Background

Our studies were designed to investigate unknown mechanistic details of ischemic organ damage, and to examine the possible ways with which to influence such events. Accordingly, the research plan was divided into three, related and interconnected parts (’mechanism’ / ‘diagnostics’ / ‘therapeutics' studies, respectively), and we used/developed specific experimental models according to the specific aims.

Models

Pericardial tamponade

Firstly, we outlined the major components of a systemic hypoperfusion-induced pro-inflammatory reaction in a porcine model of pericardial tamponade, where the post cardiogenic shock period was accompanied by impaired systemic and pulmonary hemodynamics and intestinal microcirculatory dysfunction. More importantly, these responses were associated with abrupt increases in superoxide (SOX) production, big-ET, troponin T, HMGB1, histamine, intestinal myeloperoxidase (MPO) activity and complement activation. We characterized the effects of anti-inflammatory treatments in this scenario; we have shown that a complement C5a inhibitor compound (AcPepA, developed by our collaborative partners, University of Nagoya) normalized the mean arterial pressure and preserved the cardiac output, while the superior mesenteric artery flow and mucosal average RBCV were increased significantly. The plasma superoxide, HMGB1, big-ET and intestinal MPO levels were reduced as well. These data provided the first comparative *in vivo* results for further anti-inflammatory protocols.

(Érces et al. Crit Care Med 2013, Vass et al. Eur Surg Res 2013).

Next, we have obtained further, conclusive evidence on the role of complement activation in low flow states, as C5a inhibition resulted in a significant decrease in tissue damage with a preserved villus structure and microvascular architecture, in a rat model of non-occlusive mesenteric ischemia (see later). In this experimental setup, a single iv dose of the C5a antagonist AcPepA improved the local circulatory changes and decreased the secondary mucosal damage in a relatively wide time frame, 24 hr after the insult.

(*Érces D et al. Surgery 2016*).

Irradiation-induced injury

Further, we have investigated the immediate changes in major pro- and anti-inflammatory cytokines in the peripheral circulation after irradiation of the hippocampus. We hypothesized that the consequences of irradiation might include systemic effects if the opening of the blood brain barrier is bidirectional. The hippocampus irradiation-induced pro- inflammatory stimuli not only affected the circulating cytokine concentrations, but in parallel the hepatic ATP production was significantly reduced. The results also demonstrated that a single dose of L-alpha-glycerylphosphorylcholine (GPC), a water-soluble, deacylated PC derivative (see later), can influence the changes in TNF-α, IL-6, IL-10 and histamine plasma levels and prevents the ATP depletion in the rat liver. In conclusion, these data provided evidence for the possibility of peripheral inflammatory activation after hippocampus irradiation through the production of mediators leaking from the irradiated brain.

(*Tőkés et al. Int J Rad Biol 2014).*

With this background, we have examined the later consequences of the irradiation-induced inflammation in a clinically relevant time-frame. Histopathological changes, signs of necrosis, macrophage density and reactive gliosis in the irradiated region of the brain with or without systemic GPC therapy were measured 4 months after the irradiation, the spatial orientation and learning ability were assessed with the repeated Morris water maze test. The irradiation resulted in a moderate deficit at the levels of both cognitive function and morphology and in the irradiated

hemispheres, the GPC treatment significantly decreased the histopathological changes, and the cognitive decline was ameliorated also significantly. These effects are indicative of a previously unknown, radio-neuroprotective action of the compound.
(Plangár et al. J Neurooncol 2014).

Ischemia-Reperfusion

Next, we used a standardized ischemia-reperfusion (IR) setup to investigate further details of the anti-inflammatory effectiveness of GPC - in equimolar dose with the effective anti-inflammatory doses of phospatidylcholine (PC) - to determine which part of the PC molecule is anti-inflammatory, the polar or the fatty acid parts. Our results demonstrated that intestinal IR decreased the mean arterial pressure, the superior mesenteric artery flow and the intramural RBCV, and increased the mesenteric vascular resistance significantly. At the same time, the SOX, xanthine oxidoreductase (XOR) and nitrotyrosine levels were elevated significantly in the small intestine. In contrast with this, GPC treatment stabilized the micro- and the macrocirculation, lowered SOX production and the activity of XOR. This demonstrated indirectly that PC-derived lipids do not participate in the anti-inflammatory action of PC, and the data suggested that the active component is the choline head group.
(*Tőkés et al. Eur J Nutr 2014*).

Parallel in vivo experiments were carried out to analyze the microcirculatory and mitochondrial functional changes in liver IR with or without GPC treatments, with special emphasis on the expression of NADPH-oxidase isoforms. The liver IR resulted in significant increases in NOX4 expression, increased the XOR and MPO activities and the levels of TNF-α and HMGB1. The microvascular blood flow and tissue oxygen saturation decreased by approx. 20% from control values. In this study, exogenous GPC administration did not influence the activity of the XOR system, whereas the accumulation of PMNs and the hepatic expression of NOX4 were reduced. In parallel, GPC treatment significantly reduced the postischemic microcirculatory deterioration and the release of biomarkers of functional liver damage. This suggested that PMNs and NOX4 activation may have greater impacts on the extent of postischemic liver injury than other, potentially momentous sources of superoxide production, such as XOR. Furthermore, GPC pretreatment decreased the IR-related elevation of plasma HMGB levels without affecting TNF-α release. In conclusion, we demonstrated that exogenous GPC ameliorates the inflammatory activation and preserves the postischemic microvascular perfusion and liver functions, these effects being associated with a reduced hepatic expression of NOX4.
(*Hartmann et al. J Surg Res 2014*).

Since IR injury of the liver has an increasing clinical impact and remote ischemic preconditioning (IPC) can be a modality to overcome postischemic hepatic injury, our additional aim was to investigate the effects of remote IPC on microcirculatory consequences in the postischemic liver. In this respect, we hypothesized the expression changes in the NADPH-oxidase isoforms may contribute to the efficacy of limb IPC. To address this issue, we set out to investigate the consequences of limb IPC on major intracellular SOX-generating enzyme systems in a rat model of hepatic IR injury, with special emphasis on changes in expression of NOX2 and NOX4 proteins. Remote IPC exerted marked protection against the potentially detrimental consequences of hepatic IR. The IR-related increases in the levels of necroenzymes were ameliorated and the activities of potential sources of ROS production, *i.e.* XOR and MPO were significantly reduced. This accorded with the lower levels of generation of inflammatory mediators, preserved liver blood flow and oxygenation. The induction of NOX2 was likewise weaker, but in contrast with expectations, the expression of NOX4, the main vascular NOX isoform, was not attenuated. In this sense, recent findings suggest that, unlike other ROS-generating NOXs, the tightly-assembled

active conformation of NOX4 cannot be disrupted by conventional means, because the membrane-bound subunit does not require interaction with the cytosolic subunits. In conclusion, these data demonstrate that the enhanced NOX4 expression after liver IR was not influenced by remote IPC, thus the protective effect of IPC does not extend to all NOX homologues - at least not to NOX4 (*Garab et al. Life Sci 2014*).

In this context an invited editorial paper (*Boros M: Microcirculatory Analysis in the Management of Sepsis - Occam's Razor or Achilles' Heel? Crit Care Med 2014*) was published on the use of microcirculatory analysis in experimental models and clinical settings (in this particular case the OTKA reference number was not provided - grant supports are not allowed to shown in editorials of the journal).

Non-occlusive mesenteric ischemia

To investigate the consequences of mesenteric IR further, we have developed a new model of non-occlusive mesenteric ischemia (NOMI). Our current knowledge of NOMI is based mostly on clinical reports or deduced from acute experimental studies involving the use of mesenteric artery occlusion. Our goal was, therefore, to develop a reliable in vivo rat model of NOMI to investigate the major components of local and systemic circulatory reactions in a clinically relevant time frame. The key elements of this model are a persistently decreased superior mesenteric artery flow after an extramesenteric insult (partial occlusion of the subdiaphragmatic aorta), the significantly impaired small intestinal microcirculation 24 hr after the insult, as shown by the decreased RBCV at both the serosal and mucosal surfaces, and a low level of invasiveness, which makes long-term observations possible.

(*Tuboly et al. Microbiol Immunol 2016*).

Detection/diagnostics

Our major goal was to investigate the biological significance of non-microbial methane (methane) generation and to provide data towards an understanding of the mechanism of anti-inflammatory action of methane or other, potentially methane-releasing compounds. Firstly, we have developed a diode laser-based photoacoustic (PS) system to measure methane gas signals with high sensitivity (in cooperation with the Dept. Optics and Quantum Electronics, Univ. Szeged), the sensitivity and specificity of the setup was validated with gas chromatography (GC). The detected changes were repeatable and reproducible; the instrument proved to be highly specific to methane with a wide dynamic range from a few ppm levels to several thousands of ppm (the minimum detectable methane concentration of the system is 0.3 ppm with 12s integration time). No cross sensitivity was found with water vapor and carbon dioxide and these data were validated in humans as well. This system is used as reliable tool for monitoring the in vivo dynamics of the methane production in our further studies in laboratory animals and humans. In our next study methane exhaled from the airways together with the amounts discharged through the skin and body orifices were following the reduction of the intestinal methanogenic flora by antibiotics. Acute endotoxemia was accompanied by an increasing emanation of endogenous methane throughout the experiments, and the use of rifaximin caused a decrease in methane output, but the production significantly exceeded the control and background values. In summary, the characteristics of stress-induced methanogenesis could be followed easily and non-invasively in the whole animal with the device, and we concluded that if nonbacterial methane was added to the bacterial production, this addition could occur at such a rate that it was impossible to detect it by the conventional techniques utilized to look for it to date. This work was followed by the next one, where exhaled methane concentration profiles were obtained in healthy humans during ergometer tests. Hemodynamic and respiratory parameters were determined and compared to the estimated alveolar methane concentration. The methane breath profiles were highly reproducible and showed

very consistent behaviour among the subjects. We have also shown changes in the breath methane level under exercise and non-exercise conditions.
(*Tuboly et al. J Breath Res 2013, Szabó A et al. J Breath Res 2015*).

Mechanisms

Our previous research data converged in suggesting that methane would be efficacious in influencing the inflammatory response, thus we investigated the *in vitro* and *in vivo* bioactive potential of exogenous methane in baseline conditions and in animal models of antigen-dependent and I/R-induced antigen-independent inflammation, with special emphasis on (**1**) microcirculatory and (**2**) mitochondrial reactions. The next series of studies were performed with in-depth biochemical investigations with matching in vivo analysis techniques to determine the magnitude of the whole body methane emission of sodium azide (NaN_3)-treated rats. The main effect of NaN_3 is the direct inhibition of the activity of the mitochondrial electron transport chain thus it can be considered a specific tool with which to study mitochondrial oxido-reductive stress. The NaN_3-induced global mitochondrial dysfunction was evidenced by hepatic ATP depletion, and a systemic inflammatory reaction in control rats. Direct in vivo evidence was obtained also for the deranged liver microcirculation, while the higher XOR and MPO activities indirectly demonstrated the impact of cytochrome c oxidase inhibition on ROS generation in several tissues. The microcirculation of the liver was observed by fluorescence intravital videomicroscopy and the dynamics of structural changes were assessed by in vivo histology with confocal laser-scanning microscopy. In this study we demonstrated that chronic NaN_3 administration was accompanied by an increasing emanation of endogenous methane throughout the entire duration of the experiments even with antibiotics, targeting the potentially methane-generating intestinal flora. Methane generation, the hepatic microcirculatory changes, and the increased tissue MPO and XOR activities were prevented by GPC treatment. We concluded that methane production in mammals is connected with hypoxic events associated with a mitochondrial dysfunction and GPC is protective against the inflammatory consequences of a hypoxic reaction that might involve cellular or mitochondrial methane generation.
(*Tuboly et al. Am J Physiol Cell Physiol 2013, Strifler et al. Magyar Sebészet 2016).*
Next, we studied the effects of normoxic methane on the respiratory activity and ROS production of mitochondria in vitro or after hepatic IR. The damage of the inner mitochondrial membrane was evidenced by the increased cytochrome c release, and the dysfunction of electron flow in mitochondrial electron transport leading to elevated ROS production. Both the dysfunction of mitochondrial electron transport chain and signs of mitochondria-related oxidative damage were effectively modified by the methane inhalation protocol. In line with this, IR-induced cytochrome c release together with ROS production and hepatocyte apoptosis were also reduced.
(Strifler et al. PLoS One 2016 (1).
We have summarized our findings in a book chapter on the current view on biotic methanogenesis. (*Tuboly E, Mészáros A, Boros M: Ch. 2. in Methanogenesis: Biochemistry, Ecological Functions, Natural and Engineered Environments. Gholikandi GB (ed) Nova Science Publishers, New York, USA, 2014).* Also, we have reviewed the nature of alternative, nonbacterial aerobic methane production, particularly in association with a hypoxia-induced mitochondrial dysfunction, and we have presented data on recent advances that support the notion that this reaction may play a role in cellular metabolism. In this review paper where we reported on the biological effects of methane and to which extent methane fulfils the criteria that characterise a gasotransmitter as proposed by Rui Wang (in Trends Biochem Sci 2014). The aim was to discuss the available information from this aspect - and we concluded that although the available data do not fully support the gasotransmitter concept, methane liberation (not through the resident flora) is linked to redox

regulation connected to hypoxic events leading to, or associated with, mitochondrial dysfunction, and may be an integral feature of cellular responses to changes in oxidative status in all eukaryotes.
(*Boros et al. J Breath Res 2015).*
In our next study the bioactivity of exogenous methane was investigated on the intestinal barrier function in an antigen-independent IR model of acute inflammation. Reperfusion significantly increased epithelial permeability, worsened macro- and microcirculation, increased the production of proinflammatory mediators, and resulted in a rapid loss of the epithelium. Exogenous normoxic methane inhalation maintained the superficial mucosal structure, decreased epithelial permeability, and improved local microcirculation, with a decrease in reactive oxygen and nitrogen species generation. More importantly, both the deformability and aggregation of erythrocytes improved with incubation of methane. This study demonstrated that normoxic methane inhalation effectively influences the epithelial component of transmucosal permeability, the early structural loss of the epithelial layer and provided evidence for the direct, beneficial effects of methane exerted in the oxidized biomembranes of erythrocytes. The improvement in microcirculation upon methane treatment is probably the net result of a complex mechanism, with reduced SOX production and less membrane damage (indicated by lower malondialdehyde levels).
(*Mészáros et al. Surgery 2017*).

Therapeutics

Our next aim was to investigate the possible therapeutic effects of potentially methane-releasing agents in animal models of mucosal damage in the GI tract. In this context we continued the characterization of the effects of GPC treatment on functional, structural and mitochondrial consequences of mucosal damage induced by non-steroid anti-inflammatory drugs (NSAIDs), including acetylsalicylic acid (ASA). ASA administration caused severe gastric mucosal lesions and significantly elevated the plasma and tissue level of inflammatory mediators with the functional lesions of mitochondria (decreased respiratory capacity and deterioration of oxidative phosphorylation). GPC pretreatment alleviated morphological injury scores and the functional impairment of mitochondria and decreased the inflammatory mediator levels.
(Strifler et al. PLoS One 2016(2).
Parallel to these studies, we have developed a novel compound from acetylsalicylic acid (ASA) and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) precursors (in cooperation with the Dept of Medical Chemistry, University of Szeged) and the novel 2-(acetyloxy)-N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-benzamide (ASA-Tris) compound exhibited strong bioactivity against inflammation and had a less harmful effect on the gastric mucosa than the original ASA. The administration of ASA-Tris resulted in sustained and prolonged anti-inflammation and anti-nociception effects in carrageenan-induced arthritis in rats and simultaneously prevented the injury of microvascular structures and the accumulation of polymorphonuclear leukocytes within the gastric tissues. Using this as a basis, we characterized the local and distant effects of ASA-Tris and ASA treatments in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colon damage. In order to draw meaningful conclusions, in vitro studies with isolated liver were incorporated into the protocol. ASA treatment led to mitochondrial dysfunction involving depressed mitochondrial electron transport and lower OxPhos capacity. The decreased electron flux was accompanied by the inhibition of the activities of respiratory chain enzymes, especially cytochrome c oxidase (Complex IV). The *in vitro* experimental data demonstrated the direct effects of ASA-Tris on mitochondria. The respiratory complex-specific H_2O_2 production was not affected by the compound, but the ASA-Tris conjugate reduced the disturbances associated with electron transport and increased the efficacy of oxygen consumption. Our results therefore suggest that ASA-Tris is

not a radical scavenger directly; rather the favorable effects are probably linked to its protective potential against oxidative biomembrane damage. This view is supported by our previous in vivo study, which showed that ASA increased gastric malondialdehyde levels in rats, which demonstrates the increased level of membrane lipid peroxidation. What is more, ASA caused an inhibition of the function of the mitochondrial respiratory chain, while the same phenomenon was not observed in the case of the ASA-Tris conjugate. This study has revealed that the ASA-Tris conjugate significantly decreased the degree of inflammatory damage in the large intestine by targeting local potentially destructive changes without inducing significant side-effects on the gastric mucosa. These investigations explain the anti-inflammatory action at the mitochondrial level and provide a mechanistic basis for the observations.
(*Varga G et al. Eur J Pharmacol 2016, Varga G et al. Inflammopharmacology 2017*).