reperfusion relative to that in control animals. This elevation was significantly reduced in the IR+CH_4-treated group.

Tissue MPO levels

The activity of MPO, a marker enzyme of polymorphonuclear (PMN) granulocytes, was assessed in intestinal homogenates at the end of the late reperfusion phase. A significant MPO elevation was present in both the IR and the CH_4-treated groups, indicating acute inflammation and extravasation of leukocytes into the tissue.

Structural integrity of small intestinal mucosa

Tissue samples were taken in the early reperfusion phase for conventional histology to provide structural data on the ileum mucosa. In the sham-operated group, villus morphology was normal, but in the IR group extensively damaged, denudated and progressively shrinking villi were typically found in association with increased luminal debris formation. Furthermore, the congestion of RBCs in the microvessels of the villi was seen. CH_4 treatment preserved the integrity of the mucosal layer with moderate debris formation and slight alterations of the lamina propria.

Intravital CLSEM images were recorded to get information on the structural condition of the surface of the epithelium and the microscopic histology data were evaluated using a semiquantitative scoring system. Normal villi with intact epithelial cells were observed in the control group. Relative

to the typically continuous, unbroken epithelial lining in the sham-operated animals, the mucosa was severely damaged after a 30 min reperfusion. Epithelial defects stretching across the villi were regularly seen. No epithelial disruptions on the lumen surface were present in the CH_4-treated group and the microstructural damage reflected in the injury score was significantly lower than that observed in non-treated IR animals.

3. The detection of NO by EPR without exogenously added spin-trapping molecules

EPR spectroscopy was applied to validate measurements of NO levels using endogenous trapping molecules. 16 h after an LPS injection, there was a dose-dependent increase at g = 2.075 and g = 2.042 in the complex EPR signal of liver, corresponding to hemoglobin-bound NO (NO−Hb) and Fe-bound hemoglobin (NO−Fe). In blood samples taken from the same animals, only NO−Hb signals at g = 2.075 were present. There was a good correlation of increasing LPS doses with NO−Hb and NO−Fe signals, which had been calibrated previously. The correlation was strong between NO−Hb and NO−Fe signals as well.

4. The effects of CH_4 on NO release under ischemia

Using Na-DETC and exogenously added iron to form stable, intracellular NO-Fe-DETC complexes for EPR analysis, we measured NO levels in the duodenum and ileum of rats. There was a trend showing increased NO concentrations at the end of a 45-min ischemia in both parts of the intestine as compared to those in sham-operated animals. The inhalation of a normoxic gas mixture containing 2.2% CH_4 for 10 min resulted in lower levels of NO. During the reperfusion phase, we did not see any similar trends.

To estimate whether or not CH_4 can modulate NO release from NO_2^- in anoxic tissues, liver homogenate was incubated with pure N_2 or N_2 supplemented with 2.2% CH_4. NO production was alleviated in the presence of CH_4; reaching significantly lower levels as compared to those for the N_2 only group from the 10^{th} min after adding $NaNO_2$.

5. The effects of CH_4 on the microhemorheological parameters of whole blood

The deformability of erythrocytes taken from human blood was measured using a laser-assisted optical rotational method. Oxidative stress, induced by *in vitro* treatment with the oxidizer PMS, resulted in a significantly decreased elongation index from low to moderately high shear stress rates, as compared to that for the non-treated control samples. Normoxic CH_4 incubation, applied *after* the oxidizer incubation, was able to partly counteract the decreased rigidity of RBCs at moderate levels of shear stress. Oxidative stress *in vitro* increased the aggregation of erythrocytes at low shear-stress as compared to that in control samples. After applying CH_4, these values significantly decreased to the level of non-treated control samples.

V. Discussion

Understanding the mechanism of action is the key to efficient and safe therapies, and on the basis of accumulated knowledge, the presumed physiological role of a compound can be clarified. In this work, the efficiency of a CH_4-based therapy was investigated in a small animal model of oxido-reductive stress and in a second step of accompanying *in vitro* studies, we tried to delineate a feasible mechanism of action.

1. The kinetics of CH_4 transport *in vivo*

In the case of NO or H_2S, precursors stimulating endogenous release of the gas can be administered or enzymatic synthesis of the compounds can be induced (Szczesny *et al.*, 2014; Gero *et al.*, 2016). However, with CH_4, direct delivery is currently the only feasible option. In our studies, gas inhalation was used with artificial air containing 21% O_2 and 2.2% CH_4. The solubility of CH_4 is rather low in water, but much higher in the lipid phase. The large alveolar surface of the lungs and the thin layer of alveolar cells with adjacent endothelium provide an ideal way to dissolve CH_4 in the blood. Due to the apolar property of the molecule, the majority of gas is transported by RBCs and lipoproteins. We sought to assess the kinetics of tissue and blood levels of CH_4 during and after the inhalation protocol was used *in vivo*. We found that even a brief inhalation of CH_4 resulted in high amounts of gas being dissolved and transported by the blood. Upon reperfusion, as the blood flow restarted in the tissue, the levels of the gas rose significantly. About 50 min after the end of inhalation, no CH_4 is measurable in the tissues. We can assume that upon reperfusion, the CH_4 content of the systemic blood immediately starts to equilibrate with the CH_4 level in the previously ischemic tissue, reducing the time needed to reach the target cells. There was a slight increase in CH_4 levels in the ileum during ischemia, probably as a consequence of the omnidirectional diffusion of the gas. Due to its non-polar properties, CH_4 can reach tissues without perfusion, hence it can exert effects under deep hypoxia.

2. The bioactivity of exogenous CH_4 in the intestine

The small intestine is especially sensitive to hypoxia and the gastrointestinal system is among the most vulnerable organs during circulatory redistributions (Vajda *et al.*, 2004), leading to rapid damage of the physical and immunological barriers between the interior milieu and intestinal lumen. The anti-inflammatory properties of CH_4 have already been examined in intestinal IR (Boros *et al.*, 2012) and we sought to look at the function of mucosal barrier in this setting, which is one of the key components of the process. Here we could show that even a brief, 15-min normoxic CH_4 treatment modulated epithelial component of transmucosal permeability significantly and effectively prevented the IR-induced barrier loss in the early reperfusion phase.

The assessment of the lumen-to-plasma clearance of the 4 kDa MW FITC-labelled dextrane allowed us to determine paracellular permeability, mediated predominantly by TJs (Szabo *et al.*, 2006). The maintenance of the closed conformation of TJs is energy dependent; therefore deteriorating intracellular ATP levels increase permeability (Wattanasirichaigoon *et al.*, 1999). CH_4 can in theory protect TJs from opening by directly influencing membrane fluidity or by preserving the ATP levels of epithelial cells; moreover, it can act indirectly, such as by a NO-mediated mechanism. Actually, the reduction of tissue NTyr levels does indeed suggest that CH_4 may improve the epithelial barrier function by limiting the inhibitory effect of NO on mitochondrial ETS (Brown, 2001). This, in turn, may elevate ATP production early in the reperfusion phase, allowing cells to attain sufficiently high energy levels to maintain TJ proteins in the tightly closed conformation.

In parallel with the improved barrier function, both conventional and *in vivo* histology revealed preserved structure of the most luminal layers of the mucosa. The decrease in epithelial permeability after CH_4 inhalation was associated with reduced ROS and RNS generation and decreased ET-1 levels. In addition the CH_4 treatment influenced the PMN infiltration, a specific cellular component of inflammatory reactions. CH_4 treatment improved both serosal microcirculation and the SMA flow facilitating O_2 delivery to cells and allowing effective oxidative phosphorlylation in mitochondria. The normalized ET-1 plasma levels in the CH_4-treated groups may reflect the improved local microcirculatory state on one hand and reduced inflammatory activation on the other hand.

The epithelial barrier of the ileum was already partially restored in the later phase of the reperfusion. Our data suggest that upon re-establishment of the blood and oxygen supply, the endogenous defense mechanisms cannot immediately control or counteract the damaging reactions. Salvage therapies should target the initial steps to avoid long-term or distant consequences of the barrier damage, and CH_4 treatment can fill this need.

The effects of CH_4 on the IR-induced changes in vascular permeability are less clear, since the extravasation of the circulating endothelial tracer did not increase to a great extent. Possible technical limitations cannot be ruled out. Therefore, future studies are needed to examine the influence of CH_4 on microvascular permeability directly and separately.

3. The detection of NO in biological samples

Along the above lines, one might suppose that CH_4 accumulates transiently at membrane interfaces, and a disproportionate increase in relationship with other gases, such as NO, may alter enzyme-linked processes, and ultimately mitochondrial function. Although much is known about the role of NO in physiology and pathophysiology, the determination of its actual levels in biological samples is not an easy task. EPR spectroscopy is currently the most suitable method used to measure NO directly. It has very high specificity for NO as it forms complexes with exogenously added spin

traps (e.g. Na-DETC and $FeSO_4$). Unfortunately, these compounds are toxic. Moreover, such molecules can interfere with endogenous NO utilizing pathways through their high affinity to NO. An alternative is to utilize endogenous traps of NO for this purpose. Intracellularly produced NO reacts with ferrous ions to form dinitrosyl-iron complexes (NO−Fe) (Sergent *et al.*, 2005). In contrast, in the vasculature NO forms mononitrosyl-hemoglobin complexes (NO–Hb) (Gow *et al.*, 1998) with hemoglobin. Since no ferrous ions occur in the blood, the two complexes, located at g = 2.075 and g = 2.042 are characteristic for NO−Hb and NO−Fe, respectively.

We were able to demonstrate in intact, non-processed, frozen samples that LPS increased hepatic NO levels in a dose-dependent way, both in the intracellular and intravascular compartments and NO-Hb levels in the systemic circulation. The concentrations of NO in naturally occurring complexes were substantially lower compared with previous values stated in the literature of intracellular NO using the specific NO trap iron-diethylthiocarbamate in a similar model (Kozlov *et al.*, 2005). We may conclude that only part of NO produced intracellularly is scavenged as NO–Fe and the residual NO contributes to signaling or diffuses in the blood (Kozlov *et al.*, 2001).

4. The interaction of CH_4 with NO-related nitrosative stress

NO is an exemplary double edged sword in biology. In nanomolar concentrations it is vasorelaxant and antiadhesive, but in the micromolar range NO inhibits mitochondrial respiration in a reversible way (Cooper *et al.*, 2007; Cooper *et al.*, 2008). In fact, elevated NO levels could be present during the ischemic phase, as data obtained by means of EPR indicates. With CH_4 treatment, however, the overproduction of NO could be reduced. Significantly, we detected NTyr formation in the intestinal tissue, an indicator of ONOO, which requires both $O_2^{\bullet-}$ and NO to form. The non-specific nitration of ETS proteins by ONOO is an additional, non-reversible inhibition of mitochondrial oxidative phosphorylation (Brown *et al.*, 2004).

The source of NO under hypoxia is thought to be NO_2^-, reduced mostly by enzymatic reactions and by small molecules under acidic pH. It has been shown that CH_4 inhibits XOR under normoxic conditions, a feature which has important role in alleviating ROS-induced damage during reperfusion, when XO is one of the main ROS generating enzymes. The same enzyme can function as NO_2^- reductase under hypoxia and low pH, conditions, which are fulfilled during ischemia.

Considering this information, we set out to measure NO release in real-time *in vitro* from liver tissue homogenate under anoxic conditions, mimicking ischemic tissue. After addition of $NaNO_2$ as a source of NO, in the anoxic-CH_4-incubated group, the production rate of NO was significantly lower than that with N_2 atmosphere only. Endogenous levels of XDH are similarly high in the liver and in the small intestine. Accordingly, it can be assumed that modulation of XO by CH_4 contributes to the reduced NO levels in the intestine, but other mechanisms cannot be excluded with certainty.

Taken together, we were able to demonstrate that increased CH_4 input reduces NO production and NTyr levels in hypoxic organs and in tissue homogenates under anoxia. Apparently, there is a contradiction between the reduction of anoxic/hypoxic NO generation and improved microcirculation upon reperfusion. Nevertheless, the maximal vasodilating effect of NO requires much lower levels than those needed for inhibition of cytochrome c oxidase.

5. The direct action of CH_4 on erythrocyte deformability

The tissue NTyr level is an indicator of protein nitration, associated with elevated ONOO levels. ONOO is a potent initiator of membrane lipid peroxidation (Hogg *et al.*, 1999) and it was reported earlier that IR-associated lipid peroxidation decreases membrane fluidity in various tissues (Dobretsov *et al.*, 1977) and in erythrocytes as well (Watanabe *et al.*, 1990). The membrane and cytoskeleton are together responsible for altering the shape of erythrocytes (Reinhart, 2001). Lipid peroxidation breaks the connection between the two components (Mohandas *et al.*, 1993) and, consequently, both deformability and aggregation of the RBCs is influenced in a detrimental way (Kayar *et al.*, 2001). Brath et al. reported worsened RBC deformability and increased aggregation in the early reperfusion after experimental mesenteric ischemia in the rat (Brath *et al.*, 2010). Since erythrocytes are 25% larger than the mean diameter of capillaries, RBC deformability is a prerequisite of normal microcirculation (Reinhart *et al.*, 1985; Baskurt *et al.*, 2003).

Based on this background, we obtained direct intravital data on the deranged intestinal microcirculation. CH_4 inhalation was associated with improved serosal microcirculation and the treatment also enhanced the SMA flow. Hence, we hypothesized that CH_4 can modulate erythrocyte deformability and allow them to flow freely through microvessels. With this in mind, an *in vitro* microrheological study with human whole blood was designed without the confounding *in vivo* effects of vasoactive metabolites. We demonstrated lipid peroxidation significantly reduced the elongation and increased aggregation indices of RBCs. Upon normoxic CH_4 treatment, the RBC deformability improved at low-to-moderate shear stress rates, suggesting a direct effect of CH_4, and aggregation function improved. Thus, these data provide evidence for the direct effects of CH_4 exerted on membrane fluidity and/or membrane-cytoskeleton junctions.

VI. Summary of the new findings

Studies with exogenously administered CH_4 corroborated previous findings on the anti-inflammatory effects of the molecule and provided novel data about further details of the acting mechanism.

1. CH_4 is quickly transported from the lungs to the small intestine by the circulating blood. After the inhalation of normoxic air containing 2.2% CH_4 blood concentration of CH_4 increases at least 2-3-fold over its basal levels in rats; this increase is sufficient to modulate the generation of ROS and RNS.
2. The microcirculatory and structural damage after intestinal IR is accompanied by increased epithelial permeability, demonstrating mucosal barrier damage. Normoxic CH_4 inhalation effectively prevented the elevation of intestinal epithelial permeability, maintaining structural integrity of the mucosa and improving biochemical signs of inflammation in the early reperfusion. These data support previous findings on CH_4 bioactivity and establish a mucosa-protective role for exogenous CH_4 to modulate the IR-induced pro-inflammatory activity locally in the small intestine.
3. A new, non-toxic, exogenous spin trap-free EPR spectroscopic method was developed for NO detection. The method was validated for NO measurements from both the intra- and extracellular compartments of the same samples in tissue biopsies.
4. With EPR spectroscopy reduced NO levels were detected in the intestinal tissue after normoxic CH_4 administration, and this finding was supported by *in vitro* data collected in anoxic liver tissue during CH_4 incubation. Collectively, these results confirm that exogenous CH_4 administration effectively reduces NO levels in the ischemic tissue.
5. CH_4 *in vitro* improves the deformability of red blood cells during simulated oxidative stress, which may contribute to the improvement of the effectiveness of mesenteric microcirculation in the postischemic small intestine.

VII. Acknowledgements

First and foremost, I would like to express my sincere gratitude to Professor Mihály Boros, to my supervisor and mentor for his continuous support and patience during my years in the Institute of Surgical Research. His gentle way of stimulating guidance, optimistic attitude and protecting atmosphere let me grow and follow my curiosity.

I owe special thanks to Professor Andrey V. Kozlov, my supervisor in Vienna, for his encouragement, practical and theoretical advices. His immense knowledge was indispensable to this thesis.

I would like to thank Professor József Kaszaki for his practical guidance and help over years. Likewise, supervision and help of Dr. Gabriella Varga and Dr. Borbála Fazekas during and after my university years is greatly appreciated. I express my special thanks to Professor Andrea Szabó for her always professional, friendly and extremely useful suggestions. I wish to thank Dr. Tamás Büki and Dr. Kitti Horváth for taking me to the Institute of Surgical Research on a sunny morning of September, 2008. That's where everything got started.

I would like to acknowledge the highest level professional help and personal support of Dr. Adelheid Weidinger. Special thanks go to Sergiu D. Dumitrescu, M.Sc., for being my best friend and excellent colleague in Vienna in hard times and in minutes of joy.

I am indebted to Professors Heinz Redl, Head of the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Soheyl Bahrami, Marcin F. Osuchowski and Peter Dungel for generous and continuous support of my work in Vienna.

I would like to give my sincere thanks to Professor László Tretter and his team, who engrafted me with the beautiful world of mitochondria.

This work would not have been possible without the efforts all of my coworkers, collaborators and the technical staff at the Institute of Surgical Research (Szeged), Ludwig Boltzmann Institute for Experimental and Clinical Traumatology (Vienna) and First Department of Medicine (Pécs). I especially thank to Dr. Stefan Puchner and Tanja Stögerer, B.Sc. for their skillful assistance.

Lastly, but most importantly, I would like to thank my parents and my family for their never-ending encouragement and relief. My goals could not be accomplished without such a strong background.

This thesis was supported by research grants of the Hungarian Science Research Fund OTKA K104656, the National Research Development and Innovation Office NKFI K120232 and GINOP-2.3.2-15-2016-00015