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The Role of ATP-$MgCl_2$ in Ischemia-Reperfusion and Sepsis

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"If we knew what we were doing it wouldn't be research." - Albert Einstein

1. INTRODUCTION AND CURRENT STATE

1.1. Definitions and pathophysiology of ischaemia-reperfusion and sepsis

Ischemia is defined as absolute or relative insufficiency of oxygenated blood supply to tissues resulting in cellular dysfunction. The term ischemia reflects shortage of oxygen as well as other blood components but also the limited disposal of cellular metabolism products that accumulate and may cause cellular injury. ([1]) When ischemia is corrected before irreversible alterations occur, blood flow returns providing oxygen and nutrients supply, removal of toxic products and progressive reestablishment of energy metabolism and normal cellular functions. ([2]) However, this is not a straightforward process as cellular injury is further exacerbated by the reintroduced blood flow and oxygen to the damaged tissues. Parks & Granger reported that tissue lesions during reperfusion were actually greater than those ensuing during ischemia. ([3]) In fact, tissue injury was maximal in areas with the greatest blood flow during reperfusion. This phenomenon has been termed the oxygen paradox ([4]) or reperfusion injury (I-R).

The I-R injury provokes complex interactions between the endothelium and blood cells, leading to microvascular injury, cellular apoptosis and necrosis. ([5]) Local alterations in vascular tone, permeability, complement and platelet activation, adhesion of polymorphonuclear neutrophils (PMNs), and the release of inflammatory substances with formation of both nitrogen-derived and oxygen-derived free radicals (ROS) lead to systemic inflammatory response and if severe enough to circulatory shock and death. ([6,7])

Sepsis is characterized by a failure of local inflammatory mechanisms to contain infective pathogen resulting in local and distant tissue damage and systemic activation of inflammation and coagulation. If untreated multi-organ dysfunction and death ensue. Although the principles of treatment such as source control, timely broad-spectrum antibiotics and intravenous fluid resuscitation are well established mortality from sepsis remains high.

After invading the host pathogenic bacteria and their toxins (e.g. lipopolysaccharide – LPS) interact with patient's immune system via pathogen-associated molecular pattern receptors (PAMPs) e.g. Toll like receptors. This leads to a pattern recognition activation of the innate immune system, synthesis of inflammatory cytokines by monocytes, macrophages and PMN extravasation leading to amplification of inflammation. ([8])

Simultaneously, the innate immune system interacts with the acquired immune system to mount a dynamic, sustained, and regulated response towards clearing foreign pathogen. The cytokine milieu produced by the innate immune cells plays an essential role in this process.
Endotoxemia is the presence of LPS, cellular wall component of Gram-negative bacteria, in blood stream. LPS is a crucial molecule in the induction of pathological events during sepsis, particularly, but not exclusively that of gram-negative origin and represents the most widely studied PAMP. ([8, 9]) Its interaction with the complex of CD14 and Toll like receptor 4 (TLR4) on the surface of macrophages or mononuclear blood cells triggers innate immune responses. ([10]) The downstream effects lead to activation of nuclear factors (e.g. NFκB, NF-AT) and transcription of genes involved in inflammatory response, e.g. TNFα, IL-1β, IL-6, IFNγ and IL-10.
The complex effects of inflammation on endothelium, coagulation cascade and neuroendocrine system result in vascular dysfunction of sepsis. Decreased capillary density and perfusion heterogeneity develop in the microcirculation. ([11]) Overall tissue perfusion deficit and microvascular I-R exists and are particularly prominent in the hepatosplanchnic region. Together with mitochondrial deficit in oxygen utilization, termed cytopathic hypoxia ([12]), they lead to the development of multiple organ failure in sepsis. Maintenance of perfusion pressure and recruitment of non- or malperfused capillaries is regarded as a major goal of resuscitation from septic shock. Typically fluids and vasopressors are infused to maintain perfusion pressure although vasopressors might further impair the microcirculatory flow. ([13]) Other drugs were investigated for the potential to improve microcirculation in sepsis: dobutamine, ([14]) dopexamine, ([15]) milrinone, ([16]) nitroglycerin, ([17]) and levosimendan ([18]) and in practice often both vasopressors and inodilators are employed together. ATP-$MgCl_2$ is a potent arteriolar vasodilator and inotrope theoretically fulfilling the desired quality of an agent leading to improved blood flow and recruitment of microcirculation. ([19]) Furthermore its reported salutary effects on cellular energy balance and cellular function in different shock models seemed as an attractive choice in the treatment of sepsis and I-R.

1.2 ATP in ischemia-reperfusion and sepsis

1.2.1 Role of intracellular ATP

Adenosine triphosphate (ATP) is a crucial molecule for life. It transfers energy gained during the degradation of nutrients to processes that maintain cells and organism alive. In humans ATP is covering almost 95% of body

energetic needs and is produced in a daily amount approximating the individuals' body mass (~ 1 mmol kg^{-1} min^{-1}) at rest. Under normal conditions ATP production is well adjusted to the cellular needs. This balanced situation is disturbed by I-R injury. ([20]) Cellular functions dissipate, the membrane potentials are no longer maintained, intracellular calcium levels rise and cells swell. Adenosine diphosphate (ADP) and adenosine monophosphate (AMP) levels increase transiently as ATP is used but their levels similarly fall as further breakdown to adenosine occurs. During reperfusion ATP levels are incompletely replenished and may deteriorate further. ([21]) The nucleotide precursors of ATP are being recycled via the purine salvage pathway and rarely synthesized de novo as that is an ATP demanding process. It has been suggested that the rate limiting step in ATP re-synthesis is the loss of diffusible adenine nucleotide metabolites as these purine precursors are necessary to fuel adenine nucleotide salvage pathways and the provision of external AMP or ATP during reperfusion was suggested to help replete the intracellular ATP. ([22, 19])

1.2.2 Role of extracellular ATP

Recent studies established ATP release into the extracellular space as a widespread physiological process. ([23,24]) ATP also leaks from cells through the plasma membrane damaged by inflammation, ischemia, and mechanical injury. ATP acts in autocrine, paracrine or endocrine fashion to modulate various bodily functions. ATP and other nucleotides exercise their effects via two large families of extracellular receptors for nucleotides and nucleosides, the'purinergic' P1or P2 receptors. ([25]) Compared to P2 the P1purinoceptors (A1, A2a, A2b, A3) are more responsive to AMP and adenosine than to ATP and ADP, are blocked by methylxanthines, and do in general act via adenylate cyclase.

The P2X receptors are ligand-gated ion channels responsive to ATP. This family comprises seven receptors (P2X1 through P2X7) and accounts for fast neurotransmission as well as sympathetic control of vascular tone. ([26, 27])

From the perspective of ATP-$MgCl_2$ the P2X7 receptor deserves particular mention as it acts as an ATP-activated ion channel but also forms a pore, gating passage of molecules up to 1 kDa in response to ATP. ([28]) This unique receptor plays an important role in cells involved in immunological and inflammatory responses for it is crucial in the process of IL-1β, IL-18 release, caspase-1 activation and apoptosis. ([48])

The P2Y receptors are G protein-coupled receptors that are categorized into a subfamily of receptors (P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11) coupling to Gq (activating phospholipase C) or into a family of Gi-coupled receptors (P2Y12, P2Y13, P2Y14) that usually inhibit adenylyl cyclase. ([29])

Purinoceptors are variably expressed in different organs and under various physiologic conditions, and the same receptor may trigger different responses dependent on the site of activation. For example, ATP released from erythrocytes in response to hypoxia has vasodilator properties via P2Y1 receptor stimulation thereby inducing the formation of PGI_2 and NO. ([30]) Nevertheless, when released from sympathetic nerves upon stimulation ATP may cause brief vasoconstriction. ([31])

1.2.3 Role of extracellular adenosine

Adenosine is a common degradation product of ATP, ADP, AMP and cAMP. Extracellular adenosine can either be degraded via adenosine deaminase (ADA) into inosine or transported via nucleoside transporters across the plasmatic membrane and salvaged via adenosine kinase for the rebuilt of high-energy phosphates. ([32]) Under normal conditions only small amounts (nano-moles) of adenosine are present outside of cells where they stimulate two out of four adenosine receptors A1 and A2a. ([33]) Ischemia and sepsis are associated with increased levels of ATP degradation products and adenosine. ([34, 35]) Under conditions of stress extracellular concentrations of adenosine reach micro-molar concentration and can stimulate all P1 receptors. Adenosine is thought to mediate regional vascular reactivity in response to ischemia and also replenishes intracellular nucleotide pool. It exerts strong and prolonged anti-inflammatory effect via the activation of adenosine A2a, A2b, and possibly A3 receptor. ([36]) The provision of ATP-$MgCl_2$, as will be discussed further, may partially act via adenosine receptors, reducing the cellular and tissue damage caused by I-R. Similar to the effect of ATP-$MgCl_2$, adenosine was reported to inhibit the production of TNFα, IL-6, IL-8 and IL-12, while enhancing that of IL-10 human monocytes activated by LPS. ([37, 38]) In critically ill patients adenosine levels are markedly increased, particularly in sepsis and septic shock and correlate well with the severity and outcome. ([35])

1.3 Role of magnesium in ischemia-reperfusion and sepsis

Magnesium (Mg), called the “energy ion”, is the second most abundant intracellular cation serving as a cofactor for more than 300 enzymes, some of which catalyse oxidative phosphorylation, activate energy storage and reactions of the “purine salvage” pathway. Hypomagnesemia, mainly secondary to gastrointestinal and renal losses, is common in clinical syndromes associated with I-R injury and sepsis and correlates with higher mortality in the critically ill. Intracellular free Mg^{2+} ions are natural Ca^{2+}

antagonists that are potentially capable to influence undesirable Ca^{2+} influx during I-R and septic shock. ([39]) The calcium antagonistic properties of Mg^{2+} ions are also responsible for modulation of vascular tone and immune functions including macrophage activation, lymphocyte proliferation, leukocyte adherence and bactericidal activity. ([40]) Furthermore, Mg modulates binding of agonists to purinergic P1 and P2 receptors. ([41,42]) Recent work also documented that $MgCl_2$ at milimolar concentrations non-competitively blocks the crucial inflammatory and "death" receptor P2X7. ([43]) Importantly, when $MgCl_2$ was complexed with ATP the beneficial effects of ATP were enhanced in a large number of experimental conditions. ([19]) Whether this effect could be attributed to the P2X7 receptor blockade or other plausible mechanism remains to be determined.

1.4 Pharmacology of ATP-$MgCl_2$

Although there is clear evidence that exogenously administered ATP may enter the intracellular space across the cell membrane ([44]) only in studies performed in isolated organs the extracellular concentrations of ATP-$MgCl_2$ were high enough (mM) to explain its beneficial effects simply due to supplementation of adenosine or adenosine nucleotides. ([45, 46]) By contrast the *in vivo* doses of ATP-$MgCl_2$ (μM) suffice to allow for purinoceptor activation. ([47]) Although it is not known which particular P2 and/or P1 receptors might be activated by ATP-$MgCl_2$, it is reasonable to assume that it would not be different from natural ATP, which is complexed with Mg^{2+} apart from the fact that $MgCl_2$ might in fact be in excess and lead to blockade of the P2X7 receptor ([43]) potentially preventing the production of the major pro-inflammatory cytokines IL-1β and IL-18 as well as caspase-1 activation and immune cell apoptosis during ATP-$MgCl_2$ treatment. ([48])
Similarly to ATP, ATP-$MgCl_2$ is degraded in the blood compartment to ADP, AMP and adenosine by soluble ectonucleotidases, as well as membrane bound ectoapyrase/ecto-ATPase (CD 39), ecto-ADPase and 5´ectonucleotidases (CD 73) on endothelial and blood cells. ([49]) The activition of the P1 and P2 purinergic receptors will therefore be influenced by the interplay of ATP-$MgCl_2$ metabolising enzymes, nucleoside transporters and purinergic receptor expression.
Reports studying the kinetics of intravenous ATP-$MgCl_2$ administration *in vivo* have confirmed that both P1 and P2 receptors are likely to be activated. The intravascular half-life of adenine nucleotides was calculated to be in the range of 0.2 s in blood perfusing the lung, and 10-15 min in whole blood ex vivo. ([50]) The extensive variety of nucleotides fate *in vivo* explains many of the paradoxes observed with nucleotide's pharmacodynamics. The known

ATP dual dose-dependent effects on heart rate serves as an example. In small doses ATP produces tachycardia while relatively larger doses slow the heart and induce atrioventricular nodal conduction block. ([51])

1.5 Previous experimental studies combining ATP with $MgCl_2$

1.5.1 Role of ATP-$MgCl_2$ in animal models

ATP-$MgCl_2$ has been used in animal models of hemorrhagic shock, ischemia-reperfusion and sepsis as well as in the few published clinical human studies. As synthesis of ATP was recognized as a major limiting factor of recovery during shock or ischemia ([52]) most of the published studies have been based on the premise of supplementing "energy" to ischemic tissues upon reperfusion or resolution of shock in the form of ATP and adenine nucleotides. However, other mechanism such as enhancement of mitochondrial activity and efficiency, ([53]) improvement of endothelial function, ([54]) reduction of cytokine production ([55]), hemodynamic improvement based on systemic and regional vasodilator effect, ([56, 57, 58, 59]) and improved cardiac performance ([60]) have been proposed as well and are likely to be explained by activation of the purinergic receptors.
In relation to the presented work the following studies are worth mentioning. In a model of aortic cross-clamping in rabbits ATP-$MgCl_2$ prevented paraplegia and histological damage of the spinal cord and in two rodent models of caecal ligation - puncture (CLP) peritonitis resuscitation with ATP-$MgCl_2$ restored reticuloendothelial system function, increased lymphocyte proliferative response and preserved endothelial cell function as well as vascular reactivity all of which was associated with improved survival. ([61, 62, 63, 64])

1.5.2 Overview of ATP-$MgCl_2$ use in humans

Despite the large amount of experimental data, human studies using ATP-$MgCl_2$ in the treatment of shock states are surprisingly scarce, which may relate to undesired side effects (A-V block, hypotension) when high doses of ATP-$MgCl_2$ are administered too fast. ([65]) Other possibility might be either the occurrence of potentially unreported side effects e.g. cardiac ischemia, hypoxia or lack of interest from pharmaceutical companies as ATP-$MgCl_2$ is

not a patent protected compound. Nevertheless, I will briefly review the results of published human studies with intravenous ATP-$MgCl_2$.
Appropriately titrated infusion of ATP-$MgCl_2$ in human volunteers (ATP 0.1-0.4 mg kg^{-1} min^{-1} and $MgCl_2$ 0.033-0.133 mg kg^{-1} min^{-1}) increased cardiac output without changing mean arterial pressure. The increased stroke volume and tachycardia attributed to reflex sympathetic nervous stimulation more than compensated for the fall in systemic vascular resistance. ([66])
ATP-$MgCl_2$ was also infused into the main pulmonary artery in children undergoing preoperative evaluation of pulmonary hypertension secondary to congenital heart disease. The mean pulmonary artery pressure decreased while pulmonary blood flow increased without any change in blood pressure or heart rate. ([67,68]) In the same population ATP-$MgCl_2$ also decreased pulmonary artery pressure during postoperative course without any side effects or rebound pulmonary hypertension. ([69]) In patients during coronary flow reserve measurement ATP-$MgCl_2$ displayed systemic hemodynamic effects similar to adenosine but the frequency of undesired side effects (A-V block, facial flushing, burning sensations in the chest) was diminished with ATP-$MgCl_2$. ([70,71])
In patients suffering from acute ischemic renal failure ATP-$MgCl_2$ infusion for 90-120 minutes improved kidney function. ([72]) This uncontrolled study prompted a prospective, randomised controlled trial in 30 patients with ischemic acute renal failure. ATP-$MgCl_2$ reduced the duration of extracorporeal renal support and increased survival. ([73]) In contrast, ATP-$MgCl_2$ did not improve survival in a study of 88 patients with multiple organ failure despite suggested improvement in mitochondrial redox potential. ([74])

2. AIM OF THE STUDIES

I. To investigate the effects of ATP-$MgCl_2$ during thoracic aortic clamping with particular emphasis on hepato-splanchnic and renal oxygen exchange and metabolism.
II. To investigate the potential of ATP-$MgCl_2$ during hyperdynamic porcine endotoxemia with particular emphasis on hepato-splanchnic oxygen exchange and metabolism.
III. To assess the effects of ATP-$MgCl_2$ degradation product adenosine on cytokine production in LPS stimulated whole human blood from healthy volunteers.
IV. To assess the effects of ATP on cytokine production in LPS stimulated whole human blood from healthy volunteers.

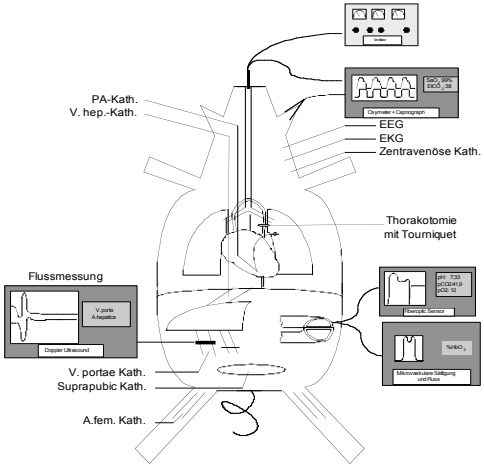
3. METHODOLOGICAL CONSIDERATION

All animal studies were performed in the Section of Pathophysiology and Process Development in Anesthesia, University Hospital, Ulm, Germany. The *ex vivo* whole blood culture study was performed in the Department of Intensive Care, Nepean Hospital, University of Sydney, Penrith, New South Wales, Australia. The details of experimental protocols and methods used, except for the aortic cross clamp study, are described in the respective original publications. The animal experiments were performed in adherence to National Institutes of Health Guidelines on the Use of Laboratory Animals. University Animal Care Committee and the federal authorities for animal research (Baden-Wurttemberg, Germany) approved the study protocols.

3.1 Porcine model of I-R injury induced by high thoracic aortic clamping

Healthy domestic pigs (n. 24) were anesthetized, mechanically ventilated and instrumented. The animals were randomized into three groups. Eight animals each into sodium nitroprusside (SNP) and ATP-$MgCl_2$ group, respectively. Six animals were assigned SNP plus esmolol treatment. The experimental setup is depicted in the following schema. The descending aorta tourniquet was inserted via left lateral thoracotomy and placed carefully so that clamping was atraumatic. A two-way ileostomy was formed via midline laparotomy and was covered to prevent drying of the mucosal surface.

Figure 1.



Baseline measurements were recorded after one-hour rest period post instrumentation. The infusion of SNP, ATP-$MgCl_2$ or esmolol was started just prior to aortic cross-clamping. When the MAP decreased to less then 75 mmHg the descending thoracic aorta was occluded by the pre-implanted tourniquet for 30 minutes. The study drug was titrated against the proximal MAP (pMAP) as follows:

SNP	8- 10 µg/ kg/ min
ATP-$MgCl_2$	5-10 µg/ kg/ min
SNP + Esmolol	130-260 µg/ kg/ min.

The drug infusion was stopped 1 minute prior to thoracic aortic clamp release in the case of ATP-$MgCl_2$ and 5 and 10 minutes in the case of SNP and esmolol, respectively. We recorded respiratory, cardiovascular, renal, and hepatosplanchnic function and metabolic parameters prior to de-clamping to assess the extent of ischemic damage. We observed the animals for further 4 hours and collected blood and measurements at 2 and 4 hours post de-clamping to assess the effects of the three regimes on I-R injury. The animals were then sacrificed under anaesthetic by an injection of KCl. All measured and derived values are expressed as median and 25 – 75 percentile. The intragroup and intergroup differences relative to pre-clamp measurements were analysed with Friedmann ANOVA and Kruskall-Wallis variance-test, respectively. The p value less than 0.05 was considered significant.

3.2 Long-term hyperdynamic porcine sepsis model

The key features of this model are hyperdynamic normotensive hemodynamics without hypovolemia and multiple organ dysfunction with

sepsis-like hypermetabolism as well as microcirculatory changes. ([75, 76]) The model allows for an access to splanchnic organs and the use of methods for monitoring hemodynamics, oxygen transport and parameters of metabolic activity similar to patients in intensive care unit.
In brief, domestic pigs were anaesthetized, intubated and mechanically ventilated. Thereafter surgical preparation was performed as previously described in detail. ([77]) Endotoxemia was induced and maintained with continuous i.v. infusion of E. Coli LPS administered for the period of 24 hrs. To simulate clinical situation, ATP-$MgCl_2$ (0.3 $\mu mol.kg^{-1}.min^{-1}$), prepared as described previously, ([78]) was initiated 12 hrs after the start of the endotoxin infusion in a „post-treatment" fashion after full-blown endotoxemia/sepsis had been established and continued throughout the experiment. The dosage was reduced to 0.1 $\mu mol.kg^{-1}.min^{-1}$ if necessary to maintain a mean arterial pressure greater then 70 mmHg. Respiratory, cardiovascular, renal, and hepatosplanchnic function and metabolic parameters were recorded after a rest period post surgical preparation, 12 hours post endotoxin infusion and at 6 hour intervals till the end of experiment. The animals were then sacrificed under anaesthetic by an injection of KCl.

3.3 Methodology of *ex vivo* LPS stimulated human whole blood cultures

The aim of these *ex vivo* studies was to investigate whether ATP or adenosine influences secretion of major cytokines in human whole blood cultures stimulated with LPS. This is an important part of pre-clinical assessment as infusing ATP-$MgCl_2$ leads to ATP and adenosine formation, which may influence cytokine secretion in humans and modulate immune system function in beneficial or detrimental way. *Ex vivo* LPS-stimulated whole human blood cytokine production assay is considered a more relevant model of human leukocyte function than the 'classical' leukocyte cultures of isolated peripheral blood mononuclear cells as it allows for more complete evolvement of the complex cross talk of immune cells with respect to local compartmentalized cytokine responses. ([79, 80])

3.3.1 Effect of adenosine on IL-10 secretion

Venous blood (5mL) was collected from 6 healthy volunteers into heparinized Vacutainers. Aliquots of whole blood were mixed with RPMI 1640 (pH 7.3), in individual hydrophobic microtubes and cultured in the presence or absence of 100ng/mL LPS and/or adenosine. The following incubating conditions were set in duplicate for each subject: unstimulated whole blood cultures (WBC), WBC with LPS, WBC with adenosine, and

WBC with LPS and adenosine. Adenosine (30μM) was added 4 times over 2 hours (120μM total) to mimic *in vivo* situation. To elucidate whether IL-10 production differs with adenosine pre or post-treatment half of LPS free WBCs pre-treated with adenosine as described above received LPS (100ng/mL) subsequently. All samples were incubated at 37°C for total of either 4 or 8 hours. For assessments of IL-10 secreted from leukocytes during incubation, plasma supernatants were harvested and stored at - 20°C until analysed. IL-10 in the plasma supernatant was measured by ELISA in accordance with the manufacturer's instructions. In addition, leukocyte count was measured (Cell Counter). The IL-10 levels were corrected for respective leukocyte counts and same individual's duplicate values were averaged. Statistical analysis was performed using Wilcoxon signed rank test and Spearmann's correlation coefficient as appropriate.

5.3.2 Effect of ATP on IL-10, TNFα and IFNγ secretion

In this whole human blood experiment we used the Instant Leukocyte Culture System (ILCS®) containing LPS, as described. ([81]) In brief, 1mL of peripheral venous blood was collected to multifunctional tubes containing cell culture medium with heparin as anticoagulant and 10 ng of LPS/mL. To test whether the culture conditions in the absence of LPS lead to cytokine release we incubated blood from 7 volunteers in tubes identical to ILCS® but without LPS. To study the effects of ATP on LPS induced cytokine production venous blood was drawn into the ILCS® tubes from 7 other healthy human volunteers and100μM ATP or RPMI 1640 (control) was injected. The ILCS® tubes were then incubated for 24 hours, thereafter serum was separated and samples were frozen at -20°C until cytokine level determination.
The concentrations of cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNFα, IFNγ, and GM-CSF) in the samples were determined using multiplex kits from Biosource. Samples were thawed at room temperature, filtered through a 0.2-μm filter at 14000 g for 30 s and placed in duplicate onto 96 well filter plates. They were prepared according to manufacturer's instruction and assayed on a robotic liquid handling workstation. Briefly, 50 μl of each sample was incubated with antibody-coupled microsphere beads (~5,000 beads per well) for 2 h followed by incubation with detection antibody for 1 h. The conjugates were then incubated with phycoerythrin-labelled Streptavidin for 30 min, washed, resuspended in buffer and vortexed prior to reading the analytes. All incubation steps were carried out at room temperature (20-°C) with shaking and protected from light. All samples were analysed on a Luminex system using the Bio-Plex Manager v4.0 software

reading a minimum of 100 beads per analyte per sample. Data on cytokine levels from the same individual's duplicate specimens were averaged and are presented as means and SEM. Statistical analysis was performed using the Wilcoxon signed rank test.

4. RESULTS

4.1. Role of ATP-$MgCl_2$ in porcine thoracic aortic cross-clamping model

4.1.1 Effects on systemic and regional hemodynamics and oxygen transport

Thoracic aortic clamping produced tachycardia in all three groups but was most pronounced in animals receiving SNP. The catecholamine serum concentrations were accordingly elevated. Despite the catecholamine surge the proximal MAP could be controlled in all groups. The effect on SVR was most pronounced with ATP-$MgCl_2$. The cardiac stroke volume increased during clamping with ATP-$MgCl_2$ treatment and remained constant in the other two groups while cardiac output roughly doubled in both SNP and ATP-$MgCl_2$ group and remained constant in the SNP/Esmolol group. The cardiac output was increased due to tachycardia in the SNP group; whereas ATP-$MgCl_2$ treated animals doubled cardiac output predominantly by increasing the stroke volume. Accordingly the oxygen delivery (DO_2) increased in the SNP and ATP-$MgCl_2$ group and remained unchanged in the SNP/Esmolol group. The whole body oxygen consumption (VO_2) was halved during the clamping phase in all three groups. The portal blood flow was diminished and no hepatic arterial flow was present during clamping. During reperfusion the portal venous blood flow increased compared to pre-clamp values, however, the hepatic artery blood flow only slowly returned to baseline level during the reperfusion phase in all groups.

Table 1: Systemic, intestinal and hepatic hemodynamics, oxygen parameters and catecholamine levels.

	Pre-clamp	End of clamp	2 hrs reperfusion	4 hrs reperfusion
HR[1/min]				
A	102 (97 – 110)	178# (159 – 195)	135# (103 – 156)	119 (88 – 144)
B	95 (91 – 102)	114# (105 – 147)	111# (94 – 116)	102 (81– 106)
C	90 (86 –110)	99 (93 – 108)	103(87 – 105)	97 (88 – 98)

pMAP [mmHg]				
A	101 (94-107)	108 (103-119)	97# (90-102)	93# (85-105)
B	97 (94-105)	83#§ (69-89)	98? (92-102)	98? (82-103)
C	96 (94-99)	90 (74-95)	94 (86-98)	94 (83-104)
Adrenaline[pg/ml]				
A	15 (15-15)	3659# (2818-4781)		17 (15-36)
B	15 (15-25)	4062# (2310-5990)	Not measured	15 (15-17)
C	15 (15-21)	1426# (479-1753)		15 (15-15)
Noradrenaline				
[pg/ml] A	20 (15-37)	5575# (2140-7091)	Not measured	59# (27-320)
B	18 (15-39)	5156# (2750-8732)		42 (25-71)
C	21 (15-28)	3403# (917-4880)		52 (28-78)
SV [ml/kg/min]				
A	1,1 (1,1 – 1,2)	1,3 (0,9 – 1,5)	1,2 (1,1 – 1,3)	1,0 (0,9 – 1,2)
B	1,2 (1,1 – 1,2)	1,8# (1,8– 2,0)	1,3? (1,2 – 1,4)	1,1? (0,9 – 1,2)
C	1,0 (0,9 –1,2)	1,2 (1,0 – 1,4)	1,2 (1,1 – 1,5)	0,9 (0,9 – 1,1)
SVR [dyn.s/cm^5]				
A	1418(1252-1518)	714# (593-820)	949# (841-1103)	1268 (1127-1433)
B	1250(1005-1363)	475# (409-532)	942# (816-1059)	1316 (1138-1463)
C	1523(1374-1683)	1153# (1004-1282)	1321# (940-1360)	1586 (1324-1802)
CO [ml/kg/min]				
A	113 (100 – 126)	223# (184 –264)	147# (123 – 182)	106 (98 – 140)
B	110 (104 – 130)	222# (204 – 254)	132# (121 – 162)	104 (97 – 115)
C	98 (94 – 111)	114 (99 – 150)	115(101 – 123)	90 (84 – 99)
DO_2 [ml/kg/min]				
A	13 (12-15)	25#§ (23-29)	15 (13-16)	12 (12-13)
B	12 (12-15)	25#§ (21-28)	14# (13-17)	12 (10-13)
C	12 (11-12)	12 (9-15)	12 (11-14)	11 (9-12)
VO2 [ml/kg/min]				
A	4 (3-4)	2# (2-3)	4 (4-5)	4 (3-5)
B	4 (4-4)	2 (2-2)	4 (4-5)	4 (4-5)
C	4 (4-5)	2# (1-3)	4 (4-4)	4 (4-4)
QPV [ml/kg/min]				
A	15 (13-23)	0#§ (0-1)	22# (18-33)	17 (16-25)
B	10 (9-22)	0#§ (0-1)	16# (15-27)	11 (9-20)
C	18 (14-20)	5# (4-13)	26# (24-27)	21 (17-22)
QHA [ml/kg/min]				
A	3 (1-4)	0# (0-0)	3 (1-4)	3 (0-3)
B	4 (2-4)	0# (0-0)	3 (2-4)	3 (2-4)
C	3 (2-3)	0# (0-0)	3 (2-4)	3 (2-3)

Values are presented as median and 25-75 percentile. #: p< 0,05 vs. Pre-clamp, §: significant intergroup difference.
A: SNP, B: ATP-$MgCl_2$, C: SNP/Esmolol.
(HR: heart rate*; pMAP:* proximal mean arterial blood pressure; *MPAP:* mean pulmonary arterial blood pressure; *SV:* stroke volume; *SVR:* systemic vascular resistance; *CO:* cardiac output; DO_2: oxygen delivery,; VO_2: oxygen consumption, Q_{PV}: portal-venous blood flow, Q_{HA},: hepatic artery blood flow; Q_{Liver}: liver blood flow)

4.1.2 Effects on acid base and lactate metabolism

There was only a mild change in systemic pH in all three groups of animals during the clamping phase but portal and hepatic venous pH decreased significantly. Metabolic acidosis did not persist in any animal at 4 hours. Lactate increased during clamping and while there was no difference in systemic lactate levels, animals receiving SNP/Esmolol or ATP-$MgCl_2$ produced significantly less lactate in the gut. Nevertheless, only in the SNP/Esmolol group significantly less lactate was present in the systemic and hepatosplanchnic circulation at 2 hours of reperfusion. The lactate/pyruvate (L/P) ratio, a marker of cellular redox state increased during clamping. Interestingly, L/P ratio was highest in the SNP/Esmolol group. The liver utilized lactate before the clamping but liver lactate clearance was grossly reduced during clamping. Lactate was increasingly utilized at 2 hours of reperfusion in all groups but an increased lactate uptake persisted in the ATP-$MgCl_2$ treated animals up to the end of experiment. (Table 2.)

Table 2: Systemic, intestinal und hepatic acid-base and redox parameters.

	Pre-clamp	End of clamp	2 hrs reperfusion	4 hrs reperfusion
A pH				
A	7,51? (7,48-7,53)	7,44# (7,42-7,47)	7,38# (7,34-7,42)	7,47# (7,41-7,49)
B	7,50 (7,50-7,53)	7,48# (7,46-7,51)	7,41# (7,37-7,44)	7,47# (7,45-7,50)
C	7,46? (7,45-7,49)	7,48 (7,45-7,49)	7,43 (7,43-7,46)	7,48 (7,45-7,48)
PV pH				
A	7,47 (7,44-7,48)	7,22# (7,16-7,28)	7,34#(7,32-7,39)	7,43# (7,37-7,45)
B	7,47 (7,46-7,47)	7,20# (7,15-7,27)	7,38# (7,34-7,41)	7,42# (7,40-7,46)
C	7,43 (7,42-7,45)	7,24# (7,20-7,32)	7,39# (7,39-7,43)	7,43 (7,41-7,44)
HV pH				
A	7,47 (7,45-7,48)	7,32# (7,24-7,34)	7,34# (7,25-7,38)	7,43# (7,37-7,46)
B	7,48 (7,47-7,50)	7,33# (7,30-7,38)	7,40# (7,35-7,43)	7,44# (7,42-7,46)
C	7,45§ (7,44-7,46)	7,27# (7,23-7,32)	7,42# (7,38-7,43)	7,44 (7,42-7,45)
A Lac [mmol/l]				
A	1,5 (1,2-1,7)	6,8# (5,8-7,6)	5,5# (3,8-7,9)	1,4 (1,2-1,6)
B	1,3 (1,1-1,7)	6,3# (5,3-7,4)	5,0# (4,1-5,8)	1,3 (1,0-1,7)
C	1,2 (1,0-1,6)	5,2# (4,2-5,9)	2,8# (1,9-3,5)	1,1 (0,8-1,4)
PV Lac [mmol/l]				
A	1,7 (1,5-1,9)	11,0# (10,0-12,0)	5,8# (3,7-8,4)	1,7 (1,3-1,9)

B	1,6 (1,3-1,8)	9,2# (8,5-9,9)	5,2# (4,1-5,8)	1,6 (1,1-1,9)
C	1,5 (1,2-1,7)	9,0# (6,0-9,8)	3,0# (1,9-3,6)	1,3 (1,0-1,6)
HV Lac [mmol/l]				
A	1,1 (0,7-1,5)	7,5# (7,2-8,1)	4,4# (3,4-8,3)	0,9 (0,5-1,9)
B	0,9 (0,8-1,2)	6,8# (6,7-8,1)	3,7# (3,3-4,5)	0,9 (0,5-1,0)
C	0,8 (0,6-1,2)	6,7# (5,6-8,0)	2,5# (1,4-2,8)	0,7 (0,4-1,6)
A Lac/Pyr				
A	11,2(10,2-11,9)	18,9#§ (18,5-20,4)	15,5# (14,2-16,3)	12,6 (11,9-14,2)
B	11,8 (9,5-13,2)	17,9#§ (16,9-19,5)	14,8#(13,1-15,2)	11,8 (9,5-12,7)
C	12,8 (8,6-14,0)	23,5# (21,7-25,2)	14,3 (9,4-18,3)	12,9 (11,8-14,4)
PV Lac/Pyr				
A	12,6(10,2-15,0)	29,1# (26,1-39,2)	17,8# (16,2-22,3)	13,4 (12,4-14,8)
B	19,6(11,4-24,7)	30,7# (25,2-57,3)	17,1 (15,0-20,4)	10,7 (9,1-15,5)
C	12,5(10,4-14,8)	38,9# (30,2-42,3)	14,0 (11,0-17,8)	14,3 (12,9-15,3)
HV Lac/Pyr				
A	11,4(10,9-12,4)	43,9# (38,8-50,2)	16,0# (14,7-19,1)	14,4# (12,9-16,3)
B	11,9(10,1-13,1)	46,0# (35,2-66,3)	16,4# (14,0-17,4)	12,8# (11,2-14,6)
C	15,8(11,9-20,2)	167,9#(66,2-196,0)	14,8 (11,4-19,5)	15,9 (13,1-16,5)
Liver $_{Lac\text{-}Balance}$ [mmol/kg/min] A	11,7 (7,6 – 12,5)	1,8 (0,3 – 1,9)	17,0 (4,7 – 23,6)	11,9 (4,9 – 13,8)
B	9,2 (5,7 – 12,1)	-0,04# (-0,2 – 0,8)	21,8 (8,8 – 30,1)	12,7 (7,3 – 13,6)
C	10,3 (8,7 – 14,2)	1,0 (-2,0 – 2,0)	15,8 (6,1 – 21,8)	10,8 (7,3 – 12,9)

Values are presented as median and 25-75 percentile. #: $p < 0,05$ vs. Pre-clamp, §: significant intergroup difference. A: SNP, B: ATP-$MgCl_2$, C: SNP/Esmolol
(A: arterial; *PV*: portal-venous; *HV*: hepatic-venous; *Lac*: Lactate; *BE*: base excess; *Lac/Pyr*: lactate/pyruvate-ratio; *Liver$_{Lac\text{-}Balance}$*: liver-lactate-balance)

4.1.3 Effect on small bowel metabolism

In contrast to the relatively balanced bowel lactate metabolism the partial CO_2 pressure (pCO_2) of the bowel mucosa rose impressively during cross clamping induced ischaemia regardless of the treatment assignment. The arterial to bowel mucosal pCO_2 gap was significantly elevated and although the gap decreased during reperfusion it remained elevated compared to pre-clamp values in all 3 groups. (Table 3)

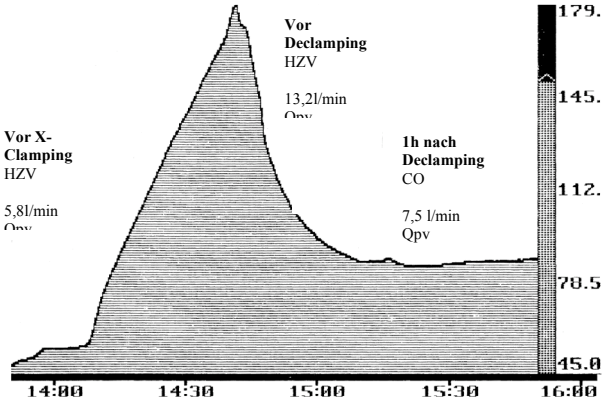
Table 3: The bowel mucosal pCO_2-gap

	Pre-clamp	End of clamp	2 hours reperfusion	2 hours reperfusion
PCO_2 gap [mmHg]				
A	18 (10 – 20)	54 # (41 – 97)	29# (30 – 41)	41# (17 – 60)
B	13 (8 – 20)	67# (32 – 79)	32# (19 –60)	26 #(19 – 57)
C	15 (9 – 17)	61# (49 – 97)	24# (20 – 29)	25# (15 – 38)

Values are presented as median and 25-75 percentile. #: p< 0,05 vs. Pre-clamp, §: significant intergroup difference. A: SNP, B: ATP-$MgCl_2$, C: SNP/Esmolol
(pCO_2 gap: CO_2 partial pressure difference between arterial blood and bowel mucosa)

The time course of a representative mucosal pCO_2 measurement is shown:

Figure 4.



4.2 Role of ATP-$MgCl_2$ in long term hyperdynamic porcine sepsis model

4.2.1 Effects on systemic and regional hemodynamics, metabolism and oxygen exchange

ATP-$MgCl_2$ or placebo was infused during long term hyperdynamic porcine endotoxemia characterised by a fall in systemic vascular resistance and increased cardiac output. ([75]) Despite a widespread interindividual variability, the two experimental groups received nearly identical amounts of LPS and intravenous resuscitation fluid. As expected from its vasodilator properties, ATP-$MgCl_2$ decreased mean arterial pressure and caused a further fall in systemic vascular resistance (Table 5).

Table 5. Time-dependent variations of systemic hemodynamic and O_2 exchange parameters.

		Before LPS	12h LPS	18h LPS	24h LPS
MAP	CON	92 (91;97)	101 (97;106)	100 (92;103)	97 (87;107)
mm Hg	ATP	94 (90;96)	99 (91;115)	82 (76;88) #	78 (74;87) # §
MPAP	CON	22 (20;27)	39 (35;43)	36 (30;38)	37 (33;39)
mm Hg	ATP	22 (19;24)	38 (33;38)	32 (30;35)	35 (34;36)
CVP	CON	7 (6;10)	17 (11;20) #	19 (13;20) #	17 (13;21) #
mm Hg	ATP	8 (6;11)	16 (11;20) #	17 (13;20) #	19 (13;21) #
PAOP	CON	9 (7;11)	18 (16;20) #	17 (16;21) #	17 (16;17) #
mm Hg	ATP	9 (7;13)	17 (17;19) #	19 (16;20) #	17 (15;22) #
ITBV	CON	32 (28;32)	36 (33;38)	35 (31;37)	35 (32;39)
mL/kg	ATP	26 (23;27) §	30 (26;35)# §	33 (28;34) #	31 (28;34) #
CI	CON	116 (106;123)	148 (117;165) #	140 (133;154) #	148 (140;171) #
mL/kg/min	ATP	112 (90;140)	134 (120;148) #	159 (142;192) #	175 (156;204) #
SVR	CON	1216 (1159;1504)	929 (784;1316) #	917 (767;1136) #	929 (788;1067) #
dyn.s/cm^5	ATP	1427 (1182;1538)	1061 (925;1276)	653 (588;797)# §	531 (525;653)# §
DO_2	CON	15 (13;16)	14 (13;18)	14 (12;16)	14 (12;16)
mL/kg/min	ATP	13 (11;16)	14 (13;14)	14 (13;17)	14 (13;18)
VO_2	CON	4.6 (4.24;5.26)	3.9 (3.46;4.84)	3.6 (3.37;4.33)	3.7 (3.51;4.01)
mL/kg/min	ATP	4.3 (4.19;4.44)	4.3 (4.19;4.60)	3.9 (3.53;4.56)	4.2 (3.52;4.80)
pHa	CON	7.49 (7.47;7.51)	7.39 (7.35;7.41) #	7.39 (7.35;7.43) #	7.38 (7.35;7.44) #
	ATP	7.45 (7.44;7.48)	7.39 (7.38;7.42) #	7.38 (7.37;7.39) #	7.36 (7.33;7.41) #
Hct	CON	28 (26;30)	23 (19;23) #	21 (17;22) #	19 (18;20) #
%	ATP	24 (22;25)	23 (19;24) #	18 (17;19) #	18 (17;19) #

LPS, endotoxin infusion; MAP, mean arterial pressure; MPAP, mean pulmonary artery pressure; CVP, central venous pressure; PAOP, pulmonary artery occlusion pressure; ITBV, intrathoracic blood volume; CI, cardiac index; SVR, systemic vascular resistance; DO_2 sys, systemic O_2 delivery; VO_2 sys, systemic O_2 uptake; pHa, arterial pH; Hct, hematocrit. Data are median and 25th and 75th quartile (CON, n=8; ATP, n=9). # Significant difference within each group versus before LPS ($p<0.05$). § Significant between CON and ATP groups at the same time ($p<0.05$).

This was more then compensated for by an increase in cardiac output leading to enhanced systemic and portal venous blood flow, whereas hepatic arterial flow remained unchanged. This phenomenon might be consistent with preservation of the hepatic arterial buffer response that is normally ablated by LPS. As a result ATP-$MgCl_2$ led to higher total liver blood flow and hepatic oxygen delivery ($p < 0.05$) at 18 and 24 hrs of endotoxin administration. There was no additional ATP-$MgCl_2$ effect on gut oxygen extraction, hepatic oxygen uptake, or CO_2 production. Both portal and hepatic venous pH decreased ($p < 0.05$) concomitantly with arterial pH, without intergroup difference. Hepatosplanchnic hemodynamics, oxygen exchange, and metabolic variables are summarized in table 6.

Table 6. Time dependent variation of splanchnic hemodynamics, O_2 transport and metabolism.

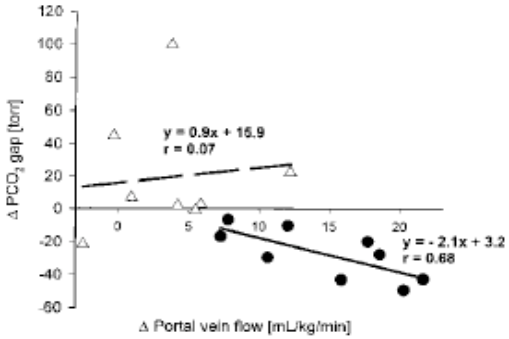
		Before LPS	12h LPS	18h LPS	24h LPS
Q ha	CON	4.1 (2.8;6.2)	2.5 (2.2;3.2)	5.3 (3.7;6) #	5 (4.2;5.5) #
mL/kg/min	ATP	2.1 (1.4;5.8)	2.2 (1.8;3.2)	3.4 (1.3;10) #	2.3 (1.5; 10.4)
Q pv	CON	22 (18;23)	25 (22;28) #	23 (19;28)	28 (26;31) #
mL/kg/min	ATP	19 (18;24)	22 (20;29)	42 (31;43) # §	42 (36;45) # §
Q liv	CON	26 (22;29)	30 (26;31) #	28 (23;37)	34 (31;40) #
mL/kg/min	ATP	22 (20;34)	26 (22;31)	46 (42;53) # §	50 (38;53) #
hDO_2	CON	2.3 (1.7;2.5)	2.2 (2.0;2.8)	2.2 (1.9;2.7)	2.4 (2.2;3.2)
mL/kg/min	ATP	1.8 (1.5;2.5)	1.7 (1.7;2.7)	3.4 (2.5;3.6) # §	2.8 (2.7;3.2) #
Gut EO_2	CON	32 (31;35)	26 (23;28) #	22 (22;26) #	22 (19;23) #
%	ATP	35 (30;45)	34 (27;36)	20 (19;23) #	20 (17;24) #
PCO_2 gap	CON	15 (10:22)	30 (21:43) #	44 (28:70)	50 (32:64)
torr	ATP	16 (14:24)	41 (32:60) #	18 (14:21) §	18 (12:22) §
hVO_2	CON	0.8 (0.6;1.1)	0.8 (0.6;1)	0.6 (0.5;0.8)	0.7 (0.5;0.9)
mL/kg/min	ATP	1.0 (0.6;1.5)	0.9 (0.8;1)	0.9 (0.8;1)	0.7 (0.6;0.9)
hv pH	CON	7.47	7.34 (7.31;7.36)	7.35 (7.33;7.39)#	7.34(7.31;7.38)
	ATP	7.43	7.34 (7.32;7.38)#	7.34 (7.33;7.39)#	7.33(7.29;7.37
pv pH	CON	7.46(7.45;7.46)	7.35 (7.31;7.36)#	7.36 (7.32;7.38)#	7.34(7.31;7.4)#
	ATP	7.42(7.39;7.43)	7.34 (7.34;7.38)#	7.35 (7.33;7.39)#	7.33(7.30;7.38
Gut lac bal	CON	-3.8 (-5.1;-3.4)	-2.5 (-4;-1.7	-3.0 (-5.4;-2.1)	-3.5 (-3.8;-2.6)
mmol/kg/min	ATP	-4.5 (-5.7;-2.6)	-2.7 (-6.7;-2.4)	-3.8 (-5.1;-0.5	-4.7 (-10;-4.4)

Liv lac bal	CON	15.4 (11.5;20)	9.8 (7.7;12.1)	6.6 (3.4;8.9) #	6.9 (4.2;11.9) #
mmol/kg/min	ATP	11.3 (9.9;16.8)	10.7 (10.1;13.1)	10.6 (9.3;12) §	14.5 (12.7;16)§

LPS, endotoxin infusion; Qha, hepatic artery flow; Qpv, portal vein flow; Qliv, total liver flow; hDO_2, hepatic oxygen delivery; hVO_2, hepatic oxygen uptake; Gut EO_2, gut oxygen extraction; HV pH, hepatic vein pH; PV pH, portal vein pH. Data are median and 25th and 75th quartile (CON, n=8; ATP, n=9). # Significant difference within each group versus before LPS ($p<0.05$). § Significant between CON and ATP groups at the same time ($p<0.05$). To convert torr to kPa, multiply by 0.1333

LPS infusion also increased the ileal mucosal–arterial pCO_2-gap. In contrast to the aortic clamping study, ATP-$MgCl_2$ restored the gap to baseline levels. The pCO_2 gap was inversely correlated with portal vein flow variations in the ATP-$MgCl_2$ group but not in control animals. (Figure 5.)

Figure 5. Changes (Δ) in the mucosal-arterial pCO_2 gap of the ileum plotted as a function of variations in portal vein blood flow.

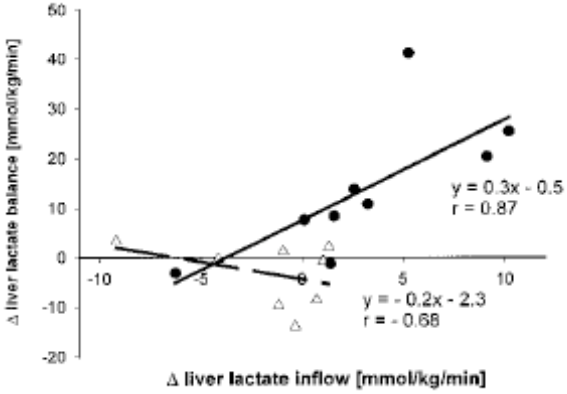


Triangles: control animals, circles: ATP-$MgCl_2$ group.

Such changes in the gastrointestinal circulation could be either due to increased microcirculatory blood flow and/or redistribution of the perfusion within the bowel wall supporting the role of ATP as a vasoregulatory molecule. ([82, 83]) Orthogonal remission spectrophotometry imaging of the ileal mucosa after endotoxemia induction showed increased number of non-perfused villi, (~50%) at the end of the experiment. ATP-$MgCl_2$ was unable to improve villous perfusion. Similarly, arterial, hepatic vein, or portal venous lactate/pyruvate ratios and amino-acid fluxes were not different between the groups. Nevertheless, ATP-$MgCl_2$ maintained the physiologic

coupling between gut lactate release and hepatic uptake that was markedly impaired by endotoxin (Figure 6).

Figure 6. Changes in the liver lactate uptake rate as a function of variations in liver lactate inflow. Triangles: control animals, circles: ATP-$MgCl_2$ group.



This result is of particular interest since it demonstrates that although ATP-$MgCl_2$ failed to improve capillary density in the ileal mucosa it did exert beneficial metabolic effects on the intestine and liver. Whether vasodilation with ATP-$MgCl_2$ occurred upstream from capillaries, which was important for the beneficial metabolic effect remains speculative as ATP-$MgCl_2$ influences several metabolic pathways per se. ([84])

4.2.2 Tissue nucleotide levels and other effects

Liver and ileal tissue concentrations of ATP, ADP, and AMP were similar in both groups at the end of the experiment; hence, ATP-$MgCl_2$ did not influence the ATP/ADP ratio or the adenylate energy charge confirming that energy provision to tissues is not the prime mechanism for the effects of ATP-$MgCl_2$. (Table 7.)

Table 7. Gut and liver tissue nucleotide concentrations.

		ATP nmol/g	ADP nmol/g	AMP nmol/g	ATP/ADP	NEC
Gut	CON	193 (177;203)	98 (81;115)	79 (70;83)	2.1 (1.7;2.5)	0.7 (0.6;0.7)
nmol/g	ATP	171 (161;244)	113 (85;136)	173 (71;255)	1.9 (1.2;2.99)	0.5 (0.5;0.6)

Liver	CON	216 (183;239)	48 (36;62)	121 (84;208)	3.9 (3.2;7.3)	0.6 (0.5;0.7)
nmol/g	ATP	191 (164;209)	43 (26;143)	187 (153;200)	4.6 (1.3;6.6)	0.5 (0.5;0.6)

NEC, nucleotide energy charge; concentrations reported are nmol/g wet weight, data are median and 25th and 75th quartile (CON, n = 8; ATP, n = 9).

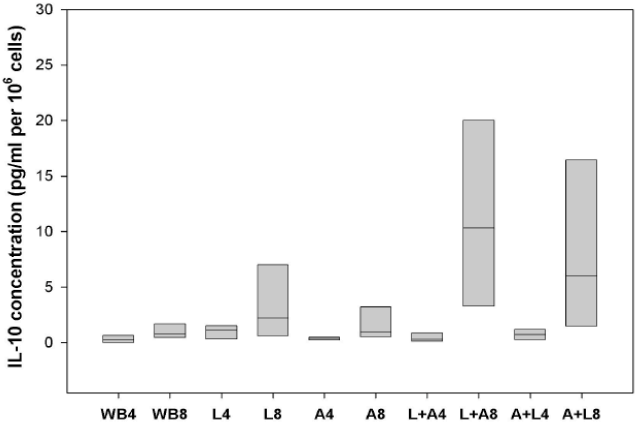
The NO^{2-} blood concentrations significantly increased ($p < 0.05$) in both experimental groups to the same extent. Likewise, the portal and hepatic venous isoprostane concentrations rose during endotoxemia with no intergroup difference. Plasma TNFα levels always remained below the detection threshold regardless of sampling location. Portal and hepatic venous interleukin-10 concentrations peaked ($p < 0.05$) after 12 hrs of LPS infusion and decreased subsequently until the end of the experiments, without differences between the groups (data not shown).

4.3 Effects of ATP and adenosine on cytokine secretion in LPS stimulated human whole blood cultures

4.3.1 Effect of adenosine on IL-10 secretion

We added adenosine in four aliquots over two hours to whole human blood diluted in 1:1 ratio with RPMI1640 culture media in a final concentration of 120μM. Minimal IL-10 production was observed in all blood cultured for four hours and those without either LPS or adenosine. Similarly, the addition of adenosine without LPS led to only negligible IL-10 secretion. As expected endotoxin led to noticeable IL-10 production in whole human blood with values increasing to 2.21pg/mL/10^6 leu. Added adenosine markedly enhanced the IL-10 production in LPS stimulated WBC regardless whether it has been present before, (5.99pg/mL/10^6 leu) or after (10.35pg/mL/10^6 leu) LPS stimulation. (Figure 7.)

Figure 7. Effects of adenosine (total 120μM) on LPS stimulated IL-10 secretion in whole human blood.

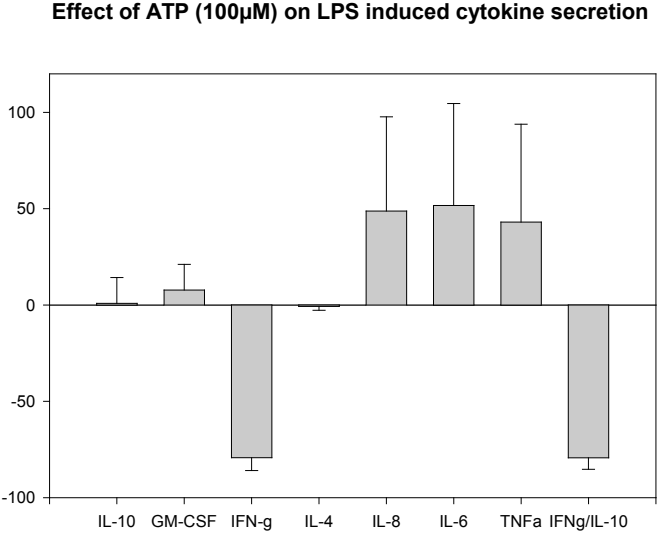


WB 4 – whole blood culture (WBC) incubated for 4 hours, WB 8 - WBC incubated for 8 hours, L4 - WBC with lipopolysaccharide (LPS) incubated for 4 hours, L8 - WBC with LPS incubated for 8 hours, A4 - WBC incubated with adenosine for 4 hours, A8 -WBC incubated with adenosine for 8 hours, L+A4 – WBC incubated with LPS and adenosine for 4 hours, L+A8 - WBC incubated with LPS and adenosine for 8 hours, A+L4 - WBC incubated with adenosine and LPS for 4hours, A+L8 - WBC incubated with adenosine and LPS for 8 hours. Data are depicted as median and range interval.

4.3.2 Effects of ATP on multiple cytokines secretion

In whole blood from seven healthy human volunteers incubated for 24-hours in the absence of both LPS and exogenous ATP we measured the following cytokines IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNFα, IFNγ, and GM-CSF. Of the 10 cytokines determined none were present at significant levels. To determine the effects of ATP on LPS stimulated cytokine production, whole blood incubated in ILCS® was treated with ATP (100 μM) or equivalent volume of RPMI 1640 (as control) at 37°C for 24 hours. Stimulation of whole blood with LPS resulted in the secretion of all cytokines tested except for IL-2, IL-4 and IL-5. Production of IL-1β in the LPS-stimulated cultures exceeded the upper limits of detection.
The addition of ATP to the LPS stimulated whole blood led to no significant change in the levels of TNFα, IL-6, IL-8, IL-10, and GM-CSF. (Figure 8.) However it caused a dramatic and consistent drop in IFNγ production in all individuals (1585±690 pg/mL vs. 246±87 pg/mL, p=0.018). (Figure 8.)

Figure 8.



Data are expressed as percentage (SEM), with zero (0) line representing individual cytokines release under stimulation by LPS without ATP.

The reduced IFNγ secretion following ATP incubation resulted in a decrease of the IFNγ/IL-10 ratio (an indicator of Th1/Th2 immune system activation) from 63 with LPS alone to 8 with LPS and ATP together (p=0.018).

5. DISCUSSION

5.1 Predominantly hemodynamic effects of ATP-$MgCl_2$ are responsible for its effects in I-R and sepsis

The aim of the animal studies was to confirm the previously reported effects of ATP-$MgCl_2$ on systemic and hepatosplanchnic hemodynamics, oxygen exchange, and metabolism as well as markers of I-R injury. Titrating proximal intravenous infusion of ATP-$MgCl_2$ during porcine high thoracic aortic cross clamping has enabled better management of systemic hemodynamics during cross-clamp period as compared to SNP. The reactive tachycardia was less pronounced, the cardiac inotropy enhanced and proximal MAP was easy to control with ATP-$MgCl_2$. Nevertheless, the best hemodynamic profile during cross clamping was achieved by the combination SNP/esmolol, confirming the beneficial effects of beta blockade on perioperative outcomes. ([85]) As opposed to SNP/esmolol treatment ATP-

$MgCl_2$ had no advantage over SNP in terms of liver perfusion during cross clamping. We were also unable to document beneficial effect of ATP-$MgCl_2$ on any of the marker of organ ischemia-reperfusion injury (AST, ALT, creatinine) in this model. Although we could demonstrate superior hemodynamic management during aortic cross clamping over standard SNP treatment, in the same respect ATP-$MgCl_2$ treatment was inferior to a combination of SNP and esmolol.

In the long-term hyperdynamic model of porcine sepsis we demonstrated that ATP-$MgCl_2$ maintained cardiac output but oxygen delivery remained the same secondary due to intrapulmonary shunting. ATP-$MgCl_2$ also decreased MAP and SVR beyond the effects of endotoxin alone. As a result, the drug markedly improved portal venous flow, whereas hepatic arterial flow remained unchanged thus, at least in part, restoring hepatic arterial buffer response. ([86, 87]) This effect might be explained by ATP-$MgCl_2$-induced improvement in endothelium-dependent relaxation ([88]) but it is tempting to speculate that this effect is related to activation of endothelial P2Y and A2a purinergic receptors, by ATP and adenosine respectively, ([89,90]) leading to prostacyclin and NO production, arteriolar smooth muscle relaxation, ([91]) and increase in portal blood flow ([92]) since both molecules are important endogenous regulators of hepatic arterial buffer response. ([93]) Interestingly, ATP-$MgCl_2$ significantly increased splanchnic blood flow, which was associated with a normalization of the ileal mucosal-arterial pCO_2 gap despite the inability of ATP-$MgCl_2$ to prevent microcirculatory derangements caused by endotoxin. ATP-$MgCl_2$ allowed maintenance of hepatic lactate uptake, which could be explained by either the perfusion effect or by preservation of metabolic coupling between liver lactate influx and utilization. Importantly, by measuring tissue adenine nucleotides concentrations we could not confirm that ATP-$MgCl_2$ treatment leads to improved tissue energy charge ([46]) thus effectively ruling out that *in vivo* energy provision was behind the mechanism of ATP-$MgCl_2$ induced changes at least in our animal model.

5.2 Extracellular adenosine and ATP lead to immune suppressive cytokine milieu in LPS stimulated human whole blood cultures

Purinergic receptors are known to significantly alter immune response. As they are implicated in the hemodynamic effects of ATP-$MgCl_2$ we have investigated the influence of adenosine and ATP on immune system. Endotoxin stimulated WBC serves as a model of human sepsis allowing for the development of complex cellular and humoral network in the blood *ex vivo*. The main findings are that adenosine enhanced early IL-10 and ATP reduced IFNγ secretion in LPS stimulated human whole blood. We noticed

no significant changes in the secretion of either of IL-1β, TNFα, IL-2, IL-4, IL-5, IL-6, IL-8, and GM-CSF. The early rise in IL-10 secretion upon exposure to adenosine (8 hours) occurs regardless of whether adenosine is added before or after the LPS challenge. Contrary to our expectation IL-10 levels at 24 hours of culture were not uniformly changed with the addition of adenosine (data not shown) or ATP with only some individuals exhibiting an increase as described by Swennen et al. ([94]) Nevertheless, our results are concordant with others who demonstrated that adenosine leads to enhanced IL-10 production in human monocytes activated by LPS ([38]) In contrast, Soop et al. could not demonstrate increased IL-10 production when infusing adenosine after LPS challenge in human volunteers. However, IL-10 levels were determined within 4hrs of adenosine infusion, whereas we could only demonstrate increased production after 8 hours. ([95]) The variations in stimulated IL-10 secretion could be explained either by differential activity of adenosine kinase or adenosine deaminase among the volunteers or by the variable age and ethnic origin of volunteers. ([96, 97])

The finding of the profound reduction of IFNγ secretion by extracellular ATP is very interesting, especially because the inter-individual variability was very small. On the contrary, the response seemed to be fairly uniform pointing towards a universal mechanism. Our data are also consistent with work of others that described inhibition of IFNγ production by ATP or ATPγS (non degradable ATP analogue) in LPS-activated monocytes and monocyte-derived dendritic cells. ([98, 99])

Human studies have clearly shown that stimulated IFNγ production *in vitro* by monocytes and lymphocytes as well as *ex vivo* in whole blood is severely depressed in critically ill patients. ([100, 101]) Decreased synthesis of IFNγ by lymphocytes ([102]) as well as of IFNγ in whole blood after cardiac surgery has been reported. ([103,104]) Moreover treatment with recombinant IFNγ has been shown to restore both HLA-DR expression on monocytes from sepsis patients and improve the diminished ability to secrete IL-6 and TNFα. ([105]) We thus hypothesize that ATP released in substantial quantities during major trauma, sepsis or shock, impairs IFNγ production by blood leukocytes and contributes to immune dysfunction, impaired bacterial clearance and susceptibility to secondary infection.

Ertel et al. suggested that there are two main mechanisms responsible for the "dramatic disturbances of the IFNγ pathway during critical illness". ([106]) Firstly, deactivation of IFNγ producing leukocytes following an insult and secondly, the presence of serum suppressive factors different from IL-4, IL-10, or $TGF\beta_1$. Our results suggest that extracellular ATP may represent the unknown serum factor mediating, at least in part, the suppression of stimulated IFNγ secretion thus causing immune suppression.

6. SUMMARY OF CONCLUSIONS

"Good tests kill flawed theories; we remain alive to guess again"
Karl Popper

The previously reported multiple beneficial effects of ATP-$MgCl_2$ were tested in two clinically relevant large animal models. We observed mainly cardiovascular effects of ATP-$MgCl_2$ likely related to purinergic receptors stimulation. Adding ATP and its metabolite adenosine to *ex vivo* LPS stimulated whole human blood cultures and measuring cytokine secretion we have further tested whether modulation of inflammation might be responsible for some of the ATP-$MgCl_2$ effects. The results are summarized as follows:

1. Infusing ATP-$MgCl_2$ intravenously in a porcine I-R injury model of thoracic aortic cross clamping provides better cardiovascular stability compared to currently used standard agent sodium nitroprusside. Although ATP-$MgCl_2$ led to reduced gut lactate release we could not demonstrate any beneficial effects on numerous markers of reperfusion injury. Moreover the combination of sodium nitroprusside with esmolol provided hemodynamic control superior to ATP-$MgCl_2$.

2. In long term hyperdynamic porcine model of sepsis ATP-$MgCl_2$ increased portal venous blood flow, reduced ileal mucosal-arterial pCO_2 gap and preserved hepatic arterial buffer response as well as metabolic coupling between lactate release from the gut and its utilization by the liver. Despite the beneficial effects of ATP-$MgCl_2$ on hepatosplanchnic hemodynamics and metabolic function we were unable to observe diminished reperfusion related structural injury. Importantly, we could not confirm increased tissue adenine nucleotides concentrations after several hours of ATP-$MgCl_2$ infusion suggesting that provision of substrates for endogenous tissue nucleotides recovery or energy is not responsible for ATP-$MgCl_2$ effects, at least in the doses used in our experiment.

3. Adding the ATP-$MgCl_2$ metabolite adenosine to LPS stimulated cultures of whole human blood leads to increased secretion of IL-10. This suggests that extracellular adenosine at clinically relevant levels may contribute to earlier and higher production of IL-10 during

endotoxemia thus potentially preventing host tissue damage but potentially impairing immune defence against pathogens.

4. Using standardized LPS stimulated human blood cultures (ILCS®) we demonstrated that extracellular ATP at moderate concentrations is able to modulate cytokine production mainly by reduced secretion of the prime T helper cell 1 (Th1) cytokine IFNγ. This is an important finding as low IFNγ levels in critically ill patients and reduced production of IFNγ upon immune stimulation are associated with nosocomial infections, poor infection clearance and increased mortality.

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8. ORIGINAL STUDIES

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