# Microcirculatory-mitochondrial resuscitation with new anti-inflammatory therapies in experimental sepsis

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## 1. INTRODUCTION

Sepsis is a potentially life-threatening condition caused by a dysregulated host response to infection (Singer et al. 2016). Along with its progression, regulatory failure is frequently associated with a mismatch between oxygen delivery ($DO_2$), oxygen consumption ($VO_2$) and a deficit in oxygen extraction ($ExO_2$) at the cellular level. The poorly functioning microvasculature reduces the delivery of oxygen to the tissues, while the mitochondrial electron transport system (ETS) is deficient, being unable to use oxygen efficiently. These processes are closely linked and ultimately lead to microcirculatory and mitochondrial distress syndrome (MMDS), which is thought to mediate end organ damage (Spronk et al. 2014).

Experimental sepsis models can provide a good basis for the development of human therapeutics. However, an effective laboratory strategy cannot always be transferred directly to clinical practice; evidence-based guidelines thus strengthen the value of pre-clinical testing. The recently published Minimum Quality Threshold in Preclinical Sepsis Studies (MQTiPSS) criteria outline a recommended method for the design of rodent sepsis experiments (Osuchowski et al. 2018). In this scheme, it is essential to investigate the dynamics and time course of the septic response to the applied stimulus, and thus to define the time window where we can observe changes most similar to human sepsis.

Today, the main strategy for the management of sepsis is the use of respiratory and circulatory support therapies, but these cannot always improve sepsis-induced functional alterations (Armstrong et al. 2017). The adequate treatment of multiple organ failure (MOF) is one of the most challenging tasks; therefore, it is necessary to find new therapeutic strategies. In recent years, the bidirectional interaction between the microcirculation and mitochondria has become a focus of investigations, and it is increasingly apparent that the mechanisms involved in microcirculatory and mitochondrial dysfunction are different from those implicated in the development of macrohemodynamic changes.

Among the many mediators involved in sepsis, the hypoxia-sensitive endothelin (ET) system plays an important role in regulating the circulation through the vasoconstrictors $ET_A$ and $ET_{B2}$ and vasodilator $ET_{B1}$ receptors ($ET_A$-R and $ET_B$-R) (Brooks et al. 1995). The metabolites and end products of the tryptophan–L-kynurenine pathway have also been implicated in several ischemic and inflammatory disorders. Kynurenic acid (KYNA), which is a metabolite of the kynurenine pathway of tryptophan catabolism exhibits pleiotropic cell-protective effects under many inflammatory conditions (Vécsei et al. 2013). We thus

hypothesized that these mediators have potential therapeutic effects on sepsis-induced MMDS.

## 2. MAIN GOALS

Our main goal was to design novel, therapeutic anti-inflammatory maneuvers to influence the microcirculation-linked oxygen debt and to minimize the energy deficit of the organs during the septic response. Specifically, we tested the hypothesis that the manipulation of ET or KYNA systems might play an important role in the resuscitation of sepsis-induced MMDS and organ failure in experimental conditions. In this context our objectives were:

- To design and characterize a novel rodent model of intraabdominal sepsis and to examine the outcome of planned therapeutic strategies. Our aim was to characterize the time course of the septic reaction to determine the optimal timing for treatment when the changes mimic the human disease.
- The modulation of $ET_A$-R- and $ET_{B1}$-R-transmitted effects may have an influence on microcirculatory recruitment and mitochondrial respiration as well. Therefore, we wanted to examine the individual and combined effects of $ET_A$-R antagonism and selective $ET_{B1}$-R activation.
- We hypothesized that administration of KYNA and its synthetic analogue SZR-72 might be a therapeutic tool to reduce microcirculatory and mitochondrial disturbances in sepsis.

We investigated the connection between the microcirculation and mitochondrial function and the efficacy of microcirculatory and mitochondrial-targeted resuscitation in our sepsis model under clinically relevant experimental conditions.

## 3. MATERIALS AND METHODS

The experiments were performed in three in vivo studies using a rat model of intraabdominal sepsis (male Sprague Dawley rats; 350 ± 30 g; $n_{\sum}$=187). In Study 1, the long-term effects of sepsis induction were characterized to determine the clinically most relevant period of sepsis progression. In Studies 2 and 3, macro- and microcirculatory as well as mitochondrial and biochemical changes were examined. In Study 2, the effects of $ET_A$- and $ET_B$-R-targeted treatments were examined, while the roles of NMDA-R antagonist KYNA and its synthetic analogue SZR-72 were assessed in Study 3.

### *3.1 Sepsis induction, monitoring and surgical instrumentation*

Rats were subjected to fecal peritonitis (0.6 g $kg^{-1}$ feces ip) or a sham operation (sterile saline ip). The general condition of the animals was evaluated with a standardized scoring system. At the time points for sickness assessment, all animals received fluid replacement and analgesic treatment subcutaneously. At the predetermined timeline after sepsis induction, the animals were anesthetized to start organ function monitoring. A tracheostomy was performed to provide spontaneous breathing, and the right jugular vein was cannulated for continuous anesthesia, drug treatments and infusion. The left carotid artery was cannulated to record mean arterial pressure (MAP) and heart rate (HR) (SPEL Advanced Cardiosys 1.4, Experimetria Ltd., Budapest, Hungary) in all the studies. In Study 2, a thermistor-tip catheter was also positioned into the right carotid artery to measure cardiac output (CO; SPEL Advanced Cardiosys 1.4, Experimetria Ltd., Budapest, Hungary).

### *3.2 Experimental protocols*

#### *Monitoring the long-term effects of sepsis induction in Study 1*

The animals were randomly assigned to sham-operated ($n_{\sum}$=49) and septic groups ($n_{\sum}$=51), which were randomly further divided into four independent groups each (sham-operated: $n_{12h}$=13, $n_{24h}$=12, $n_{48h}$=12, $n_{72h}$=12; septic: $n_{12h}$=13, $n_{24h}$=13, $n_{48h}$=13, $n_{72h}$=12) according to a termination timeline set between 12 and 72 h. Invasive monitoring was started after 12, 24, 48 and 72 h, and blood samples were taken to characterize the main components of sepsis progression as a function of time.

#### *Treatment protocol and invasive monitoring in Study 2*

The animals ($n_{\sum}$=55) were randomly assigned into sham-operated (n=10) and septic (n=45) experimental groups. After a 30-min recovery period from anesthesia induction and baseline measurements, MAP, HR and CO monitoring was performed every 30 min for 90 min. Septic animals were randomly allocated to one or another of the following groups. The 1. septic group served as a vehicle-treated septic control and received 1 mL of saline (n=13). The 2, and 3. groups of septic animals received the $ET_A$-R antagonist ETR-p1/fl peptide (100 nmol kg $h^{-1}$; Kurabo Ltd., Osaka, Japan; n=11) (Baranyi et al. 1995) or the highly selective $ET_{B1}$-R agonist (IRL-1620; 0.55 nmol $kg^{-1}$ $h^{-1}$; Sigma-Aldrich, St. Louis, MO, USA; n=11) (Brooks et al. 1995). The 4. septic group received a combination of the $ET_A$-R antagonist and $ET_{B1}$-R agonist compounds in the same doses (n=10). All treatments were administered in a continuous 60-min iv infusion in 1 mL volume.

***Treatment protocol and invasive monitoring in Study 3***

Animals ($n_{\sum}$=32) were randomly divided into sham-operated (n=8) and sepsis (n=24) groups. Septic animals were divided further into KYNA- (Sigma-Aldrich Inc., St. Louis, Mo., USA; 160 μmol $kg^{-1}$ ip; n=8) or SZR-72- (2-(2-N,N-dimethylaminoethyl-amine-1-carbonyl)-1H-quinolin-4-one hydrochloride, synthesized by the Institute of Pharmaceutical Chemistry, University of Szeged, Hungary; 160 μmol $kg^{-1}$ ip; n=8) treated and vehicle-treated control (saline ip; n=8) groups. Treatments were performed in two steps (80 μmol $kg^{-1}$; in 1 mL $kg^{-1}$ saline each) 16 h and 22 h after sepsis induction. After surgery and a 30-min stabilization, MAP and HR monitoring was performed every 15 min for a 60-min observation period.

***3.3 Microcirculatory measurements***

After the observation period, microcirculation of the ileal serosa was monitored by intravital video microscope imaging. In Study 2, the orthogonal polarization spectral (OPS) imaging technique was used (Cytoscan A/R, Cytometrics, Philadelphia, PA). A quantitative assessment of the red blood cell velocity (RBCV) and capillary perfusion rate (CPR; perfused/non-perfused area) was performed offline with a computer-assisted image analysis system (IVM Pictron, Budapest, Hungary). In Study 3, the incident dark field (IDF) imaging system was used. Images of serosal microcirculation were captured with the CytoCam Video Microscope System (Braedius Medical, Huizen, the Netherlands), while the proportion of perfused vessels (PPV) and microvascular heterogeneity (MVH) were quantified offline by automated analysis (AVA 3.0, Automated Vascular Analysis, Academic Medical Center, University of Amsterdam).

***3.4 Metabolic, inflammatory and organ function-related markers***

After the intestinal microcirculatory measurements, a liver tissue biopsy was taken to evaluate mitochondrial respiratory functions. Tissue samples from the terminal ileum were harvested for biochemical measurements, and blood samples were taken. After tissue samplings, animals were sacrificed under deep anesthesia. Whole blood lactate levels were measured from venous blood samples (Accutrend Plus Kit, Roche Diagnostics Ltd., Rotkreuz, Switzerland) to determine metabolic imbalance. Plasma ET-1 and IL-6 levels were determined with standard ELISA kits (Biomedica Ltd., Vienna, Austria, and Cusabio Biotechnology Ltd., Wuhan, China, respectively) following manufacturer's instructions. Kidney injury was determined from plasma urea level, whereas liver dysfunction was

assessed by measuring plasma alanine aminotransferase (ALT) levels, using a Roche/Hitachi 917 analyzer (F. Hoffmann-La Roche AG, Switzerland). In Study 3, xanthine oxidoreductase (XOR) activity and nitrotyrosine content were determined from ileal biopsies with a fluorometric kinetic assay and enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI, USA), respectively.

### *3.5 Rat organ failure score assessment*

The severity of cardiovascular, respiratory, liver and kidney failure was determined with a scoring system adapted for rats (i.e. rat organ failure assessment - ROFA) considering the principles of the Sepsis-3 international consensus. ROFA values were calculated by summing up the scores in each element of the scoring system.

### *3.6 Assessment of mitochondrial respiratory function in liver homogenates*

Mitochondrial $O_2$ consumption in Studies 2–3 was assessed from liver homogenates using high-resolution FluoRespirometry (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria). Measurements were performed in a Mir05 respiration medium at 37°C. After a stable basal respiration (without exogenous substrates and ADP), complex I (CI)- and complex II (CII)-linked capacities of oxidative phosphorylation (OXPHOS I and OXPHOS II) and respiratory control ratio (RCR), an index of coupling between respiration and phosphorylation, were determined. DatLab software (Oroboros Instruments, Innsbruck, Austria) was employed for online display, respirometry data acquisition and analysis.

### *3.7 Statistical analysis*

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Normality of data distribution was analyzed with the Shapiro–Wilk test. The Friedman analysis of variance on ranks was used within groups. Time-dependent differences from the baseline for each group were assessed with Dunn's method. Differences between groups were analyzed with the Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn's method. Median values and $75^{th}$ and $25^{th}$ percentiles are provided in the figures; $P$ values $<0.05$ were considered significant.

## 4. RESULTS

### *4.1 Long-term changes in plasma ET-1 levels and oxygen extraction in Study 1*

There were no significant changes in $ExO_2$ between the sham-operated and septic animals at 12 h. In contrast, $ExO_2$ values for the septic animals started to decrease 24 h after the septic insult. In the 48$^{th}$ and 72$^{nd}$ hours, $ExO_2$ remained at a lower level in the septic animals, indicating that oxygen delivery did not completely meet the oxygen demand of tissues, although this decrease was not significant at these later time points. Plasma ET-1 concentration was significantly elevated 24 h after sepsis induction.

### *4.2 Results of Study 2 – hemodynamic changes*

In the sham-operated group, there were no significant hemodynamic changes during the observation period. Sepsis, however, was accompanied by a significant hypotension, an increase in CO and a decrease in TPR. As compared to the vehicle-treated septic group, ETR-p1/fl treatment had no effect on hemodynamics, but IRL-1620 significantly increased the MAP and TPR and decreased CO and SVI during the 90-min observation period. The combined $ET_A$-R antagonism and $ET_{B1}$-R agonism caused significant increases in MAP and TPR values as compared to the baseline and to the respective values of the vehicle-treated sepsis group.

### *4.3 Changes in oxygen dynamics*

The 24-h septic period caused significant changes in oxygen dynamics, including an increase in $DO_2$, deterioration of $VO_2$, and reduced $ExO_2$ values and $PaO_2/FiO_2$ ratio as compared to the sham-operated group. Lung injury, characterized by the $PaO_2/FiO_2$ ratio, was significantly lower in the septic animals, and the ETR-p1/fl treatment or IRL-1620 treatment did not influence this change. $VO_2$ and $ExO_2$ were significantly higher after ETR-p1/fl treatment as compared to the non-treated sepsis group. A significant increase in $ExO_2$ was observed in response to IRL-1620 treatment compared to that of the vehicle-treated sepsis group. When these treatments were combined, the oxygen dynamics parameters showed a statistically non-significant trend of improvement as compared to the vehicle-treated sepsis group.

### *4.4 Metabolic changes and organ dysfunctions*

Plasma ALT activity and urea levels were significantly higher in the non-treated septic group as compared to the sham-operated animals. The ETR-p1/fl treatment or IRL-1620 treatment did not influence the sepsis-induced changes in plasma ALT or urea levels.

However, when the $ET_A$-R antagonists and $ET_{B1}$-R agonists were combined, the plasma ALT level was significantly lower than in the vehicle-treated septic group. Likewise, polymicrobial sepsis was associated with significantly increased lactate levels in the saline-treated sepsis group. In response to the ETR-p1/fl and the combined treatments, the lactate values were not different from those of the sham-operated group.

### *4.5 Microcirculatory changes*

The 24-h septic insult caused significant microcirculatory deterioration in the intestinal serosa. The CPR reached 55%, while RBCV was approx. 60% lower than those in the sham-operated animals. When compared to the vehicle-treated sepsis group, ETR-p1/fl therapy caused a significant improvement in CPR, but not in RBCV. Agonism of the $ET_{B1}$-R with IRL-1620 caused a slight, non-significant perfusion recovery without affecting changes in RBCV. A combination of the ETR-p1/fl and IRL-1620 treatments, however, was effective in restoring both parameters of the intestinal microcirculation.

### *4.6 Changes in inflammatory markers*

In the septic animals, plasma IL-6 and ET-1 levels reached higher values compared to those of the sham-operated group. IRL-1620 did not influence the sepsis-induced elevations in IL-6 and ET-1, but ETR-p1/fl treatment caused a significant reduction in plasma ET-1 level. The combined therapy significantly reduced the sepsis-induced elevation in plasma ET-1 and reduced IL-6 values as well ($P$=0.051).

### *4.7 Changes in mitochondrial respiration and membrane integrity*

Intra-abdominal sepsis significantly decreased substrate oxidation as compared to the sham operation. Compared to the vehicle-treated sepsis group, the $ET_A$-R antagonist and $ET_B$-R agonist therapies resulted in an increasing tendency in oxygen flux, and this change was statistically significant after the combination of therapies. As a result of septic insult, both OXPHOS capacity and RCR values were significantly lower than in the sham-operated animals. Compared to the non-treated sepsis group, ETR-p1/fl treatment preserved both CII RCR and CII-linked OXPHOS values, and an increasing trend was observed in CI-linked respiration. Treatment with IRL-1620 resulted in a slight, non-significant amelioration in CI- and CII-linked OXPHOS capacity. In response to the combined treatment, CI- and CII-linked OXPHOS as well as RCR values were significantly increased. Sepsis resulted in significant disintegration of the outer membrane based on the effect of exogenous

cytochrome c, but preserved membrane integrity was observed in the animals subjected to the combined treatment.

### *4.8 Results of Study 3 – hemodynamics and oxygen dynamics*

Sepsis resulted in significant hypotension during the observation period, which was not altered by the treatments. As compared to the sham-operated animals, a decreased $PaO_2/FiO_2$ ratio was observed in the vehicle-treated sepsis group, while no significant changes were found in the other groups. Sepsis reduced $ExO_2$ values as compared to the sham-operated group, whereas both KYNA and SZR-72 resulted in a significant improvement in this parameter.

### *4.9 Changes in metabolic and organ dysfunction markers*

In the vehicle-treated sepsis group, plasma urea levels significantly increased, but urea levels in both treated groups were similar to those seen in the sham animals. Hepatic cellular damage was evident in the vehicle-treated and SZR-72-treated sepsis groups, whereas this ratio did not differ between the sham-operated and KYNA-treated animals. Compared to the sham-operated group, all of the groups challenged with sepsis showed a similar extent of elevation in blood lactate levels. The ROFA score was significantly higher in the vehicle-treated and SZR-72-treated sepsis groups than in the sham-operated group. The ROFA values in the KYNA-treated group were not significantly different from those in the sham-operated group.

### *4.10 Changes in inflammatory and oxidative/nitrosative stress markers*

Sepsis led to significant elevations in ET-1, IL-6, nitrotyrosine levels and XOR activity. All of these parameters remained, however, at the levels seen in the sham group in both the sepsis+KYNA and sepsis+SZR-72 groups.

### *4.11 Microcirculatory changes*

Sepsis-induced microcirculatory perfusion disorders manifested in lower levels of capillary perfusion and increased perfusion heterogeneity as compared to those in the sham-operated group. The values of these parameters did not differ between the sepsis and sepsis+SZR-72 groups and between the sham-operated and sepsis+KYNA groups. KYNA was significantly more effective in ameliorating sepsis-related changes than SZR-72.

### *4.12 Changes in mitochondrial respiration*

Baseline respiration without external substrate and respiration following the oxidation of complex I- and complex II-linked substrates were significantly decreased in sepsis. KYNA

administration did not modify sepsis-induced changes in these parameters. In this respect, treatment with SZR-72 preserved mitochondrial respiration with and without NADH- and $FADH_2$-linked substrates. In addition, sepsis significantly decreased complex I- and II-linked OXPHOS. Both KYNA and SZR-72 increased complex II-linked OXPHOS capacity, while SZR-72 was able to restore complex I-linked OXPHOS completely. As a result of septic insult, CI-RCR and CII-RCR were markedly decreased. These parameters were significantly improved by KYNA and completely reversed by SZR-72 treatment.

## 5. DISCUSSION

### *5.1 Overview of sepsis pathophysiology – evaluation of the model and sepsis-induced organ failure*

In our studies, analgesia, proper fluid resuscitation, control of temperature and humane endpoints were employed according to the MQTiPSS guidelines (Osuchowski et al. 2018). In Study 1, the inflammatory immune response was manifested in distinctive, time-dependent increases in plasma ET-1 levels in parallel with the decreasing $ExO_2$. These changes were most pronounced at 24 h after the induction of sepsis, suggesting that the septic reaction in rats is most severe in this time point. Therefore, a test of any treatments should be done around 24 h after induction in this model. Sepsis-induced organ injuries were characterized by our developed ROFA scoring systems. The numerical parameters of cardiovascular, pulmonary, kidney and liver functions established the onset of the septic reaction.

#### *Role of microcirculation*

Restoration of tissue perfusion and subcellular oxygen utilization are fundamental goals in sepsis therapy (Balestra et al. 2009). Vasodilator manipulation of the microcirculation can open the shunted or closed microcirculatory units and improve the imbalance between tissue oxygen supply and utilization. However, several studies on vasodilator therapies have shown conflicting results (Boerma et al. 2010; Trzeciak et al 2014). Therefore, the ideal sepsis therapy could increase the systemic driving pressure parallel to the opening of insufficient microcirculation through vasodilation.

#### *Importance of mitochondria in sepsis*

The decisive role of mitochondrial dysfunction leading to oxygen utilization disorders has been demonstrated in various models of sepsis. In these studies, a considerable decrease in substrate- and ADP-stimulated respirations was accompanied by a decrease in RCR after

sepsis insult (Arulkumaran et al. 2016). An increase in mitochondrial respiratory oxygen flux after exogenous cyt c administration following OXPHOS stimulation demonstrated that the outer mitochondrial membrane was injured in the septic animals (Bar-Or et al. 2018). This change in membrane integrity may contribute to reduced ADP-ATP conversion coupled to the electron transport system resulting in decreased RCR values. Due to the fact that a marked elevation in lactate was present, it may well be that a metabolic switch from mitochondrial oxidative phosphorylation to glycolysis was present (Bkaily et al. 2008). Although an $O_2$-independent glycolytic pathway for ATP production has been documented, this alternate route may not be effective for energy production in sepsis. Taken together, all these processes are intimately involved in the progression of sepsis and contribute to MOF.

### *5.2 Endothelin receptor-targeted therapies in sepsis. Circulatory effects of treatments. Effects of ET-A receptor antagonist treatment*

We examined the $ET_A$-R antagonist therapy on the macro- and microhemodynamics and the main components of the cellular energy-providing mechanism. In this condition, the $ET_A$-R antagonist ETR-p1/fl peptide effectively improved oxygen dynamics and the splanchnic microcirculation, and displayed a tendency toward amelioration of ROFA score values. Selective blockage of the $ET_A$-R has an anti-inflammatory effect, and previous studies have already shown potentially beneficial vasodilator effects of $ET_A$-R antagonism in circulatory shock and sepsis (Gulati et al. 2016).

### *Effects of endothelin-B receptor agonist treatment*

Administration of IRL-1620 induced a systemic pressure response in the hypotensive septic animals with increased total peripheral resistance and improved oxygen dynamics. The intestinal capillary perfusion was also improved in this case. The beneficial effects of $ET_B$-R agonism have already been demonstrated in CNS disorders and in the peripheral circulation (Gulati et al. 2016; Matsuura et al. 1996). Administration of IRL-1620 also leads to tissue-dependent vasodilation or vasoconstriction; it causes a transient decline in MAP, followed by a long-lasting pressure effect (McMurdo et al. 1994). There are two functional subtypes of $ET_B$-Rs: $ET_{B1}$-Rs are expressed by the vascular endothelium and mediate nitric oxide-dependent vasodilatation, while $ET_{B2}$-Rs have a long-lasting vasopressor effect possibly due to dominant $ET_A$-R activity. Only the $ET_{B1}$-R is IRL-1620-sensitive, while the $ET_{B2}$-R is IRL-1620-insensitive (Brooks et al. 1995). IRL-1620 improved the ileal capillary

perfusion rate due to its local vasodilator effect or possibly through the increased microcirculatory driving pressure gradient as well.

***Effects of combined endothelin-A receptor antagonist and endothelin-B receptor agonist therapy***

Combining the treatments resulted in an additive effect as compared to ETR-p1/fl and IRL-1620 treatment along the global oxygen supply–demand imbalance, and the microcirculatory parameters improved further. As a final result, organ dysfunction was significantly attenuated. Our combined $ET_A$-R antagonist and $ET_{B1}$-R agonist treatment design has targeted this goal. Our results suggest that if microcirculatory failure occurs, the specific inactivation of vasoconstrictor $ET_A$-Rs can amplify the vasomodulator effects of circulating ET-1 through the $ET_B$-Rs, leading to a potentially beneficial outcome at the subcellular level. ETR-P1/fl is an intramolecular complementary peptide of the $ET_A$-R and can specifically bind and block ET-1 in the circulation. In addition, activation of $ET_{B1}$-R stimulates the reuptake of circulating ET-1, which also results in reduced ET-1 levels (Davenport et al. 2016; Baranyi et al. 1998).

***Mitochondrial effects of ET receptor-targeted therapies***

In this scenario, the sepsis-associated decrease in substrate oxidation and OXPHOS was ameliorated by $ET_A$-R antagonist treatment. Moreover, the coupling between respiration and phosphorylation was improved, and the functional damage to the outer membrane was also mitigated. The presence of $ET_B$-Rs in the nuclear membrane has recently been demonstrated, suggesting more complicated intracellular mechanisms, such as opening of calcium channels and $Na^+/Ca^{2+}$ exchangers, which can establish a complex intracellular $Ca^{2+}$ homeostasis, and mitochondria might be part of this mechanism (Elustondo et al. 2015). Accumulation of $Ca^{2+}$ may lead to OXPHOS failure and a depolarization of the inner mitochondrial membrane, which can lead to cell death. ET-1 was found to increase the cytosolic $Ca^{2+}$ transient and mitochondrial ROS production and to enhance the consumption of ATP. Inhibition of $ET_A$-Rs markedly decreased mitochondrial $Ca^{2+}$ deposition and attenuated abnormalities in $Ca^{2+}$ sequestration (Brunner et al. 2002; Boveris et al. 2007). Interestingly, IRL-1620 alone did not significantly affect the mitochondrial respiratory function, but supplementation with the $ET_A$-R antagonist resulted in a marked improvement in ADP-stimulated respiration. The direct mitochondrial effect of ET-1 on elevated

mitochondrial ROS production has been reported previously (Yuki et al. 2001), and the presence of ET receptors has only been confirmed in the nucleus to date.

### *5.3 Effects of kynurenic acid and SZR-72 on septic organ failure*

KYNA and SZR-72 treatments reduced lung and kidney dysfunctions to a similar extent due to the decreased level of inflammatory markers and their enhanced antioxidant/anti-nitrosative effect. However, only KYNA treatment reduced hypoxia-sensitive ET-1 levels, as well as displaying a tendency toward amelioration of liver injury and ROFA score values.

#### *Effects of kynurenic acid and SZR-72 on ileal microperfusion*

We observed that both KYNA and SZR-72 prevented a sepsis-induced decrease in oxygen extraction to the same extent, but only KYNA ameliorated sepsis-related microcirculatory perfusion deficit. It has been shown that KYNA increases global and cortical renal blood flow under physiological conditions and reduces renal oxidative stress during ischemia–reperfusion injury (Badzynska et al. 2014; Pundir et al. 2013). Antagonism of NMDA receptors expressed on the surface of smooth muscle cells results in smooth muscle relaxation. On the other hand, a reduction of the levels of IL-6, XOR activity and vasoconstrictor ET-1 release by KYNA may attenuate the cytokine- and ROS-induced vasoconstriction of microvessels. Taking into account that KYNA is an endogenous ligand for GPR35 (Turski et al. 2013) and that only KYNA (but not the synthetic analogue) was able to restore the perfusion disturbances in the ileum, a GPR35-mediated mechanism cannot be ruled out.

#### *Effects of kynurenic acid and SZR-72 on mitochondrial respiration*

Ameliorating effects of SZR-72 on mitochondrial function were more pronounced compared to KYNA, which was able to improve CI OXPHOS. Previous studies with the KYNA analogue demonstrated a more pronounced anti-inflammatory response than with KYNA, but both KYNA and SZR-72 reduced oxidative/nitrosative stress marker levels and stimulated the Nrf2-antioxidant response pathway (Kaszaki et al. 2012; Ferreira et al. 2018). Administration of SZR-72 resulted in a remarkable increase in CI–II OXPHOS, and RCR was found in liver homogenate after sepsis induction. Physico-chemical properties of KYNA and SZR-72 are different. SZR-72 is possibly able to cross the blood–brain barrier and therefore may affect intracellular signaling. More recently, NMDA-Rs were shown to be present in the inner mitochondrial membrane (mtNMDA-R), where they may play a regulative role in $Ca^{2+}$ transport, ROS production and metabolic switching during hypoxia

(Nesterov et al. 2018). Under these circumstances, it may well be that SZR-72 has a much higher affinity for either plasma membrane or mtNMDA-R than KYNA.

## 6. SUMMARY OF NEW FINDINGS

1 We have designed and characterized a clinically-relevant rat model of intraabdominal sepsis, with organ failure and complex macro-/microcirculatory and mitochondrial dysfunction.

2 ET receptors could indirectly influence mitochondrial function through the mechanism of tissue perfusion and restoration of the intracellular oxygen supply. The selective $ET_B$-R agonist countervailed peritonitis-induced hypotension, while the $ET_A$-R antagonist maintained microcirculation and oxygen dynamics. A mixed ET receptor-targeted treatment regime may offer a novel possibility for a simultaneous microcirculatory and mitochondrial resuscitation strategy by also reducing circulating ET-1 levels and ameliorating inflammatory indices of sepsis.

3 Treatments with KYNA and its synthetic analogue attenuated the deleterious consequences of oxidative/nitrosative stress and resulted in lower inflammatory mediator release. Administration of SZR-72 may directly regulate mitochondrial respiration and ATP synthesis, whereas treatment with KYNA primarily ameliorates microcirculatory dysfunction and therefore restores organelle function.

4 Despite compartmentalization, the microcirculatory and mitochondrial functions are closely linked under physiological circumstances. The common denominator in both mechanisms may be the capillary-mitochondrial oxygen gradient, which may be a decisive factor in mitochondrial function in sepsis. Therefore, the efficacy of microcirculatory resuscitation therapies should be apparent at the subcellular level as well.

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## LIST OF FULL PAPERS RELATED TO THE SUBJECT OF THE THESIS

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II. Juhász L, **Rutai A**, Fejes R, Tallósy SP, Poles MZ, Szabó A, Szatmári I, Fülöp F, Vécsei L, Boros M, Kaszaki J. Divergent Effects of the N-Methyl-D-Aspartate Receptor Antagonist Kynurenic Acid and the Synthetic Analog SZR-72 on Microcirculatory and Mitochondrial Dysfunction in Experimental Sepsis. Front Med (Lausanne). 2020 Nov 27;7:566582. doi: 10.3389/fmed.2020.566582. PMID: 33330526; PMCID: PMC7729001 **IF: 5.091**

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