

PhD THESIS

**EFFECT OF METHANE INHALATION ON THE INTESTINAL SEGMENT DEPENDENT FUNCTIONAL,
STRUCTURAL AND MOLECULAR CHANGES CAUSED BY ACUTE MESENTERIC ISCHEMIA AND
EARLY REPERFUSION IN RATS**

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ABBREVIATIONS

AMS	arteria mesenterica superior
HuC/HuD	human neuronal protein
I/R	ischemia/reperfusion
MPO	myeloperoxidase
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
ROS	reactive oxygen species
XOR	xanthine oxidoreductase

INTRODUCTION

Arteria mesenterica superior (AMS) is the main artery that supplies the whole length of small intestine and the proximal colon. Acute mesenteric ischemia occurs when mesenteric arteries are occluded or the blood flow is reduced which leads to inadequate oxygen levels and formation of hypoxia in the gut wall. Due to the compensatory mechanisms and the extensive collateral network of arterioles, the gut wall can tolerate longer hypoperfusion to some extent. Therefore symptoms of the acute mesenteric ischemia are non-specific, and its diagnosis is often possible only after formation of irreversible functional, structural and molecular changes in the gastrointestinal tract. On the grounds of late diagnosis mortality rate of acute mesenteric ischemia is still about 60-80%.

Many of the survivors of non-lethal mesenteric ischemia have unmanageable intestinal motoric symptoms. This suggest that myenteric neurons responsible for the intestinal motor control are badly affected during ischaemia and reperfusion (I/R).

In the mammalian gastrointestinal tract absorption, secretion and motility are controlled by the enteric nervous system. The nitrergic neurons using nitric oxide (NO) as neurotransmitter have determinative role in the control of intestinal peristalsis. Under physiological conditions production of NO is catalysed by nitric oxide synthase (NOS). Because of the oxygen-dependent function of NOS, hypoperfusion disturbs NO synthesis. The severity of NO deficiency depends on the rate of vascular occlusion. While selective loss of nitrergic neurons at the late phase of reperfusion were well documented, no informations are available on changing quantitative properties of nitrergic myenteric neurons in the acute ischemic insult or in the early phase of reperfusion when enhanced production of NO is characteristic.

At molecular level ischemia causes reductive stress. During reperfusion returning oxygen causes the release of large amount of reactive oxygen species (ROS) and formation of oxidative stress which exacerbate ischemic damages of the tissues. ROS causes lipid peroxidation of membranes thus release of arachidonic acids which activate and attract leukocytes to the damaged tissues. Activated leukocytes produce more ROS and exacerbate the oxidative stress. The enhanced production of NO at the beginning of reperfusion is the main defense mechanism against oxidative stress. NO increases the blood flow of inflamed tissues, increases the production of protective mucus, decreases the adhesion of leukocytes to

the mesenteric endothelium and protects the tissues from secondary infections. Reaction of NO with ROS refers to its antioxidant role. However, at the early phase of reperfusion the increased amount of ROS prevent the accumulation of NO accordingly the protective role of NO is also disturbed. Moreover, reaction of NO with ROS leads to formation of reactive nitrogen species and nitrosative stress. Levels of oxidative and nitrosative stress markers significantly influence the I/R sensitivity of the different intestinal segments.

We have recently demonstrated the anti-inflammatory effects of inhaled methane after mesenteric I/R. Methane inhalation applied at the early phase of reperfusion decreased the microcirculatory disorders, the intestinal mucosal damages and the tissue levels of oxidative and nitrosative stress markers. However no informations are available concerning the effects of methane inhalation on the functional, structural and molecular changes caused by acute ischemic insults or by early reperfusion.

AIMS

Severe damages of the gut wall in the late phase of I/R were well documented. However little is known about the structural, functional and molecular disturbances of the intestine caused by acute ischemic insults and/or by early reperfusion. Our aim therefore was to investigate these early events in a rat model of I/R.

The questions to be answered were: are there any gut segment-specific changes during the acute ischemic insult and during the early phase of reperfusion

- in the frequency and amplitude values of intestinal smooth muscle contractions?
- in the quantitative properties of the total myenteric- and the myenteric nitrergic neuronal populations?
- in the levels of oxidative and nitrosative stress markers?

If there are detectable changes in these parameters,

- whether methane inhalation can prevent these changes?

MATERIALS AND METHODS

To investigate the effect of ischaemic insults and early phase of reperfusion separately we used four short-term (sham-operated, ischemic, methane-treated sham-operated and methane-treated ischemic) and four long term (sham-operated, I/R, methane-treated sham-operated and methane-treated I/R) experimental animal groups. Ischemia was induced by the occlusion of AMS and lasted for 50 minutes. In the long term groups ischemia was followed by 120 minutes reperfusion. Rats in the methane-treated groups inhaled artificial air with 2.2% methane at the last ten minutes of ischemia. In the long term groups methane inhalation continued in the first five minutes of reperfusion.

To study the changes in the motoric activity of the gut wall, myoelectric signals were recorded from the outer smooth muscle layer of the intestine. Recordings were filtered via their frequency ranges into the signals originated from the small or the large intestine, then were analysed with fast Fourier-transformation. We analysed both the dominant frequency and the amplitude of the contractions of the gut wall.

To measure the quantitative properties of myenteric neurons, total myenteric neuronal number was counted by using HuC/HuD immunohistochemistry as pan-neuronal marker on whole-mount preparations. To study the quantitative parameters of nitrergic myenteric neurons the number of nNOS-immunopositive neurons was counted. After counting each immunoreactive neurons per field of view, the proportion of nitrergic myenteric neurons was calculated.

For monitoring the oxidative stress we measured the activity of xanthine oxidoreductase (XOR) which has a key role in generating reactive oxygen species. We also studied the levels of infiltrated leukocytes by measuring the activity of myeloperoxidase (MPO) which is released from the activated leukocytes congregating in the gut wall. As the stable end product of nitric oxide (NO) oxidation, levels of nitrite and nitrate were measured to monitor the levels of tissue NO. To determine the nitrosative stress levels we measured the tissue levels of nitrotyrosine.

RESULTS AND DISCUSSION

After the occlusion of the AMS, the frequency values decreased, while the amplitude values of contractions increased in both the small and large intestine. The tendency of myoelectric changes was the same in both intestinal segments. However responses to the induction of ischemia or reperfusion appeared later in the colon than in the small intestine which suggests that the colon has greater resistance to hypoxia.

At the beginning of the reperfusion phase, frequency values returned to near the baseline levels, but in the small intestine, amplitude values of contractions stayed at significantly lower level. This suggests that the changes in the myoelectric activity of the gut are partly affected by the increased levels of oxidative and nitrosative stress during ischemia, therefore this process is partly reversible.

The time of methane inhalation at the last ten minutes of ischemia and the first five minutes of reperfusion was set based on our previous study. We hypothesized that the methane inhalation would have the most effect on I/R injuries when the accumulation of oxidative and nitrosative stress markers just begun. Although methane inhalation decreased the frequency values of smooth muscle contractions in both small and large intestine, it did not affect significantly the amplitude values in any of the intestinal segments. Because of the changes in the frequency and amplitude values of the contractions appeared at the early phase of ischemia, maybe methane inhalation should be applied earlier, at the time of occluding the AMS. More studies need to confirm this hypothesis.

The total number of myenteric neurons did not change in any of the intestinal segments during ischemia or early reperfusion.

After ischemia, the proportion of nitrergic myenteric neurons decreased significantly both in the duodenal and ileal samples. After methane inhalation, proportion of nitrergic neurons reached control level in the ileum but not in the duodenum. In the early reperfusion, proportion of nitrergic neurons compared to control values was significantly lower in the duodenal and significantly higher in the ileal samples, and these values reached control levels in both small intestinal segments when methane inhalation was linked to reperfusion. This suggests that methane inhalation facilitated the adaptive changes of nitrergic neurons to reperfusion in both intestinal segments. However to reach their control values nitrergic neurons show different adaptational strategies in the duodenum and in the ileum. In the

duodenum density of nitrergic neurons increased while in the ileum it decreased. In the colon there was a temporary and reversible increase in the proportion of nitrergic neurons during ischemia which reached control levels after methane inhalation and during reperfusion. These findings also confirm the higher I/R tolerance of colon.

In accordance with the literature data our results suggest that nitrergic neurons react to ischemic and reperfusion insults in two phases. Before the selective loss of nitrergic neurons at the late phase of reperfusion there is an adaptive phase during ischemia and early reperfusion when nitrergic neurons undergo adaptive changes which are associated with the dynamic changes of nNOS content of nitrergic neurons. In the second phase, at the late phase of reperfusion, selective loss of nitrergic neurons occurs.

Under physiological conditions, there were notable differences in the levels of oxidative and nitrosative stress markers between the intestinal segments. XOR activity in the duodenum was five times higher than in the ileum, while XOR activity in the ileum was twenty times the values found in the colon. During ischemia XOR activity did not change in any of the intestinal segments. However activity of MPO in the duodenum increased significantly and this increased activity did not return to control level despite of the methane inhalation. We suggest, that the high endogenous XOR activity of the duodenum might be responsible for the higher I/R sensitivity of this gut segment and therefore for the irreversible increased MPO activity here, during the acute ischaemic insult. In the early phase of reperfusion the activity of XOR and MPO increased significantly in each intestinal segments. When methane inhalation was linked to reperfusion these values returned to control levels. This supports our hypothesis on the possible mechanism of the action of inhaled methane. We suppose that methane is able to decrease the production of reactive oxygen species via direct interaction with the XOR enzyme.

Tissue nitrite and nitrate levels increased significantly only in the ileal samples after ischemia and after reperfusion in all intestinal segments remained at control levels. We hypothesize therefore that in our experimental conditions tissue NO levels are just enough to maintain the “adaptation window” of the nitrergic neurons. However the increased levels of nitrite and nitrate in the ileum during ischemia may explain the differences in the adaptation strategies of nitrergic neurons between the duodenum and ileum.

Tissue levels of nitrotyrosine increased only in the duodenum during ischemia which also shows the higher sensitivity of duodenum to hypoperfusion. In addition it may explain the slower adaptation of nitrergic neurons in this intestinal segment. At the early phase of reperfusion nitrotyrosine levels increased significantly in each intestinal segments. This

indicate that at the early phase of reperfusion the harmful effects of NO more dominant than it's protective role. When methane inhalation was linked to reperfusion nitrotyrosine levels reached control values in each intestinal segments. This supports another hypothesis on the action of the inhaled methane through decreasing the reductive products of NO thus moderating the levels of nitrosative stress.

CONCLUSIONS

Our results answered all the questions posed in our aims. We demonstrated that acute hypoxia and reoxygenation changes the frequency and amplitude values of myoelectric activity in the whole length of intestines. These changes in the small intestine were partially reversible.

We demonstrated that quantitative properties of nitrergic neurons changed in a gut segment-specific manner during ischemia and reperfusion. We hypothesized that before the previously demonstrated selective loss of nitrergic neurons occurs at the late phase of reperfusion, there is an earlier „adaptation phase” during ischemia and early reperfusion when depending on the gut segment and the oxidative circumstances nitrergic neurons can undergo adaptive changes.

We demonstrated, that the endogenous levels of the oxidative and nitrosative stress markers we measured here showed gut segment-specific differences, which might explain the different sensitivity of the duodenum, ileum and colon to the ischaemic insults and the early phase of reperfusion.

We demonstrated here, that methane inhalation prevented the gut tissue from the increase of the oxidative and nitrosative stress markers in the early phase of reperfusion. We suggest that this is the main mechanism behind the protective effects of inhaled methane against I/R injury in the intestine.

PUBLICATIONS

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Thesis related publications:

M. Z. Poles, N. Bódi, P. Talapka, M. Bagyánszki, M. Boros, É. Fekete, J. Kaszaki (2015) Methane inhalation enhances adaptive responses of nitrergic neurons to ischemia and reoxygenation in gut region-dependent way in rats. (manuscript in preparation)

N. Bódi, Zs. Jancsó, P. Talapka, A. Pál, **M. Z. Poles**, M. Bagyánszki, E. Hermes, É. Fekete (2014) Gut region-specific rearrangement of the cellular and subcellular compartments of nitric oxide synthase isoforms after chronic ethanol consumption in rats. *Histol Histopathol.* 29(12):1547-1555. **IF: 2.096**

*Z. Máté, ***M.Z. Poles**, G. Szabó, M. Bagyánszki, P. Talapka, É. Fekete, N. Bódi (2013) Spatiotemporal expression pattern of REDT3/CCK gene construct during postnatal development of myenteric plexus in transgenic mice. *Cell and Tissue Research* 352(2): 199-206. **IF: 3.333**

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Other publications:

P. Talapka, L. Nagy, A. Pál, **M. Poles**, A. Berkó, M. Bagyánszki, L. G. Puskás, É. Fekete, N. Bódi (2014) Alleviated mucosal and neuronal damage in a rat model of Crohn's disease. *World J Gastroenterol.* 20(44):16690-16697. **IF: 2.369**

N. Bódi, P. Talapka, **M. Poles**, E. Hermes, Zs. Jancsó, Z. Katarova, F. Izbéki, T. Wittmann, É. Fekete, M. Bagyánszki (2012) Gut region-specific diabetic damage to the capillary endothelium adjacent to the myenteric plexus. *Microcirculation* 19(4):316-326. **IF: 2.763**

Thesis related abstracts published in scientific journals:

M. Z. Poles, N. Bódi, P. Talapka, G. Varga, A. Pál, M. Bagyánszki, R. Gáspár, J. Kaszaki, É. Fekete, M. Boros (2014) Effects of methane inhalation on the nitrergic myenteric neurons and intestinal myoelectrical activity during mesenteric ischemia-reperfusion in rats. Joint Meeting of the Federation of European Physiological Societies (FEPS) and the Hungarian Physiological Society, Budapest, Hungary, 27-30th august 2014.; *Acta Physiologica* (ISSN: 1748-1708) 211: (S697) p. 11.

M. Z. Poles, A. Mészáros, N. Bódi, M. Bagyánszki, G. Varga, É. Fekete, J. Kaszaki, M. Boros (2014) Effect of methane on gastrointestinal motility in ischemia-reperfusion. 49th Congress of the European Society for Surgical Research, Budapest, Hungary, 21-24th may 2014.; *Magyar Sebészet* 67(3): 235-236.

Thesis related abstracts published in conference proceedings:

N. Bódi, P. Talapka, G. Varga, T. Tőkés, **M.Z. Poles**, A. Pál, M. Bagyánszki, J. Kaszaki, É. Fekete, M. Boros (2013) Inhaled Methane is Preventing an Increase in Nitrergic Myenteric Neuron Density and Enhanced Oxidative and Nitrosative Stress After Mesenteric Ischemia-Reperfusion in Rats. 21th United European Gastroenterology Week, Berlin, Germany, 12-16th october 2013.; *UEG Journal* 1(1):p. A393.

M. Z. Poles, N. Bódi, P. Talapka, G. Varga, A. Pál, M. Bagyánszki, J. Kaszaki, É. Fekete, M. Boros, N. Bódi (2014) Effects of Methane Inhalation on the Nitrergic Myenteric Neurons and Intestinal Motility During Mesenteric Ischemia-reperfusion in Rats. 1st Innovation in Science 2014 – Doctoral Student Conference, Szeged, Hungary, 2-3rd may 2014.

M. Z. Poles, P. Talapka, G. Varga, A. Pál, M. Bagyánszki, J. Kaszaki, É. Fekete, M. Boros, N. Bódi (2014) Exogén metán hatása a myentericus nitrerg neuronok kvantitatív sajátosságaira és a bélcsatorna motilitására mesenterális ischaemia-reperfúzió alatt kísérletes patkánymodellen. 17. Spring Wind Conference, Debrecen, Hungary, 21-23th march 2014.

M. Poles, N. Bódi, P. Talapka, G. Varga, T. Tőkés, A. Pál, M. Bagyánszki, J. Kaszaki, É. Fekete, M. Boros (2013) Exogén metán hatása a myentericus nitrerg neuronok denzitására mesenterális ischaemia-reperfúzió alatt. Joint meeting of the Hungarian Physiological Society, Hungarian Society for Experimental and Clinical Pharmacology and Hungarian Society for Microcirculation and Vascular Biology, Budapest, Hungary, 5-8th june 2013.