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The role of ATP-$MgCl_2$ in ischemia-reperfusion and sepsis

Doctoral dissertation

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ORIGINAL STUDIES

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PREFACE

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

Ischemia-reperfusion (I-R) injury is involved in the pathogenesis of many human diseases including stroke, myocardial infarction, ruptured abdominal aneurysm, traumatic brain injury as well as sepsis. The deprivation of tissues of oxygenated blood during ischemia leads to a range of adaptive processes aimed at protecting cells from lack of oxygen and metabolic substrates. Nevertheless, cellular depletion of high-energy phosphates proportional to the degree of ischemia ensues. If the insult is severe enough to outweigh the adaptive mechanisms cellular malfunction and tissue damage occur. Although reperfusion restores the supply of oxygen and nutrients to ischemic organs it imposes further stress on tissues as well as the whole organism. Firstly, the blood flow to reperfused tissues is increased putting demand on the circulatory system. Secondly, stress response is activated leading to profound hormonal and metabolic changes. Thirdly, an inflammatory response in ischemic tissues is initiated. The extent of ischemic damage, the degree of reperfusion, the severity of inflammatory response and the physiological reserves of the organism eventually determine patient outcome.

Both sepsis and major aortic surgery are associated with significant morbidity and mortality and despite advances in therapy outcomes are still far from satisfactory. Novel treatment that would prevent or cure the damage associated with I-R injury in both conditions is desirable. Intracellular levels of the energy molecule adenosine triphosphate (ATP) are diminished at the time of reperfusion. Therefore the idea of external ATP provision to restore cellular energy stores is intriguing. Large body of previous experimental work, including human studies, suggested that if ATP is supplemented in complex with magnesium chloride ($MgCl_2$) the extent of cellular damage and inflammation caused by I-R is limited ultimately leading to improved organ function and survival. The following work was undertaken with the hope that ATP- $MgCl_2$ might prove to be such promising agent.

We evaluated the role of ATP-$MgCl_2$ in a porcine model of thoracic aortic cross-clamping induced I-R injury and a porcine endotoxemia model of sepsis. Because the hepatosplanchnic region is particularly vulnerable to I-R in both conditions our aim was to evaluate the ATP-$MgCl_2$ effects on bowel and liver perfusion, metabolism as well as markers of inflammation. Firstly, ATP-$MgCl_2$ was infused to treat hemodynamic changes induced by high thoracic aortic cross-clamping. The effectiveness of ATP-$MgCl_2$ was compared to sodium nitroprusside alone or in combination with an intravenous beta-blocker esmolol. Secondly, we infused ATP-$MgCl_2$ intravenously in a long-term hyperdynamic fluid resuscitated porcine model of sepsis in a post-treatment fashion.

Next, our results from the animal studies pointed to the fact that the recently discovered purinergic receptors likely mediated several of the observed effects. As purinergic receptors assume important immune modulating role during I-R and sepsis we investigated the influence of their main endogenous ligands ATP and its degradation product adenosine on cytokine production in *ex vivo* lipopolysaccharide (LPS) stimulated whole human blood cultures.

3.
CURRENT STATE

3.1. Definitions and pathophysiology of ischaemia-reperfusion

Ischemia (from Greek, isch- restriction, hema blood) is defined as absolute or relative insufficiency of oxygenated blood supply to tissues that results in cellular dysfunction or damage. Rather than in hypoxia a more general term denoting a shortage of oxygen, the term ischemia reflects shortage of other blood nutrients but also the limited disposal of cellular metabolism products that accumulate and may directly or through mediators cause cellular injury ([i]). Nevertheless, the hallmark of ischemia is the limitation of oxygen supply and diminished aerobic energetic metabolism. The depletion of intracellular levels of ATP follows leading to disturbed homeostasis, progressive cellular alterations and eventually necrosis. Corresponding microvascular circulatory disturbance and coagulation system activation occur. If damage is irreversible and tissullar necrosis ensues microvascular flow ceases and the no reflow phenomenon is observed ([ii]). 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 ([iii]). 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 ([iv]) In fact, tissue injury was maximal in areas with the greatest blood flow during reperfusion. This phenomenon has been termed the oxygen paradox ([v]) or reperfusion injury.

Reperfusion injury generally has two components: the local response (e.g. tissue swelling) and the systemic response (e.g. tachycardia) and both may contribute to resultant organ failure and death. The I-R injury provokes complex interactions between the endothelium and different cell types, leading to further microvascular injury, cellular apoptosis and necrosis ([vi]). Local alterations in vascular tone, vascular 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 ([vii,viii]) However, the degree of inflammatory response following reperfusion varies greatly. With severe ischemia causing irreversible damage to parenchyma and vasculature the no reflow phenomenon causes little inflammatory response and tissues become non viable. Where the degree of ischemic damage has not been sufficient to cause cellular death the magnitude of the inflammatory response will be determined by the proportion of reperfused tissue ([2]).

The inflammatory reaction to I-R is firstly promoted by resident tissue macrophages and the activation of complement. Intracellular generation of ROS by the enzyme xanthine oxidase and in particular by mitochondria contribute to tissue dysfunction and cell injury ([ix,x]). Recent observations point to Toll-like receptors on innate immune system cells as sensors of cellular I-R injury bridging I-R injury and inflammation ([xi]). CD4+ T helper cells become activated amplifying inflammatory signalling and attraction of leukocytes to reperfused area, where they adhere to vascular endothelium, roll along its surface to subsequently extravasate into interstitial fluid and further into the reperfused tissue. Leukocyte adherence leads to vessels narrowing and activation of platelets which then together with leukocytes plug

small capillaries causing obstruction and leading to area demarcation with further ischemia. Leukocytes also release proteases that are cytotoxic to endothelial and parenchymal cells ([xii]). The whole process is orchestrated by the secretion of cytokines and chemokines which have local as well as systemic effects ([xiii]).

3.2 Definition, epidemiology and pathophysiology of sepsis

Sepsis (in Greek, putrefaction) 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 it leads quickly to multiorgan dysfunction and death. 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.

3.2.1 Epidemiology of sepsis

Sepsis occurs in 2% of all hospitalisations and accounts for as much as 25% of intensive care unit (ICU) bed utilization. It is a major cause of death in intensive care units worldwide, with mortality rates that range from 20% for sepsis to 40% for severe sepsis to >50% for septic shock. ([xiv]) In the United States, sepsis is the tenth most common cause of death overall according to 2000 data from the Centre for Disease Control and Prevention. Similar situation is found in Europe. More then 10 000 patients suffering severe sepsis were admitted annually to intensive care units from 1996 to 2004 in England, Wales and Northern Ireland, which accounted for 27% of all ICU admissions. Although hospital mortality for admissions with severe sepsis decreased from 48% in 1996 to 45% in 2004, the total number of deaths increased from an estimated 9,000 to 14,000. This corresponds to the severe sepsis associated number of hospital deaths per 100,000 inhabitants rising from 23 to 30. ([xv]) Although sepsis on admission to ICU is common numerous patients develop sepsis while actually in intensive care. In the report on sepsis prevalence in French ICU's 42% of patients had severe sepsis at admission and 20% acquired severe sepsis during their ICU stay. Importantly, mortality was doubled in patients with ICU-acquired infection, independent of the presence of severe sepsis on admission ([xvi]). In this study severe sepsis acquired in ICU was associated with greater than 3-fold increases in workload and costs. Although this data from western world are alarming, sepsis is quietly killing countless patients in the developing countries as well. In Papua New Guinea Duke et al. reported infection was associated with up to three quarters of death in children, sepsis accounting for the majority. The commonest causes of death from sepsis are intractable shock, distant multi-organ failure or secondary infection ([xvii]).

3.2.2 Interaction of bacteria and the immune system

After invading the host pathogenic bacteria and their toxins interact with patient's immune system via so called 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 neutrophil extravasation leading to amplification of inflammation ([xviii]). 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. It is generally perceived that the host immune system response is either pro- or anti-inflammatory in nature. However, both components are essentially intertwined ([xix]) and their interplay is a key to a

successful infection clearance. Nevertheless, the mechanisms of pro- and antiinflammatory process control and coordination are poorly described. It has been proposed by Matzinger that the control role might lie within the host tissues. So called "danger molecules" or damage-associated molecular pattern (DAMPs) arising from damaged host tissues seem to regulate immune responses ([xx]). These may include unmethylated CpG sequences of promotor DNA, high mobility group box-1 protein (HMGB-1) as well as nucleotides such as ATP ([23,21,xxi]).
It is further thought that once pathogens cause tissue damage and immune system activation spills over systemic response to infection also known as "systemic inflammatory response syndrome" (SIRS) ensues ([xx]). Although the role of SIRS is generally aimed at disposing of the invading pathogen, it is capable, depending on its extent and location, to induce further injury, neuroendocrine disturbances as well as to activate coagulation and fibrinolytic systems. The anti-inflammatory branch of the immune response is aimed at feedback controlling the extent of SIRS and is described at the systemic level as the "compensatory anti-inflammatory response syndrome" (CARS). If the fine balance between SIRS and CARS is lost the host is endangered either by his own immune system effectors or by the poorly opposed pathogen and its toxins. ([xxii])
To further illustrate the complexity of host pathogen interaction two other important factors come in play. Firstly, pre-existing co-morbidities and age may limit the necessary physiological reserves that determine one's ability to sustain the demands posed by both SIRS and CARS response. Secondly, patient's genetic make-up may results in excessive or poorly regulated immune response to the offending organism. The clinician's task is even more complicated as individual patients present to intensive care units at different stages of this complex pathogen-host interaction.

3.2.3 Endotoxemia as a model of sepsis

Endotoxemia is the presence of endotoxin (LPS - lipopolysaccharide, 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. ([xviii,xxiii]). Levels in the nanogram per milliliter range have been reported in blood of patients admitted to intensive care unit ([xxiv]). 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 ([xxv]). 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. Endotoxin was traditionally used in large doses to establish short- term animal models that would replicate sepsis. However, such models are characterized by a hypodynamic circulation with decreased cardiac output and do not resemble the clinical picture of human sepsis, particularly when fluid resuscitation is not used. The experiment described here represents a clinically relevant long- term porcine model of endotoxemia, which has been developed and established in the Section of Pathophysiology and Process Development in Anesthesia, University Hospital Ulm, Germany. The intravenous endotoxin infusion in anesthetized domestic pigs closely mimics human sepsis with fullblown systemic inflammatory reaction and fulfils the criteria for human sepsis model as defined by Fink ([xxvi]). The microcirculatory alterations associated with local I-R phenomenon, particularly in the gastrointestinal tract, are the hallmark of this model of prolonged endotoxemia. As discussed above

TLR4 is activated by I-R ([xxvii]) and both endotoxin and gut I-R injury triggered TLR4 activation are likely responsible for the inflammatory response encountered in this particular model.

3.2.5 Role of circulatory disturbance in sepsis

The complex effects of inflammation on endothelium, coagulation cascade and neuroendocrine system result in vascular dysfunction of sepsis. The circulatory derangements are characterized by increased vascular permeability, excessive production of vasoactive substances like nitric oxide (NO), prostaglandins and adenosine ([xxviii]), inappropriately low levels of vasopressin as well as decreased sensitivity of vascular smooth muscle to endothelin, catecholamines and angiotensin II. Moreover activation of potassium K^+ATP sensitive channels contributes to distorted regulation of vascular tone and permeability ([xxix]). Decreased capillary density and perfusion heterogeneity develops in the microcirculation with vessels blocked by microthrombi, constricted, others dilated causing shunting of perfusion and some having relatively normal perfusion ([xxx]). 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 ([xxxi]), they lead to the development of multiple organ failure in sepsis. Maintenance of perfusion pressure and recruitment of non perfused and under perfused capillaries is regarded as a major goal of resuscitation from septic shock. To achieve the goal, range of vasoactive drugs have been studied both in animal models and in critically ill patients. Typically vasopressors are used to maintain perfusion pressure although they might further impair the microcirculatory flow ([xxxii]). On the other hand, the list of drugs investigated for the potential to improve microcirculation in sepsis includes dobutamine, ([xxxiii]) dopexamine, ([xxxiv]) milrinone, ([xxxv]) nitroglycerin, ([xxxvi]) and levosimendan ([xxxvii]). In clinical practice often both groups of drugs 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 ([xlix]). Furthermore its reported salutary effects on cellular energy balance and cellular function in different shock models make it an attractive choice in sepsis and I-R.

3.2.5 Role of gastrointestinal tract in the pathogenesis of sepsis

It has long been proposed that following trauma bacteria and their products are absorbed from the gastrointestinal tract (GIT) and are responsible for the development of shock. ([xxxviii]) Later the term “bacterial translocation” was adopted ([xxxix]) and Marshall hypothesized that in shock the bacterial and endotoxin translocation from GIT acts as a source of infection, as an “undrained abscess”, thus perpetuating ongoing inflammation and multiple organ failure ([xl]). Recent data suggest that colon might be the major site of translocation into systemic circulation ([xli]). The implicated pathogenetic mechanisms are the disruption of the normal ecology of the indigenous GIT microflora resulting in bacterial overgrowth, impairment of the host’s immune defenses, and physical disruption of the mucosal barrier of the gut ([xlii]). However, the interplay between GIT, circulation and the immune system is much more complex. Firstly, the pathogenetic importance of bacterial as opposed to endotoxin translocation from the GIT into systemic circulation in critically ill patients is doubtful ([xliii]),

secondly the GIT associated lymphatic system may drain pathogen and their products to systemic circulation and more importantly act as a source of inflammatory mediators triggered by pathogen's interaction with immune cells of the lymphatics ([xliv]) and thirdly, studies have confirmed ominous microvascular derangements in the gastrointestinal tract in sepsis with heterogenous perfusion deficit and variable degree of I-R injury ([xlv,xlvi]). The ischemia-reperfusion of the GIT microcirculation leads to mucosal damage, which triggers gut associated lymphatic tissue to mount an ongoing inflammatory response with the possibility of mimicking the "undrained abscess". In contrast the systemic immune response as assessed by monocyte and blood response to further inflammatory stimuli becomes flat or anergic consistent with "endotoxin tolerance" or "immune paralysis" ([xlvii]). Thus the ongoing inflammation in the GIT might in fact be contributing to systemic immune dysfunction leading to secondary, acquired infections with negative consequences for the critically ill patient ([xlviii]).

3.3 ATP in ischemia-reperfusion and sepsis

3.3.1 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, a purine nucleotide, covering almost 95% of body energetic needs, is produced and immediately consumed in a daily amount approximating the individuals' body mass (~ 1 mmol kg^{-1} min^{-1}) at rest. During exercise or stress this amount can increase up to 20 times. Most of the ATP is produced by mitochondrial oxidative phosphorylation in the presence of oxygen and complexes with Mg^{2+} or Mn^{2+} ions. The phosphate potential of ATP is used for three main purposes 1: active transport of molecules and ions across biological membranes 2: performance of mechanical work 3: synthesis of biomolecules. Under normal conditions ATP production is well adjusted to the cellular needs and cells are able to maintain their structure, function and membrane integrity.
The balanced situation is disturbed by I-R and sepsis. Intracellular ATP levels fall proportionally to the degree of ischemia to levels insufficient to match utilization of high-energy phosphate bonds ([xlix]). Fall in oxidative phosphorylation and mitochondrial dysfunction ([l]) are responsible for diminished ATP production while ATP is utilized in ongiong adaptive and reparative processes. For example, non-lysosomal ATP consuming proteolysis is elevated four fold in septic skeletal muscle to provide aminoacids for hepatic synthesis of acute phase reactants ([li]). 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 diffusible metabolites, mainly adenosine occurs. During reperfusion ATP levels are often incompletely replenished and may deteriorate further ([lii]). ATP is either produced via residual oxidative phosphorylation or via the glycolytic pathway. The nucleotide precursors of ATP are being recycled via the purine salvage pathway and rarely synthesized de novo as that is an energy (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 ([liii]). The

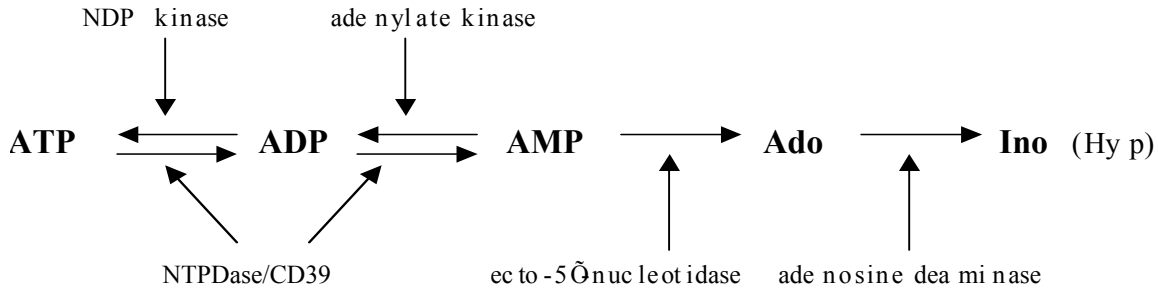
provision of external AMP or ATP during reperfusion was suggested to help replete the intracellular ATP and best results have been reported using ATP-$MgCl_2$ complex ([xlix]). However, the amount of ATP-$MgCl_2$ used in the *in vivo* experiments would hardly be sufficient to supplement all the necessary nucleotides for ATP resynthesis. It has become apparent that the repletion of ATP is not the major mechanism behind the beneficial effects of ATP-$MgCl_2$ and indeed a novel mechanism involving signalling via purinergic receptors emerged.

3.3.2 Role of extracellular ATP

Given the complex mechanisms for synthesizing, maintaining and restoring the intracellular level of ATP under most conditions, one might expect that release of ATP to the extracellular milieu would occur only after cellular death. Traditional reasoning suggests that cells should protect their intracellular ATP at all costs, and the established view was that ATP does not cross the plasma membrane of viable cells, at least not inside out. The suggestion that the selective release of cytoplasmic ATP might be part of a physiological regulatory mechanism was not long ago on the verge of heretical ([liv]). However, recent studies established that the release of ATP into the extracellular space by exocytosis, membrane transporters, and connexin hemichannels is a widespread physiological process ([lv,lvi]). Moreover, ATP may also leak from cells through their plasma membrane damaged by inflammation, ischemia, and mechanical injury. Current interest is therefore concentrated on the physiologic, regulatory or pathological role of extracellular ATP. It appears that ATP acts in autocrine, paracrine or endocrine fashion to modulate various bodily functions. For example, it is established that erythrocytes release large quantities of ATP when sensing hypoxia, high shear forces or when undergoing deformation ([lvii,lviii]). Released ATP increases, via purinergic receptor signaling (see below), nitric oxide (NO) and prostacyclin production by endothelial cells, which then act on the vascular smooth muscle to dilate. Moreover, ATP is then degraded by ectonucleotidases located on endothelial cells, membrane of erythrocytes or adjacent leukocytes, to ADP that acts as a negative feedback for further ATP release from the red cells ([lix]). Therefore erythrocytes function as an important regulator of regional blood flow by means of ATP release.
How ATP and other nucleotides exercise their effects became apparent with the discovery of two large families of extracellular receptors for nucleotides and nucleosides. In the early seventies Geoffrey Burnstock was the first to realise that nucleotides act on membrane receptors and proposed the subclassification of ‘purinergic’ receptors to P1or P2 receptors ([lx]). During the last two decades it was shown that extracellular ATP via purinergic receptors influences many biological processes and ATP in nanomolar concentrations provides „purinergic tone“ to tissues ([lxi]). Two classes of ubiquitously present purinoceptors mediate the effects of extracellular nucleotides: P1 (A1, A2a, A2b, A3) and P2 (P2Y and P2X) receptors ([lx,lxii]). The difference between the two classes is that compared to P2 the P1purinoceptors 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 that gate extracellular cations in response to ATP. This family comprises seven receptors (P2X1 through P2X7), and the possibility exists that these receptors form hetero-oligomers exhibiting pharmacological properties distinct from those of the seven different homo-oligomeric forms. P2X receptors account for fast neurotransmission as well as sympathetic control of vascular tone and largely, but not exclusively, are found on excitatory tissues ([lxiii,lxiv]). From the perspective of ATP-$MgCl_2$ the P2X7 receptor

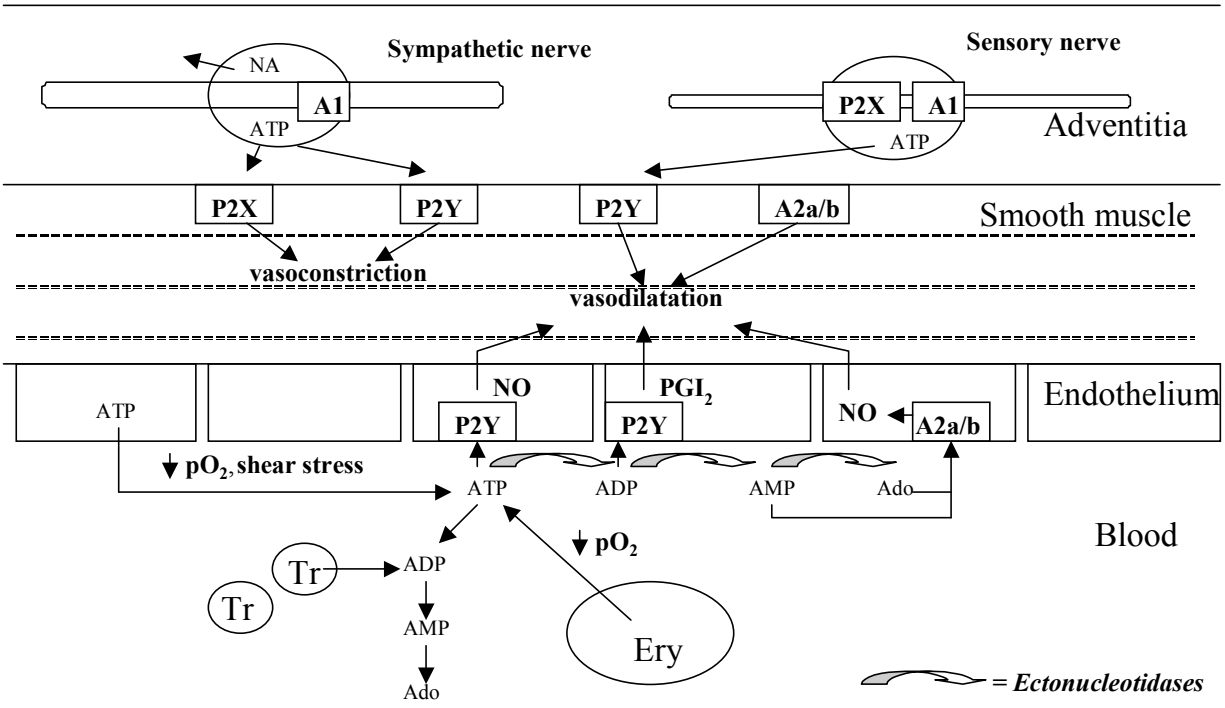
deserves particular mention as it acts as an ATP-activated ion channel but also forms a pore, gating passage of molecules up to 1 kDa in response to ATP ([lxv]). This unique receptor plays an important role in cells involved in immunological and inflammatory responses for it is crucial in the process of IL-1β, IL-18 release, caspase-1 activation and apoptosis ([ci]).
The P2Y receptors are G protein-coupled receptors that are categorized into a subfamily of receptors (P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11) that predominantly couple to Gq therefore activating phospholipase C or as in the case of P2Y11 adenylyl cyclase and intracellular calcium rise, ([lxvi]) and into a family of Gi-coupled receptors (P2Y12, P2Y13, P2Y14) that usually inhibit adenylyl cyclase and regulate ion channels but the complexity is high as for example the $P2Y_{12}$ receptor also activates phosphatidylinositol 3-kinase ([lxvii]).
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, as discussed above, ATP released from erythrocytes in response to hypoxia has vasodilator properties via P2Y1 receptor stimulation thereby inducing the formation of PGI_2 and NO ([lvii,lxviii]). Nevertheless, when released from sympathetic nerves upon stimulation ATP may cause brief vasoconstriction ([lxix]). The end result of purinergic signalling is even more complicated since ATP-degrading ecto-nucleotidases form a cascade of endothelial and blood cell surface-located enzymes converting ATP into ADP, AMP and adenosine (Fig.1), which acting via both classes of purinoceptors mediate respective vasoregulatory effects ([lxxi,lxi]).

Figure 1. Schema of ecto-enzymatic purine turnover on cellular surface



As purinoceptors in the vasculature were likely involved in the effects of ATP-$MgCl_2$ used in both the animal models a brief description of the distribution and function of purinoceptors on the vasculature follows. (Fig.2)
The ion gated P2X receptors are present on the smooth muscle and mediate a rapidly desensitizing vasocontractile response. Similarly, P2Y2, P2Y4 and P2Y6 receptors are expressed on the vascular smooth muscle and mediate vasoconstriction, while P2Y1 and P2Y2 receptors are present on the endothelium and mediate vasorelaxation, ([lxx,lxxi]) P1 (A2a, A2b) receptors are expressed on both the vascular smooth muscle and the endothelium. Depending on anatomical location and physiological conditions adenosine, similar to ATP, can also mediate either vasodilation or vasoconstriction ([lxxii,lxxiii]).

Figure 2.



3.3.3 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 ([lxxiv]). Under basal condition only small amounts (nano-moles) of adenosine are present outside cells where they stimulate two out of four adenosine receptors A1 and A2a ([lxxv]). Ischemia and sepsis are associated with increased levels of ATP degradation products and adenosine ([lxxvi,lxxvii]) as they are released from various blood cells, endothelial cells as well as from ischemic tissues. Under conditions of stress extracellular concentrations of adenosine reach micro-molar concentration and can stimulate all P1 receptors. Under such conditions adenosine actions have been described as retaliatory. Adenosine is thought to mediate regional vascular reactivity in response to ischemia and act as a replenisher of intracellular nucleotide pool. However, adenosine exhibits a range of effects on inflammation, especially during reperfusion injury. Although the immediate actions might be pro-inflammatory adenosine exerts strong and prolonged anti-inflammatory effect via the activation of adenosine A2a, A2b, and possibly A3 receptor ([81]). Based on these presumptions 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 ischemia and reperfusion and shock. In short-term animal models and in vitro experiments adenosine, adenosine analogues, inosine or adenosine deaminase inhibitors (increasing adenosine by preventing its degradation) have suppressed the acute inflammatory response not dissimilar to effects observed with

ATP-$MgCl_2$ ([lxxviii,lxxix]). Adenosine and inosine blunted the inhibition of mitochondrial respiration and blocked excessive NO production of macrophages stimulated with IFNγ + LPS ([lxxx]). In neutrophils low extracellular adenosine concentrations induce superoxide anion generation, phagocytosis, and adhesion to endothelial cells via the activation of the A1 receptor, whereas higher concentrations of adenosine (>500nM) saturating A2 receptors elevate cAMP and thereby inhibit free oxygen radical release, leukotriene synthesis, and platelet aggregation ([lxxxi,lxxxii]). 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 ([lxxxiii,lxxxiv]). In addition, it was demonstrated that inosine inhibited the production of proinflammatory cytokines (TNFα, IL-1, IL-12, MIP-1α, IFN-γ) and attenuated the LPS-induced gut mucosal injury in rat endotoxin shock ([lxxxv,lxxxvi]). Finally, adenosine and inosine are also natural inhibitors of the poly-(ADP-ribose)-polymerase (PARP) the activation of which has been shown to play a pivotal role in the development of organ failure in several experimental models ([lxxxvii]).
In critically ill patients adenosine levels are markedly increased, particularly in sepsis and septic shock and correlate well with the severity and outcome. Martin et al. reported mean plasma concentrations of 4μmol/L in patients with severe sepsis and 8μmol/L in septic shock ([83]). It is unclear whether high adenosine merely reflects the severity of the insult, acts as a retaliatory mediator, or whether high levels could actually harm the host by partially mediating the immune dysfunction associated with clinical sepsis. Such mechanism is supported by the finding of increased A2a receptor expression in LPS stimulated monocytes ([lxxxviii]) that is accompanied by attenuated monocytes cytokine secretion in response to further LPS stimulation ([lxxxix]) and also by our results as discussed further.

3.4 Role of magnesium in ischemia-reperfusion and sepsis

Magnesium (Mg), called the “energy ion”, is the second most abundant intracellular positively charged ion 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. The body normally contains approximately 25g of Mg of which 53% are stored in bones, 27% in muscle, 19% in soft tissues, 0.5% in erythrocytes and 0.3% in serum. Intracellular Mg is mostly bound to proteins, RNA, DNA, citrate as well as to ATP and ADP. Only 5-10% of cellular Mg is ionized ([xc]). 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. Ionized serum hypomagnesaemia correlates with cellular Mg depletion and has been reported both in experimental endotoxemia ([xci]) as well as in the critically ill ([xlix]). 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 ([xcii]). For example, $MgCl_2$ maintained cellular homeostasis and reduced cortical cell loss in experimental brain injury ([xciii]), while in rat endotoxic shock model Mg supplementation restored deranged mitochondrial respiration ([xciv]). Intracellular Mg^{2+} is complexed to ATP therefore its degradation in I-R and sepsis consequently leads to increase in free Mg^{2+} and efflux from cells. At the same time Ca^{2+} entry through dysfunctional sarcolemmal ion channels is increased leading to loss of calcium compartmentalization and cellular dysfunction. 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 ([41]). Furthermore, Mg modulates binding of agonists to purinergic P1 and P2 receptors ([xcv,xcvi]). Recent work also documented that $MgCl_2$ at milimolar concentrations non-competitively blocks the crucial inflammatory and "death" receptor P2X7 ([xcvii]).
We have used ATP-$MgCl_2$ based on multiple previous studies where the beneficial effects of ATP were enhanced when $MgCl_2$ was in complex with ATP in a large number of experimental conditions ([xlix]). Whether this effect could be attributed to the P2X7 receptor blockade or other plausible mechanisms was not investigated yet and remains to be determined.

3.5 Pharmacology of ATP-$MgCl_2$

Although there is clear evidence that exogenously administered ATP may enter the intracellular space across the cell membrane ([xcviii]) only in studies performed in isolated organs the extracellular concentrations of ATP-$MgCl_2$ were high enough (in the mM range) to explain its therapeutic effects simply due to supplementation of adenosine or adenosine nucleotides ([xcix,c]). By contrast the *in vivo* doses of ATP-$MgCl_2$ (in the μM range) suffice to allow for purinoceptor activation ([lxxi]). 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 ([xcvii]). This may potentially prevent the production of the major pro-inflammatory cytokines IL-1β and IL-18 as well as caspase-1 activation and immune cell apoptosis ([ci]).
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 ([cii]). The activition of the P1 and P2 purinergic receptors depends on the levels of adenosine degrading enzyme adenosine deaminase (ADA) but also ecto-nucleotide kinases, adenylate kinase, and nucleoside diphosphate kinase, potentially an extracellular ATP generating enzyme ([ciii,civ]). ATP-$MgCl_2$ metabolic products are taken up by nucleoside transporters by facilitated diffusion ([cv]) and are either metabolized intracellularly to inosine, hypoxanthine, xanthine and, ultimately, uric acid ([cvi]) and/or salvaged - rephosphorylated to AMP as well as other nucleotides, with ATP accounting for the majority ([cvii]). Although this is unlikely to be the major effect of exogenous ATP-$MgCl_2$, phosphorylation of some ectokinases by ATP-$MgCl_2$ might act as a trigger for salvage of endogenous purine nucleotides ([cviii]). That salvage of purine nucleotides occurs in humans is supported by a study showing that when labeled NAD is added extracellularly, most of the label is subsequently found intracellularly as ATP ([cix]).
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 ([cx]). In rabbits, only 1% of the dose injected intravenously (5 μmol/kg ATP-$MgCl_2$ over 1 minute) was present as ATP at 40 seconds, and only inosine remained at 280 seconds. After 1 hour of continuously infusing ATP-$MgCl_2$ (30μmol/kg) mainly inosine (37%), AMP (17%),

ATP (5%) and only a minute amount of adenosine were detectable in the circulating blood ([cxi]). Nucleotides are largely metabolized by endothelial and blood cell CD39 and CD73 ectonucleotidases, nevertheless, even the plasma membrane of cardiac myocytes rapidly hydrolyzes ATP ([cxii]). Accordingly, 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 ([cxiii]). These negative chronotropic effects are likely to be the result of ATP degradation to adenosine ([cxiv]) and the action of the latter on sinoatrial and atrioventricular nodes ([cxv]). This has also been shown during administration of ATP into the sinus node coronary blood supply in dogs ([cxvi]), in which model the negative chronotropic action of ATP was attenuated by theophylline, a known antagonist of adenosine at the P1-receptor site ([cxvii]). Therefore several undesired side effects (A-V block, cardiac arrest, hypotension) are to be expected when high doses of ATP-$MgCl_2$ are infused too quickly ([cxviii]).

3.6 Previous experimental studies combining ATP and $MgCl_2$

Before the discussion of papers, which form the basis of this thesis, following chapters will overview the reported effects of ATP-$MgCl_2$ in animal models of hemorrhagic shock, ischemia-reperfusion and sepsis as well as in the few published clinical human studies. Given the fact that synthesis of ATP was recognized as a major limiting factor of recovery during shock or ischemia ([cxix]) the majority of the studies were based on the premise of supplementing "energy" in the form of ATP and adenine nucleotides to ischemic tissues upon reperfusion ([xlix,cxx]). However, other mechanism such as enhancement of mitochondrial activity and efficiency ([cxxi]), improvement of endothelial function ([cxxii]), reduction of cytokine production ([cxxiii]), hemodynamic improvement based on systemic and regional vasodilator effect ([cxxiv,cxxv,cxxvi,cxxvii]), and improved cardiac performance ([cxxviii]) were documented and are likely to be explained by activation of purinergic receptor.

3.6.1 Role of ATP-$MgCl_2$ in haemorrhagic shock

In the early seventies Irshad Chaudry and colleagues showed that survival of animals subjected to hemorrhagic shock was enhanced by the administration of ATP-$MgCl_2$ during resuscitation ([cxxix]). When ATP-$MgCl_2$ was added to crystalloid resuscitation 60% of animals survived after 3 days in contrast to 30% with $MgCl_2$, and 20% when only crystalloids were infused. Two decades of subsequent studies provided evidence that ATP and cAMP levels in the liver, kidney, muscle and brain from ATP-$MgCl_2$ treated animals were significantly increased, which was associated with restoration and maintenance of cardiac output, coronary, portal, total hepatic and renal blood flow and attenuated tissue oedema ([cxxv,cxxviii,cxxx,cxxxi]). ATP-$MgCl_2$ induced vasodilation of systemic resistance arterioles and restored cardiac performance, which was associated with increased myocardial lactate utilization [(cxxv,cxxxii)]. Also the shock-induced depression of the vascular responsiveness and vasoregulation were restored ([cxxxiii]). Addition of ATP-$MgCl_2$ to resuscitation regimen improved renal blood flow as well as microcirculatory perfusion, glomerular filtration rate and urine output.

Renal tissue oedema was also reduced ([cxxxiv]). Similarly ATP-$MgCl_2$ improved microcirculatory blood flow in the liver, restored hepatic excretory function, and prevented tissue oedema formation ([cxxxv,cxxxvi]). Haemorrhagic shock-induced insulin resistance was reversed in skeletal muscle ([cxxxvii]) and ATP-$MgCl_2$ exerted beneficial effects on immune competence after haemorrhage. Interestingly, in one animal model shock-related immune anergy of splenocytes and macrophages was restored with ATP-$MgCl_2$ administration while systemic TNFα, IL-6 levels decreased. ([cxxxviii,cxxxix]) In summary, ATP-$MgCl_2$ addition to resuscitation after haemorrhage improved cardiac performance, enhanced organ blood flow and function as well as immune competence, although the decrease in perfusion pressure related to marked vasodilatation may caution against its use human haemorrhagic shock.

3.6.2 Role of ATP-$MgCl_2$ in ischemia and reperfusion

The protective effects of ATP-$MgCl_2$ in hemorrhagic shock prompted studies in various ischemia-reperfusion models. It was suggested that first of all the ATP-$MgCl_2$-induced vasodilatation might beneficially influence the microcirculation after ischemia ([cxl]) but the effects were also described in isolated organs thus excluding causality of the vasoactive properties of ATP-$MgCl_2$. For example isolated ischemic rat kidneys reperfused with ATP-$MgCl_2$ displayed complete recovery of glomerular filtration rate (GFR), improved renal perfusion, urine flow and fractional absorption of sodium, ([cxli,cxlii]) effects similar to systemic administration of ATP-$MgCl_2$ after renal ischemia ([cxliii,cxliv]). Interestingly, more recent study documented improved renal recovery, namely increased new DNA synthesis and augmented expression of genes critical to cellular proliferation with ATP (without $MgCl_2$) and suggested P2 receptor stimulation as the major mechanism ([cxlv]). Furthermore, Lee et al. recently suggested that the protective effect of ATP on renal ischemic injury is, in part, related to inhibition of the nuclear transcriptor activator factor NF-κB activation via P2Y receptors in isolated rabbit proximal renal tubular cells ([cxlvi]). As ATP-gamma-S, a stable ATP analogue activating P2Y11, had similar effect, the cAMP elevating P2Y11 receptor was likely responsible for the inhibition of NF-κB activation ([cxlvii]).
To test whether other adenine nucleotides were also able to attenuate organ dysfunction $MgCl_2$ was infused together with AMP, ADP, or ATP 24 hours following an ischemic renal injury in rats ([xcix]). Although all compounds increased renal blood flow, GFR and improved tubular functions ATP-$MgCl_2$ compared favourably with the other nucleotides in terms of preservation of morphologic integrity and the persistence of functional improvement. In contrast to the later study, ([cxlv]) the beneficial effect disappeared when AMP, ADP or ATP were infused without $MgCl_2$ ([xcix,cxlviii]). This fact may either underscore the importance of magnesium as a co-factor of external ATP or point to another mechanism like the inhibition of P2X7 receptor as comparably higher ATP doses likely to stimulate P2X7 were used ([xcvii]).
The effects of ATP-$MgCl_2$ were also investigated in models of hepatic I-R. The survival rate following complete liver ischemia for 60 minutes in rats was doubled when ATP-$MgCl_2$ was infused during reperfusion. The release of transaminases was attenuated, liver cell energy charge normalized, and both hepatic blood flow and indocyanine green (ICG) clearance in the treated group returned to control values ([cxlix,cl]). This was further underscored by an improved hepatic ultrastructure and mitochondrial function ([cli,clii]). However, other groups have reported mixed or

negative effects of post-ischemic treatment with ATP-$MgCl_2$ in rat liver I-R model. Frederiks and Fronik observed that ATP-$MgCl_2$ given at reperfusion nearly completely blunted liver tissue necrosis in fasted rats, while the extent of necrotic areas was increased in fed animals ([cliii]). Hasselgren et al. reported no beneficial effect of ATP-$MgCl_2$ on the liver membrane potential or protein synthesis. On the contrary, the post-ischemic restitution of these cellular metabolic variables was slower in animals receiving ATP-$MgCl_2$. At the end of the ischemic period, ATP, glucose and lactate in liver tissue were the same in animals receiving ATP-MgCl2 or saline before ischemia ([cliv]). The reasons for the disparity are not clear but the liver metabolic status before ischemia or the degree of hepatic and porto-systemic shunting at reperfusion might be responsible.

The effects of ATP-$MgCl_2$ on myocardial I-R injury were addressed in a number of studies. Intracoronary ATP-$MgCl_2$ infusion during myocardial reperfusion in dogs produced a dose-dependent coronary vasodilatation with high doses causing profound systemic hypotension precluding the recovery of myocardial function achieved by the low dose ([clv]). In dogs subjected to 150 min of aortic cross clamping ATP-$MgCl_2$ infused upon reperfusion allowed for complete functional recovery of the myocardium whereas control animals exhibited tissue oedema and impaired myocardial performance ([clvi]).

In a rat lung transplant model with cold I-R injury peak airway pressure, intrapulmonary shunt fraction, wet to dry lung weight ratio and histological injury were reduced with ATP-$MgCl_2$ treatment while no improvement was observed with ATP or $MgCl_2$ alone ([clvii]). In isolated rat lung I-R model pre-treatment with ATP-$MgCl_2$ and adenosine but not ATP or $MgCl_2$ alone (all at 10μM) significantly attenuated the degree of acute lung injury. Interestingly, the protective effects of ATP-$MgCl_2$ were diminished when promazine (an ecto-ATPase inhibitor) or 3, 7-dimethyl-1-propargylxanthine (an A2-receptor antagonist) were added prior to ATP-$MgCl_2$. The authors hypothesised that the protective effect of ATP-$MgCl_2$ was in part mediated via adenosine, which is produced by the magnesium dependent ecto-ATPase on the surface of neutrophils and reacts with neutrophil A2 receptors to inhibit the production of O_2 radicals ([clviii]). Subsequent study indirectly confirmed the results showing that leukocyte presence in the lung perfusate was necessary for the protective effect of ATP-$MgCl_2$. The authors speculated that the degradation of ATP-$MgCl_2$ to adenosine by the leukocyte ecto-nucleotidase was mandatory for lung protection against I-R injury ([clix]).

Finally, the effects of ATP-$MgCl_2$ were investigated in a model of aortic cross-clamping in rabbits. Treatment with ATP-$MgCl_2$ prevented paraplegia and histological damage of the spinal cord ([clx]). Based on these experiments we evaluated the therapeutic potential of ATP-$MgCl_2$ infusion during high thoracic aortic cross-clamping in pigs concentrating on the effects in the hepatosplanchnic region.

3.6.3 Role of ATP-$MgCl_2$ in sepsis

The utility of ATP-$MgCl_2$ has also been tested in experimental septic shock. In a rat model of caecal ligation and puncture (CLP) peritonitis resuscitation with ATP-$MgCl_2$ along with hypertonic glucose resulted in restoration of reticulo-endothelial system function and improved survival ([clxi,clxii]). In mice CLP peritonitis ATP-$MgCl_2$ at the onset of sepsis increased lymphocyte ATP levels and proliferative response, preserved

endothelial cell function as well as vascular reactivity all of which was associated with improved survival ([clxiii,clxiv]). In Group B streptococcal sepsis in piglets ATP-$MgCl_2$ started 15 min after the onset of sepsis-induced pulmonary hypertension reduced mean pulmonary artery pressure and improved cardiac performance, gas exchange as well as acid-base status ultimately leading to improved survival ([clxv]). In addition, intravenous ATP-$MgCl_2$ induced bronchodilation, which was in contrast to the well-known bronchoconstrictive effects of inhaled ATP in asthmatics ([clxvi]). Major drawbacks of this study were the short duration and the hypodynamic shock state characterised by a markedly decreased cardiac output. In fact, it has been underscored that therapies of septic shock be investigated in animal models that mimick the features of human sepsis, e.g. a hyperdynamic circulatory state associated with hypermetabolism ([clxvii]). We therefore examined the role of ATP-$MgCl_2$ in a long term, fluid resuscitated hyperdynamic endotoxemia model of sepsis in pigs ([clxviii]).

3.6.4 Overview of ATP-$MgCl_2$ use in humans

Despite 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 ([clxix]). Other possibility might be either the occurrence of potentially unreported side effects such as cardiac ischemia or lack of interest from pharmaceutical companies as ATP-$MgCl_2$ is not a patent protected compound. On the other hand, 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 compensated for the fall in systemic vascular resistance ([clxx]).
Based on experimental results in piglets demonstrating that ATP-$MgCl_2$ was able to attenuate hypoxia-induced pulmonary hypertension without major undesired side effects on the blood pressure ([clxv,clxxi]) ATP-$MgCl_2$ (0.01-0.2mg kg^{-1} min^{-1}) was 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. Two children who inadvertently received a large bolus injection of ATP-$MgCl_2$ (app. 5mg) developed transient (30s) second-degree A-V block without hemodynamic compromise, suggesting an effect similar to intravenous action of adenosine ([clxxii,clxxiii]). In the same population ATP-$MgCl_2$ also preferentially decreased pulmonary artery pressure during postoperative course and pulmonary hypertensive crises were controlled by ATP-$MgCl_2$ without any side effects or rebound pulmonary hypertension ([clxxiv]). In contrast, the ATP (not ATP-$MgCl_2$) induced decrease of mean pulmonary artery pressure in COPD patients led to a fall in arterial O_2 saturation probably as a result of aggravated ventilation/perfusion-mismatch regularly seen with intravenous vasodilators in the pulmonary circulation ([clxxv]). The rebound pulmonary hypertension after ATP discontinuation was attributed to induction of acute hypoxic pulmonary vasoconstriction affiliated with fall in arterial PO_2. Although the systemic hemodynamic effects of ATP-$MgCl_2$ are similar to adenosine the frequency of undesired side effects (A-V block, facial flushing, burning sensations in the chest)

was reported less with ATP-$MgCl_2$ during coronary flow reserve measurement ([clxxvi,clxxvii]).
Hirasawa et al. were the first to try ATP-$MgCl_2$ in addition to standard treatment of shock-induced organ failure in humans. In patients suffering from acute ischemic renal failure ATP-$MgCl_2$ infusion for 90-120 minutes improved kidney function and ultimately 13 out of 16 patients survived ([clxxviii]). This uncontrolled study prompted a prospective, randomised controlled trial in 30 patients with ischemic acute renal failure: compared to controls ATP-$MgCl_2$ reduced the duration of extracorporeal renal support (9.8 ± 1.8 vs. 11.9 ± 2.7 days) and increased survival ([clxxix]). Beneficial effects were also documented in a study of 88 patients with multiple organ failure. ATP-$MgCl_2$ increased the arterial ketone body ratio and shifted the respiratory quotient in favour of carbohydrate oxidation and glucose utilisation suggesting improved mitochondrial redox potential. However, improved patients survival was not observed ([clxxx]).
Taken together, a large body of experimental evidence would supports the use of ATP-$MgCl_2$ in situations where ischemia-reperfusion contributes to morbidity and mortality.

4.
AIM OF THE STUDIES

I. To investigate the previously reported beneficial effects of intravenous infusion of ATP-$MgCl_2$ in multiple models of I-R injury during thoracic aortic clamping in pigs. Particular emphasis was put on hepato-splanchnic and renal vasoregulation, oxygen exchange and metabolism.
II. To investigate the potential of intravenous infusion of ATP-$MgCl_2$ used in a post-treatment fashion during hyperdynamic porcine endotoxemia with particular emphasis on hepato-splanchnic blood flow, oxygen exchange and metabolism.
III. To assess the effects of an ATP-$MgCl_2$ metabolite adenosine on cytokine production in LPS stimulated whole human blood from healthy volunteers.
IV. To assess the effects of ATP and other P2 receptor agonists on multiple cytokines production in LPS stimulated whole human blood from healthy volunteers.

5.
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. All methodological aspects of the

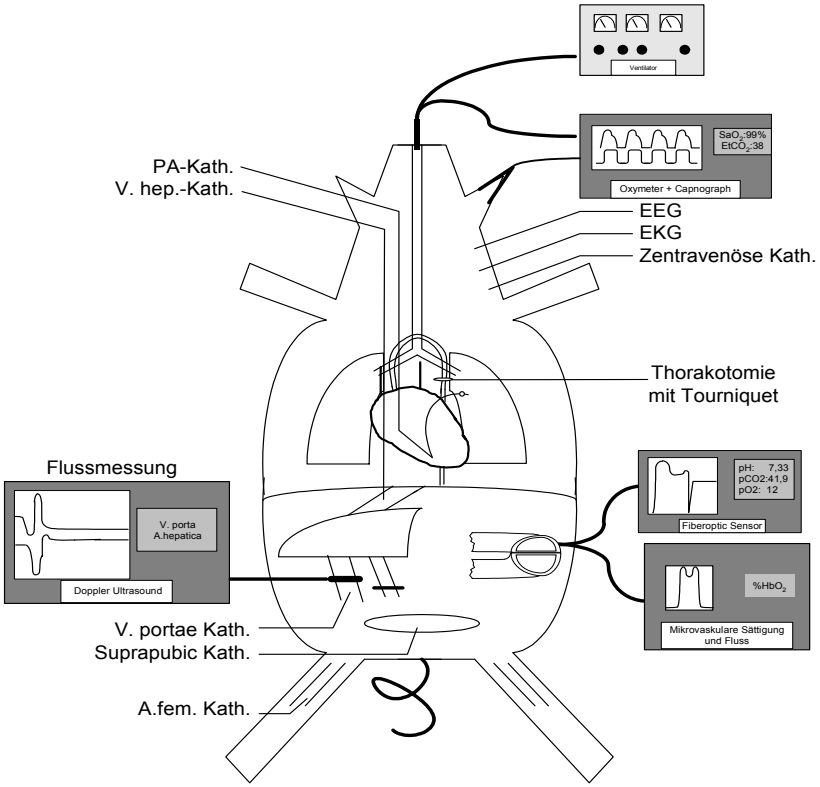
animal trials are described first while the methodology and rationale for the *ex vivo* experiments are presented subsequently.

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

The porcine model of high thoracic aortic clamping was developed and established in the Department of Anaesthesia, Section of Pathophysiology and Process Development in Anesthesia, University Hospital Ulm, Germany. The 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 of Regierungspraesidium Tuebingen (Baden-Wurttemberg, Germany) approved the study protocol. Twenty-four healthy domestic German landrace pigs were anesthetized with pentobarbital (200-300 mg/kg) and paralyzed with alcuronium (14 mg/h). Validated continuous EEG recording assured depth of anesthesia. The pigs were mechanically ventilated and the tidal volume and respiratory rate were adjusted to maintain arterial PCO_2 between 35-45 mmHg. The animals were instrumented and randomized into three groups. Eight pigs in the sodium nitroprusside and ATP-$MgCl_2$ group each and six animals into sodium nitroprusside plus esmolol treatment.

Under aseptic conditions the common carotid artery was dissected and cannula for continuous mean arterial pressure (pMAP) monitoring above the aortic clamp was inserted. Thermodilution pulmonary artery catheter was inserted into the adjacent right submandibular vein and floated into the pulmonary artery for continuous monitoring of central venous pressure (CVP), mean pulmonary artery pressure (MPAP) and intermitent pulmonary artery occlussion pressure (PAOP), (model 93A 754 7 F, Fa. Baxter Healthcare, Irwine CA, USA). A 7 French catheter (Multipurpose A-1, Cordis, Roden, NL) was introduced under ultrasound guidance into the common hepatic vein and placement confirmed with agitated saline test. The catheter served for blood sampling. Another catheter was inserted into the right femoral artery for continuous mean arterial pressure monitoring below the aortic clamp (dMAP) as well as for blood sampling. Thereafter the descending thoracic aorta was dissected via left posterolateral thoracotomy and an occlusion band was placed over the aorta immediately below the branching of the left subclavian artery. The occlusion tourniquet and a chest drain tube were placed and the thoracotomy wound was closed in two layers. Subsequently median laparotomy was performed and hepatic artery and portal vein dissected. A Doppler sonographic flow probe (Transonics Systems, Ithaca, NY, USA) was placed around both vessels. Moreover a 4 French catheter was inserted into the portal vein (Arrow, Reading, USA) to enable blood sampling. Loop of the ileum was isolated and brought onto surface via midline laparotomy and a two-way ileostomy was formed. The stoma was covered with a stoma bag to prevent drying of the mucosal surface. A fiberoptic catheter for continuous intramucosal pCO_2-measurement (Paratrend 7®, Biomedical Sensors, Highwycombe, UK) was advanced through the stoma to be placed above the ileal mucosa. A suprapubic urinary catheter was placed percutaneously to derive urine (Cystofix, Fresenius, Bad Homburg, Germany). The experimental setup is depicted in figure 3. (decription in German).

Figure 3.



After one-hour rest period the baseline measurements were taken. The infusions of sodium nitroprusside (SNP), ATP-$MgCl_2$ or esmolol and SNP were 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 investigation drug dose was titrated against the proximal MAP (pMAP) as follows:

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

The drug infusion was stopped 1 minute prior to thoracic aortic clamp release (de-clamping) in the case of ATP-$MgCl_2$ and 5 and 10 minutes in the case of sodium nitroprusside and esmolol, respectively. We recorded respiratory, cardiovascular, renal, and hepatosplanchnic function and metabolic parameters 5 minutes prior to de-clamping to assess the extent of ischemic damage. Following de-clamping of thoracic aorta noradrenaline in small bolus doses was carefully titrated to achieve MAP of 60 mmHg. Thereafter we observed the animals for further 4 hours and collected blood and measurements as described above at 2 and 4 hours post de-clamping to assess the effects of the three drugs on reperfusion injury. The animals were then sacrificed under anaesthetic by an injection of 20mmol KCl.

Figure 4.
Schematic diagram of the experiment

Anaesthesia/ Induction - 1h	Surgical preparation 3h	Recovery phase -1h	Clamping 30min	Reperfusion phase I - 2h	Reperfusion phase II - 2h

Measurements and derived parameters

Systemic hemodynamics

Proximal, distal mean arterial and central venous as well as mean pulmonary artery blood pressure were measured by standard pressure transducers (model 0129oA, Hewlett Packard, Rockville, MD, USA). Heart rate was derived from ECG trace and cardiac output was measured in triplicate by thermodilution using intermittent saline bolus via the pulmonary artery catheter (66S-Monitor, Hewlett Packard, Palo Alto, CA, USA) and the mean of the three measurements was recorded.

Regional hemodynamics

Regional macrocirculation was measured continuously using transit time ultrasound flowmetry (Transonic Systems T206, Ithaca, NY, USA). In this technology utilizing the Doppler principle, two transducers pass ultrasonic signals back and forth, alternately intersecting the flowing liquid in upstream and downstream directions. The difference in transit time between upstream and downstream signals is a measure of particle/cell velocity. In our experiments, pre-calibrated flow probes were placed around the common hepatic artery and the portal vein during surgical preparation. The recorded portal venous and hepatic arterial blood flow rates were summed to obtain the total hepatic blood flow.

Small intestinal tonometry

Intestinal tonometry was used to monitor ileal intramucosal PCO_2 ($PiCO_2$). The tonometrically derived $PiCO_2$, assuming that PCO_2 is the same in both the surrounding tissue and the lumen of a hollow visceral organ, represents an index of ileal mucosal perfusion and cellular energy balance ([clxxxi]). $PiCO_2$ was measured continuously using a precalibrated fiberoptic PCO_2 sensor, PARATREND^{TM} (Paratrend Sensor, Trend Care, Diametrics Medical Inc. Roseville, USA) ([clxxxii]). Simultaneously, an arterial blood sample was obtained for arterial PCO_2 ($PaCO_2$) measurement, and the calculation of systemic acid base independent variable, the ileal mucosal-arterial PCO_2 gap ([clxxxiii]).

$$\Delta PCO_2 = P_iCO_2 - PaCO_2$$

Additionally, hepatic arterial, portal and mixed central venous blood was sampled to obtain pH, BE, PO_2 und PCO_2 using a standard blood gas analyser (Nova Stat Profile Ultra, Nova Biomedical, Waltham). Hemoglobin concentration and hemoglobin oxygen saturation were also determined by cooximetry, especially calibrated for porcine blood (IL 682 CO-Oximeter, Instrumentation Laboratories) The arterial and portal venous blood as well as portal venous and hepatic arterial CO_2 content difference was then calculated as follows: ([clxxxiv])

$$CCO_2 = pCCO_2\left[1 - \frac{0{,}0289 * [Hb]}{(3{,}352 - 0{,}456 * SO_2) * (8{,}142 - pH)}\right] \text{[ml/100ml]},$$

$pCCO_2 = 2{,}226 * S * pCO_2 * (1 + 10^{pH-pK})$,

where $S = 0{,}0307 + 0{,}00057 * (37-T) + 0{,}00002 * (37-T)^2$

and $pK = 6{,}086 + 0{,}042 * (7{,}4-pH) + (38-T) * (0{,}00472 + 0{,}00139 * (7{,}4-pH))$

The oxygen content and oxygen balances were calculated accordingly.
Oxygen content:

$$CaO_2 = (Hb * 1{,}36) * SaO_2 + (PaO_2 * 0{,}003) \quad [ml/100ml]$$

$$CvO_2 = (Hb * 1{,}36) * SvO_2 + (PvO_2 * 0{,}003) \quad [ml/100ml]$$

$$C_{PV}O_2 = (Hb * 1{,}36) * S_{PV}O_2 + (P_{PV}O_2 * 0{,}003) \quad [ml/100ml]$$

Systemic oxygen kinetic
Oxygen delivery (DO_2)

$$DO_2 = 10 * CaO_2 * CO \quad [ml/min/kg].$$

Oxygen consumption

$$VO_2 = 10 * \text{a-v}DO_2 * CO \quad [ml/min/kg].$$

Regional oxygen balance (intestinal O_2-extraction)
The intestinal O_2-extraction in the portal vein drained organs was calculated as follows.

$$iO_2Ex[\%] = \frac{CaO_2 - C_{PV}O_2}{CaO_2}$$

Assessment of energy metabolism and cytosolic redox state

The lactate/pyruvate ratio reflects the cellular energy balance. The lactate/pyruvate ratios were measured in the arterial, portal and hepatic venous blood to assess regional energy balances.
Theoretical principles:

(a) $\text{Lactate} + NAD^+ \xrightleftharpoons{\text{LDH}} \text{Pyruvate} + NADH + H^+$

(b) $\text{Pyruvate} + \text{L-Glutamat} \xrightleftharpoons{\text{GTP}} \text{L-Alanin} + \text{Ketoglutarate}$

$\text{Lactate} + NAD^+ \xrightleftharpoons[\text{Normoxia}]{\text{Hypoxia}} \text{Pyruvate} + NADH + H^+$

The utility of reaction (b) is to constantly remove excess pyruvate to keep the equilibrium for reaction (a) in favour of lactate to pyruvate conversion even at low lactate concentrations. The cytosolic concentration ratio between lactate and pyruvate is 10:1 and is influenced by the intracellular content of NADH as the enzymes catalysing the reactions are in NADH dependent equilibrium. Under physiological conditions the equilibrium lies largely on the site of lactate.
We used enzymatic UV-test for lactate and pyruvate concentration measurement. (Boehringer GmbH, Mannheim, Germany). The actual measured component is NADH, which can be determined photometrically as it absorbs light at 365 nm wavelength. The resulting quantity of NADH production or consumption is then equivalent to lactate and pyruvate concentrations, respectively. Rising lactate points towards increased intracellular NADH. Under physiological circumstances pyruvate is metabolised in the liver through oxidation in the tricarboxylic acid (TCA) cycle, while lactate is utilised in the gluconeogenetic pathway. In case of hypoxia the oxidation of pyruvate in the TCA cycle is deranged and the equilibrium of the pyruvate/lactate reaction shifts towards lactate. The NAD thus produced is then used

to provide proton receptor for anaerobic energy production. This processes of anaerobic glycolysis is less efficient in terms of ATP gain for a given substrate as for 1 Mol of glucose only 2 Mol of ATP are produced as compared to 36 Mol of ATP when glucose is oxidated in the presence of oxygen. As a result cellular energy metabolism is disturbed during hypoxia, proton concentration rises and so-called "lactic acidosis" ensues.

Statistical analysis

All results in the tables are expressed as median and 25 – 75 percentile. The intra-group differences relative to pre-clamp measurements were analysed with Friedmann ANOVA test. The inter-group differences were analysed using Kruskall-Wallis variance-Test. The level for significant differences of <0.05 was considered.

5.2 Long-term hyperdynamic porcine sepsis model

This model, similarly utilizing anesthetized domestic pigs, closely mimics human sepsis and fulfils the criteria for human sepsis model as defined by Fink ([clxxxv]). The key features of this model are firstly resuscitation of the animals with large volumes of i.v. fluids (hydroxyethylstarch), which mimics clinical practice for the management of sepsis and septic shock. The infusion rate is titrated to the pulmonary artery occlusion pressure resulting in hyperdynamic normotensive hemodynamics characterized by sustained increase in cardiac index and reduced systemic vascular resistance. It is thus ensured that hypovolemia is not a factor contributing to the hemodynamic and metabolic derangements. Secondly, the model exhibits sepsis-like hypermetabolism documented by markedly increased endogenous glucose production, as well as systemic and regional lactate and lactate/pyruvate ratio. Thirdly, microcirculatory changes identical to clinical sepsis and the development of multiple organ dysfunction is observed ([clxxxvi,clxxxvii]). Moreover, the porcine model allows for an access to splanchnic organs and for monitoring systemic hemodynamics, oxygen transport and parameters of metabolic activity similar to patients in intensive care unit.

In brief, domestic pigs with a median body weight of 46 kg were fasted for 24 hrs, with free access to water. They were anaesthetized, intubated and mechanically ventilated. Thereafter surgical preparation was performed as previously described in detail ([clxxxviii]). Endotoxemia was induced and maintained with continuous i.v. infusion of E. Coli lipopolysaccharide 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, ([clxxxix]) 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.

Assessment of regional macro- and microcirculation

Regional macrocirculation was measured continuously using transit time ultrasound flowmetry (Transonic Systems T206, Ithaca, NY, USA) as described above for the previous experiment. In addition, the ileal mucosal microcirculation was evaluated with recently developed orthogonal polarization spectral (OPS) imaging using the CYTOSCAN™ (Cytometrics, Inc.; Philadelphia, PA). This device instrument consists of a small endoscopic-like light guide attached to a light source with filters providing polarized green light. Because haemoglobin in the red blood cells maximally absorbs

light of the filtered wavelength (550 nm), the red blood cells can be clearly observed flowing through the microcirculation ([cxc]). This technique provides microcirculatory images comparable to intravital capillary microscopy without requiring transillumination. We videotaped mucosal villus microcirculatory flow in five different, randomly chosen, locations of the ileal mucosa. An investigator blinded to the experimental group analyzed the recorded video images. As the pictures' quality allowed for separation of single villi, their number was counted and semi quantitatively classified as perfused, non-perfused, or heterogeneously perfused ([clxxxvi]).

Small intestinal tonometry

Intestinal tonometry was used to monitor ileal intramucosal PCO_2. The device and methods were used in the same fashion as in the previous experiment.

Assessment of energy metabolism and cytosolic redox state

Lactate and pyruvate concentrations and lactate/pyruvate ratio were determined in arterial as well as in portal and hepatic venous blood as described for the previous experiment. Hepatic lactate clearance was subsequently calculated as the product of portal venous and hepatic arterial blood flow times the portal-hepatic venous and the arterial-hepatic venous concentration differences, respectively.

Other measurements

We also determined arterial, portal and hepatic vein glucose concentrations, hepatic and gut lactate and amino-acid fluxes. Arterial, portal, and hepatic venous NO^{2-} + NO^{3-} concentrations were determined. Portal and hepatic venous blood was collected to measure tumor necrosis factor-alpha (TNFα) and IL-10 levels (ELISA for TNFα; Endogen, Woburn, MA, IL-10: BioSource International, Camarillo, CA). The detection limit of TNFα was 10 pg/mL, and the limit was 3 pg/mL for IL-10. Portal and hepatic venous 8-isoprostane (8-epi Prostaglandin F2α) levels were determined as a direct marker of lipid peroxidation ([cxci]) by using a commercial enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). The measured concentrations of NO^{2-} + NO^{3-}, cytokines, and isoprostanes were normalized per gram of total plasma protein content to correct for any dilutional effects of intravenous fluids ([cxcii]). Further we determined nitric oxide exhalation, as a surrogate of total body NO production, by using a chemiluminescence analyser (NOA™ 280 NO Analyzer, Sievers Medical Instruments, Boulder, CO, USA). The same analyser was also used for the determination of arterial, portal and hepatic venous nitrate concentrations. Liver and ileal tissue adenyl nucleotide content in biopsies, frozen in liquid nitrogen immediately after sampling, was measured after separation by high performance liquid chromatography by using a 5-µm ODS Sperisorb column (0.46 x 18 cm) at 30°C. Elution was with 25 mM sodium pyrophosphate/pyrophosphoric acid, pH 5.75, with a flow rate of 1.2 mL/min. Absorbance was measured at 254 nm. From these data, we calculated the liver and ileal ATP/ADP ratio and nucleotide energy charge (ATP + 0.5 ADP]/[ATP + ADP + AMP) ([cxciii]).

Statistical analysis

All results in the tables are expressed as median and 25 – 75 percentile. The intra-group differences relative to measurements post LPS prior to ATP-$MgCl_2$ or placebo infusions were analysed with Friedmann ANOVA test. The inter-group differences

were analysed using Kruskall-Wallis variance-Test. The level for significant differences of <0.05 was considered.

5.3 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 IL-10, TNFα, IFNγ and other cytokines in human whole blood cultures stimulated with LPS. This is an important part of pre-clinical assessment as infusing ATP-$MgCl_2$, ATP or its breakdown product adenosine may influence cytokine secretion in humans and depending on circumstances modulate immune system function in beneficial or detrimental way. *Ex vivo* LPS-stimulated whole human blood cytokine production assay is considered a relevant model of human leukocyte function rather than the 'classical' leukocyte cultures of isolated peripheral blood mononuclear cells. Although it cannot fully mimic the interactions *in vivo* the system allows for more complete evolvement of the complex cross talk of immune cells with respect to local compartmentalized cytokine responses ([cxciv,cxcv]). It is characterized by stable leukocyte counts and high leukocyte viability throughout the experimental period. Oxygen consumption in the whole blood decreases slowly whereas carbon dioxide partial pressure increases accordingly throughout the culture time thus there is no need to keep cultures in 5%CO_2 atmosphere ([cxciv]).

5.3.1 Effect of adenosine on IL-10 secretion

Venous blood (5mL) was collected from 6 healthy volunteers (3 females, 3 males, aged 22-44yrs) into heparinized Vacutainers (Becton Dickinson Pty. Ltd., Lane Cove, NSW, Australia). Aliquots of whole blood (300µL) were mixed with same amount of serum free culture media RPMI 1640 containing 20 mM HEPES (pH 7.3), (Sigma-Aldrich Pty. Ltd, Castle Hill, NSW, Australia) in hydrophobic microtubes (SSI, Lodi, CA, USA) and cultured in the presence or absence of 100ng/mL LPS (Escherichia coli, Serotype 055:B5, Sigma-Aldrich Pty. Ltd, Castle Hill, NSW, Australia). Adenosine (30µM) was added 4 times over 2 hours (120µM total) to mimic *in vivo* situation of ongoing adenosine release during sepsis. 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. 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 by centrifugation and stored at - 40°C until analysed. We measured IL-10 in the plasma by ELISA (R&D Systems, Inc. Minneapolis, MN, USA) in accordance with the manufacturer's instructions. In addition, leukocyte numbers in whole blood were measured with an automated Cell Counter (ADVIA®120, Bayer Health Care, Dublin Ireland).

Statistical analysis

The IL-10 levels were corrected for respective blood leukocyte counts and levels from same individual's duplicate specimens were averaged. The values for each incubating

condition are presented as median and range interval. For statistical analysis, we used the Wilcoxon signed rank test and Spearmann's correlation coefficient as appropriate, p value of < 0.05 was considered significant.

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

In this experiment we used commercial whole human blood model Instant Leukocyte Culture System (ILCS®) containing LPS (derived from Escherichia coli serotype O55:B5) obtained from EDI GmbH, (Reutlingen, Germany) as described ([cxcvi]). In brief, 1mL of peripheral venous blood was collected to multifunctional tubes that were supplied pre-filled with a specialized cell culture medium (using RPMI 1640 as basis), supplemented with heparin as anticoagulant and 10 ng of LPS/mL. The ILCS® tubes firstly serve as syringes to draw blood, which is thus immediately stimulated by LPS, and thereafter as non-adherent culture vessels thereby avoiding additional cellular manipulations.
To test whether the culture conditions in the absence of LPS lead to cytokine release we incubated venous blood from 7 volunteers with 50 μL of RPMI 1604 medium (nucleotide diluent) in tubes identical to ILCS® but without LPS. To study the effects of ATP (Sigma, St. Louis, MO, USA) on LPS induced cytokine production venous blood was drawn into the ILCS® tubes from 7 other healthy human volunteers (4 females, 3 males) and 50μL of ATP or RPMI 1640 medium was injected to the ILCS® within the next 30 minutes. The final concentration of ATP was 100μM. The ILCS® tubes were then incubated for 24 hours. At the end of the incubation period, serum separators (a valve septum that hermetically separates leukocytes from the supernatant and avoids sample centrifugation) were assembled, carefully inserted and positioned 2-3mm above the blood sediment level. After closing the tubes with a screw cap samples were frozen at -20^{o}C until cytokine levels were determined.
The concentrations of cytokines and chemokines in the samples were determined using multiplex cytokine kits obtained from Biosource (Luminex® 10-plex, Biosource, Camarrillo, CA) able to determine IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNFα, IFNγ, and GM-CSF. 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. Samples also were diluted in assay diluent in order to obtain cytokines readings that were expected to exceed the standard curve. Standard curves were constructed as per the manufacturer's instructions. Samples and standards were assayed on a robotic liquid handling workstation (epMotion 5075, Eppendorf, Germany) with built-in vacuum manifold for washing the bead conjugates according to the manufacturer's instructions. 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-^{o}C) with shaking and protected from light. All samples were analysed on a Luminex system using the Bio-Plex Manager v4.0 software (Bio-Rad, Hercules, CA) reading a minimum of 100 beads per analyte per sample. Data on cytokine and chemokine levels from the same individual's duplicate specimens were averaged and are presented as means and SEM in 7 healthy subjects. Statistical analysis was performed using the Wilcoxon signed rank test.

6.
RESULTS

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

6.1.1 Effect on systemic and regional hemodynamics and oxygen transport

Thoracic aortic clamping produced tachycardia in all 3 groups of animals and reached dangerous levels in the sodium nitroprusside (SNP) group. Tachycardia was less in the SNP/Esmolol group while ATP-$MgCl_2$ caused moderately elevated heart rate. Accordingly, the catecholamine (adrenaline, noradrenaline) serum concentrations were elevated, especially in the SNP and ATP-$MgCl_2$ groups. Despite the catecholamine surge the proximal MAP could be controlled in all three groups and even mildly lowered in the ATP-$MgCl_2$ and SNP/Esmolol group. Systemic vascular resistance distal to clamp decreased during clamping and returned back to pre-clamp levels after reperfusion in all three groups. The effect on SVR was pronounced with ATP-$MgCl_2$ and was associated with stroke volume increase during clamping while remaining constant in the other two groups. Cardiac output approximately doubled in both SNP and ATP-$MgCl_2$ group and remained constant in the SNP/Esmolol group. In contrast to the SNP group, ATP-$MgCl_2$ treated animals doubled cardiac output by increasing the stroke volume rather then heart rate. 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 as expected. The portal blood flow was diminished during clamping, although residual portal venous flow was demonstrated in the SNP/Esmolol group (5ml/kg/min) No hepatic arterial flow was present while the aorta was clamped. 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 and was not increased during the reperfusion phase in either group.

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

	Pre-clamp	End of clamp	2 hours reperfusion	4 hours 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)
MPAP [mmHg]				

A	23 (21-25)	24 (23-27)	24 (22-29)	23 (21-26)
B	24 (22-26)	27 (25-28)	26 (21-32)	25 (22-28)
C	22 (20-24)	22§ (21-23)	23 (23-27)	22 (21-23)
Adrenalin [pg/ml]				
A	15 (15-15)	3659# (2818-4781)	Not measured	17 (15-36)
B	15 (15-25)	4062# (2310-5990)		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)

VO_2 [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)
Q_{PV} [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)
Q_{HA} [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)
Q_{Liver} [ml/kg/min]				
A	18 (14-27)	0 (0-1)	25 (19-37)	20 (16-29)
B	14 (12-26)	0 (0-1)	19 (17-31)	14 (11-23)
C	21 (16-23)	5 (4-13)	29 (26-31)	24 (18-26)

Values are presented as median and 25-75 percentile.
#: $p < 0,05$ vs. Pre-clamp, §: significant inter-group 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)

6.1.2 Effect 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. In the same time portal and hepatic venous pH decreased significantly and the acidosis persisted for 2 hours of reperfusion. The SNP/Esmolol and the ATP-$MgCl_2$ group tended to better restore acid-base status. Metabolic acidosis resolved in all three groups of animals at the end of experiment. Lactate increased in all sampled sites during clamping and while there was no difference in systemic lactate levels, animals in the SNP/Esmolol and ATP-$MgCl_2$ group produced significantly less lactate in the gut draining into portal vein. 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 ratio, a marker of cellular redox state increased during clamping in systemic circulation as well as in the hepatosplanchnic region. Interestingly, L/P ratio was highest in the SNP/Esmolol group. The liver utilized lactate before the clamping but lactate metabolism by the liver was grossly reduced during aortic 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: Systemic, intestinal und hepatic acid-base and redox parameters.

			2 hours	4 hours

	Pre-clamp	End of clamp	reperfusion	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-BE				
A	5 (4-6)	-1# (-3-0)	1# (-2-2)	4# (3-4)
B	5 (5-7)	-1# (-2- 0)	0# (-1-2)	5 (3-6)
C	3 (2-3)	-3# (-4 –1)	0# (-1-2)	2 (2-3)
PV-BE				
A	6 (6-8)	-3# (-6- -3)	1# (-2-3)	5# (4-6)
B	7 (6-8)	-3 (-5- -2)	1 (-1-2)	5 (3-7)
C	6 (4-6)	-4# (-6-0)	1 (0-3)	3 (2-4)
HV-BE				
A	8 (7-8)	-1# (-5- 0)	3# (-1-4)	6# (5-7)
B	7 (6-10)	1# (-2-1)	2# (1-3)	7 (6-7)
C	6§ (4-6)	-2# (-4- -1)	2# (0-3)	4#§ (2-5)
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 inter-group 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)

6.1.3 Effect on small bowel metabolism

In contrast to the relatively balanced bowel lactate metabolism (data not shown), 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	4 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 inter-group 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 in figure 4.

Figure 4.

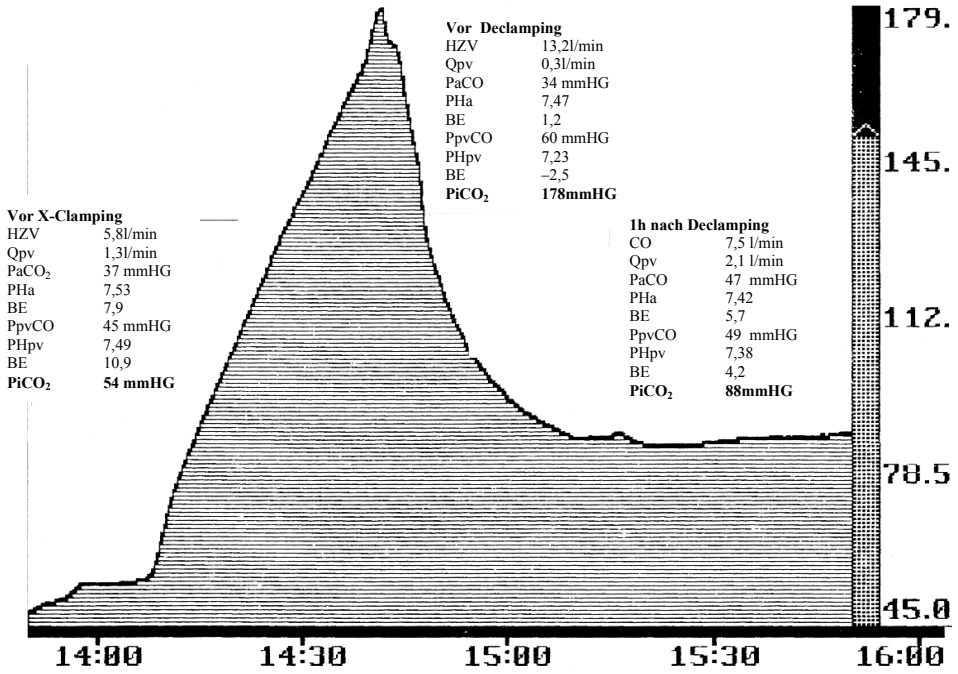


Fig 4. Interstitial pCO_2 tonometry of the jejunal mucosa during thoracic aortic clamping and reperfusion.

6.1.4 Effect on hepatosplanchnic biochemical parameters

As expected the liver enzymes AST and ALT were reduced in the ischemic phase while liver reperfusion was associated with rise of transaminases that reached significance for AST in the SNP and ATP-$MgCl_2$ groups at both measurement times. In contrast the ALT levels were normalized by 4 hours of reperfusion. A possible explanation is that skeletal muscles contributed to the high AST release. The bilirubin levels were reduced during ischemia in the SNP/esmolol group and decreased during the reperfusion phase in all groups. In contrast creatinine levels rose during the clamping phase in all groups and remained elevated at 2 hours of reperfusion in SNP and ATP-$MgCl_2$ group. The urea levels were unchanged regardless of experimental phase and treatment.

Table 4: Biochemical parameters related to organs below the clamp level

	Pre-clamp	End of clamp	2 hours reperfusion	4 hours reperfusion
AST [U/l]				
A	23 (18-30)	19# (14-24)	29# (23-38)	32# (26-45)
B	19 (16-22)	16 (14-22)	30# (19-81)	38# (23-86)
C	48§ (29-50)	34# (23-39)	50 (39-72)	58 (42-90)
ALT [U/l]				
A	24 (20-30)	18# (15-23)	21 (18-26)	24 (21-28)
B	22 (21-28)	16# (15-19)	19# (17-22)	21 (17-26)
C	32 (29-35)	21# (18-25)	27# (23-31)	29 (25-34)
Bilirubin [mg/dl]				
A	1,5 (1,2-2,1)	1,6 (1,3-2,3)	1,1-1,0-1,3)	1,1# (0,9-1,3)
B	2,5§ (1,6-3,3)	2,1§ (1,8-2,6)	1,5#§ (1,0-1,6)	1,3# (1,0-1,4)
C	1,2 (1,1-1,3)	1,0# (0,9-1,3)	0,8# (0,7-0,9)	0,8# (0,7-1,1)
Creatinine [mg/dl]				
A	1,4 (1,1-1,4)	1,7# (1,3-1,8)	1,7# (1,4-2,3)	1,4 (1,2-1,6)
B	1,4 (1,2-1,6)	1,5# (1,4-1,8)	1,5# (1,3-1,7)	1,5 (1,3-1,7)
C	1,3 (1,3-1,4)	1,5# (1,5-1,7)	1,4 (1,3-1,6)	1,3 (1,2-1,6)
Urea [mg/dl]				
A	0,3 (0,2-0,3)	0,3 (0,2-0,3)	0,3 (0,2-0,3)	0,3 (0,2-0,3)
B	0,3 (0,3-0,3)	0,3# (0,2-0,3)	0,3# (0,3-0,3)	0,3#§ (0,3-0,3)
C	0,2 (0,2-0,3)	0,2 (0,2-0,3)	0,2 (0,2-0,3)	0,2 (0,2-0,2)

Values are presented as median and 25-75 percentile.
#: $p < 0,05$ vs. Pre-clamp, §: significant inter-group difference.
A: SNP, B: ATP-$MgCl_2$, C: SNP/Esmolol
(*ALT*: alaninaminotransferase*, AST:* aspartataminotransferase)

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

6.2.1 Systemic hemodynamic and oxygen exchange effects

ATP-$MgCl_2$ or placebo was infused during long term hyperdynamic porcine endotoxemia characterised by a fall in systemic vascular resistance and a sustained increase in cardiac output ([clxxxiii,clxxxvi]). Despite a widespread inter-individual variability, the two experimental groups received nearly identical amounts of endotoxin 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.

	Before LPS	12 Hrs After LPS	18 Hrs After LPS	24 Hrs After LPS
MAP, mm Hg				
CON	92 (91, 97)	101 (97, 106)	100 (92, 103)	97 (87, 107)
ATP	94 (90, 96)	99 (91, 115)	82 (76, 88)[a]	78 (74, 87)[a,b]
MPAP, mm Hg				
CON	22 (20, 27)	39 (35, 43)	36 (30, 38)	37 (33, 39)
ATP	22 (19, 24)	38 (33, 38)	32 (30, 35)	35 (34, 36)
CVP, mm Hg				
CON	7 (6, 10)	17 (11, 20)[a]	19 (13, 20)[a]	17 (13, 21)[a]
ATP	8 (6, 11)	16 (11, 20)[a]	17 (13, 20)[a]	19 (13, 21)[a]
PAOP, mm Hg				
CON	9 (7, 11)	18 (16, 20)[a]	17 (16, 21)[a]	17 (16, 17)[a]
ATP	9 (7, 13)	17 (17, 19)[a]	19 (16, 20)[a]	17 (15, 22)[a]
ITBV, mL/kg				
CON	32 (28, 32)	36 (33, 38)	35 (31, 37)	35 (32, 39)
ATP	26 (23, 27)[b]	30 (26, 35)[a,b]	33 (28, 34)[a]	31 (28, 34)[a]
CI, $mL \cdot kg^{-1} \cdot min^{-1}$				
CON	116 (106, 123)	148 (117, 165)[a]	140 (133, 154)[a]	148 (140, 171)[a]
ATP	112 (90, 140)	134 (120, 148)[a]	159 (142, 192)[a]	175 (156, 204)[a]
SVR, $dyn \cdot sec \cdot cm^{-5}$				
CON	1216 (1159, 1504)	929 (784, 1316)[a]	917 (767, 1136)[a]	929 (788, 1067)[a]
ATP	1427 (1182, 1538)	1061 (925, 1276)	653 (588, 797)[a,b]	531 (525, 653)[a,b]
$\dot{D}o_2$, $mL \cdot kg^{-1} \cdot min^{-1}$				
CON	15 (13, 16)	14 (13, 18)	14 (12, 16)	14 (12, 16)
ATP	13 (11, 16)	14 (13, 14)	14 (13, 17)	14 (13, 18)
$\dot{V}o_2$, $mL \cdot kg^{-1} \cdot min^{-1}$				
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)
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)[a]	7.39 (7.35, 7.43)[a]	7.38 (7.35, 7.44)[a]
ATP	7.45 (7.44, 7.48)	7.39 (7.38, 7.42)[a]	7.38 (7.37, 7.39)[a]	7.36 (7.33, 7.41)[a]
Hct, %				
CON	28 (26, 30)	23 (19, 23)[a]	21 (17, 22)[a]	19 (18, 20)[a]
ATP	24 (22, 25)	23 (19, 24)[a]	18 (17, 19)[a]	18 (17, 19)[a]

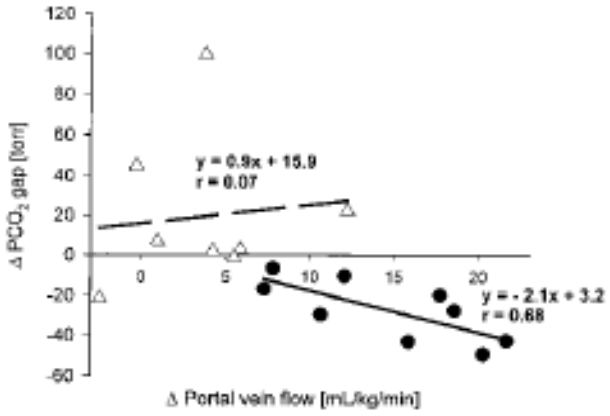
This was more then compensated for by an increase in cardiac output leading to enhanced systemic and portal venous blood flow, whereas hepatic arterial flow remained unchanged. This phenomenon is consistent with preservation of the hepatic arterial buffer response, which is normally ablated by LPS and resulted in a higher total liver blood flow as well as 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 inter-group differences. Hepatosplanchnic hemodynamics, oxygen exchange, and metabolic variables and hepatic and portal venous pH are summarized in Table 6.

Table 6.

	Before LPS	12 Hrs After LPS	18 Hrs After LPS	24 Hrs After LPS
Qha, mL·kg^{-1}·min^{-1}				
CON	4.1 (2.8, 6.2)	2.5 (2.2, 3.2)	5.3 (3.7, 6)[a]	5 (4.2, 5.5)[a]
ATP	2.1 (1.4, 5.8)	2.2 (1.8, 3.2)	3.4 (1.3, 10)[a]	2.3 (1.5, 10.4)
Qpv, mL·kg^{-1}·min^{-1}				
CON	22 (18, 23)	25 (22, 28)[a]	23 (19, 28)	28 (26, 31)[a]
ATP	19 (18, 24)	22 (20, 29)	42 (31, 43)[a,b]	42 (36, 45)[a,b]
Qliv, mL·kg^{-1}·min^{-1}				
CON	26 (22, 29)	30 (26, 31)[a]	28 (23, 37)	34 (31, 40)[a]
ATP	22 (20, 34)	26 (22, 31)	46 (42, 53)[a,b]	50 (38, 53)[a]
hDo_2, mL·kg^{-1}·min^{-1}				
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)
ATP	1.8 (1.5, 2.5)	1.7 (1.7, 2.7)	3.4 (2.5, 3.6)[a,b]	2.8 (2.7, 3.2)[a]
Gut Eo_2, %				
CON	32 (31, 35)	26 (23, 28)[a]	22 (22, 26)[a]	22 (19, 23)[a]
ATP	35 (30, 45)	34 (27, 36)	20 (19, 23)[a]	20 (17, 24)[a]
Pco_2 gap, torr				
CON	15 (10, 22)	30 (21, 43)[a]	44 (28, 70)	50 (32, 64)
ATP	16 (14, 24)	41 (32, 60)[a]	18 (14, 21)[b]	18 (12, 22)[b]
Gut Co_2 a-v, mmol/min				
CON	6.7 (5.3, 8)	5.6 (2.7, 6.5)	5.8 (3.1, 7.2)	2.1 (1.8, 3.5)
ATP	5.3 (4.3, 6.3)	3.5 (1.4, 4.3)	2.9 (0.7, 4.2)	1.5 (1.2, 3.6)
hVo_2, mL·kg^{-1}·min^{-1}				
CON	0.8 (0.6, 1.1)	0.8 (0.6, 1)	0.6 (0.5, 0.8)	0.7 (0.5, 0.9)
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 (7.45, 7.48)	7.34 (7.31, 7.36)[a]	7.35 (7.33, 7.39)[a]	7.34 (7.31, 7.38)[a]
ATP	7.43 (7.40, 7.46)	7.34 (7.32, 7.38)[a]	7.34 (7.33, 7.39)[a]	7.33 (7.29, 7.37)[a]
pv pH				
CON	7.46 (7.45, 7.46)	7.35 (7.31, 7.36)[a]	7.36 (7.32, 7.38)[a]	7.34 (7.31, 7.4)[a]
ATP	7.42 (7.39, 7.43)	7.34 (7.34, 7.38)[a]	7.35 (7.33, 7.39)[a]	7.33 (7.30, 7.38)[a]
Gut lactate balance, μmol·kg^{-1}·min^{-1}				
CON	−3.8 (−5.1, −3.4)	−2.5 (−4, −1.7)	−3.0 (−5.4, −2.1)	−3.5 (−3.8, −2.6)
ATP	−4.5 (−5.7, −2.6)	−2.7 (−6.7, −2.4)	−3.8 (−5.1, −0.5)	−4.7 (−10, −4.4)[b]
Liver lactate balance, μmol·kg^{-1}·min^{-1}				
CON	15.4 (11.5, 20)	9.8 (7.7, 12.1)	6.6 (3.4, 8.9)[a]	6.9 (4.2, 11.9)[a]
ATP	11.3 (9.9, 16.8)	10.7 (10.1, 13.1)	10.6 (9.3, 12)[b]	14.5 (12.7, 16)[b]

Endotoxin infusion also increased the ileal mucosal – arterial pCO_2-gap, which in contrast to the aortic clamping study, was restored to baseline levels by ATP-$MgCl_2$ until the end of the experiment. These changes in the pCO_2 gap were inversely correlated with portal vein flow variations in the ATP-$MgCl_2$ group but not in control animals (Fig. 5).

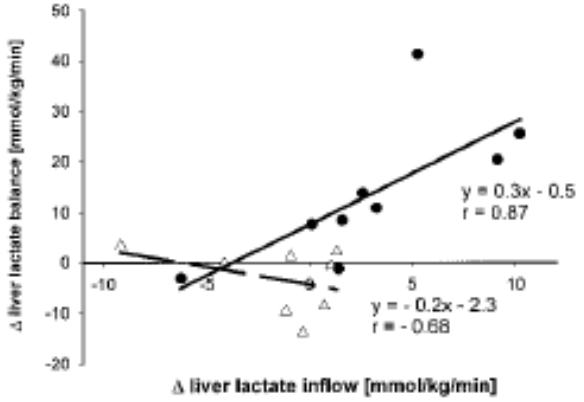
Figure 5. Changes (Δ) in the mucosal-arterial pCO_2 gap of the ileum plotted as a function of variations in portal vein blood flow. Triangles: control animals, circles: ATP-$MgCl_2$ group.



Such changes in the gastrointestinal circulation could be either due to an increased microcirculatory blood flow, and/or a redistribution of the perfusion within the bowel wall thus supporting the role of ATP as a vasoregulatory molecule ([xlix,cxcvii,cxcviii]). However, infusion of ATP-$MgCl_2$ did not increase the number of perfused ileal mucosal villi assessed by orthogonal remission spectrophotometry video imaging. Also arterial, hepatic vein, or portal venous lactate/pyruvate ratios and amino-acid fluxes were not different between the groups (data not shown). Nevertheless, ATP-

$MgCl_2$ maintained the physiologic coupling between gut lactate release and hepatic uptake that was markedly impaired by endotoxin (Table 6., Fig. 6).

Figure 6. Changes (Δ) in the liver lactate uptake rate plotted 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 ([cxcix]).

6.2.2 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.

	ATP, nmol/g	ADP, nmol/g	AMP, nmol/g	ATP/ADP	NEC
Gut, nmol/g					
CON	193 (177, 203)	98 (81, 115)	79 (70, 83)	2.1 (1.7, 2.5)	0.7 (0.6, 0.7)
ATP	171 (161, 244)	113 (85, 136)	173 (71, 255)	1.9 (1.2, 2.99)	0.5 (0.5, 0.6)
Liver, nmol/g					
CON	216 (183, 239)	48 (36, 62)	121 (84, 208)	3.9 (3.2, 7.3)	0.6 (0.5, 0.7)
ATP	191 (164, 209)	43 (26, 143)	187 (153, 200)	4.6 (1.3, 6.6)	0.5 (0.5, 0.6)

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 inter-group 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).

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

6.3.1 Effect of adenosine on IL-10 secretion in WBC

We added adenosine in four aliquots over 2 hours to whole human blood diluted in 1:1 ratio with RPMI1640 culture media in a final concentration of 120μM. The samples were then cultured for 4 and 8 hours, respectively. As there was negligible IL-10 production at 4 hours of culture time regardless of incubating conditions those results are presented only in a table (table 8.) and graphic format and the results for 8 hours are discussed here. Minimal IL-10 production was observed in cultured blood without either LPS or adenosine. Similarly, the addition of adenosine without LPS led to only minimal 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.

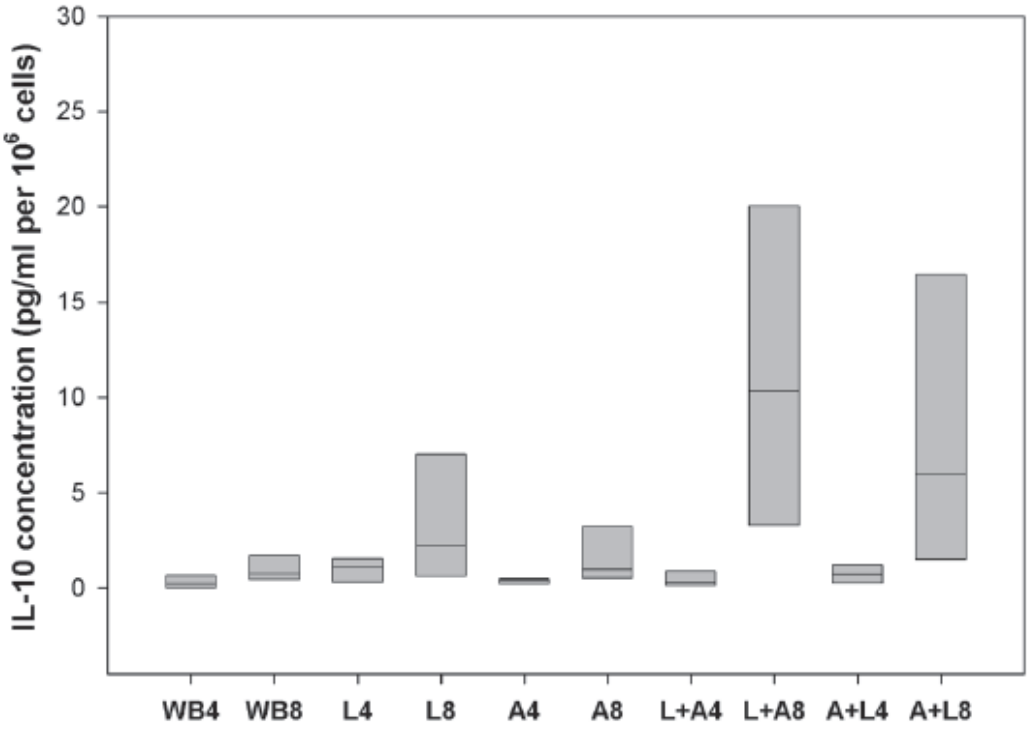


Table 8.

IL-10 concentrations from individual samples expressed in pg/ml/106 leukocytes in WBC under different conditions

	WB 4	WB 8	L 4	L 8	A 4	A 8	L+A 4	L+A 8	A+L 4	A+L 8
1	0.96	0.21	0.45	0.00	0.66	6.35	0.19	25.78	1.28	26.75
2	0.00	0.69	0.00	1.71	0.18	0.89	0.00	0.96	0.02	1.85
3	0.02	h	1.68	0.82	0.40	0.54	1.25	18.12	0.90	12.99
4	0.55	0.74	0.75	2.72	0.27	1.08	0.76	4.06	1.17	5.16
5	0.36	0.78	1.48	6.71	0.43	2.17	0.25	11.47	0.35	6.82
6	0.09	2.60	1.47	8.02	0.42	0.49	0.37	9.23	0.53	0.50
median	0.23	0.74	1.11	2.21	0.41	0.99	0.31	10.35	0.71	5.99

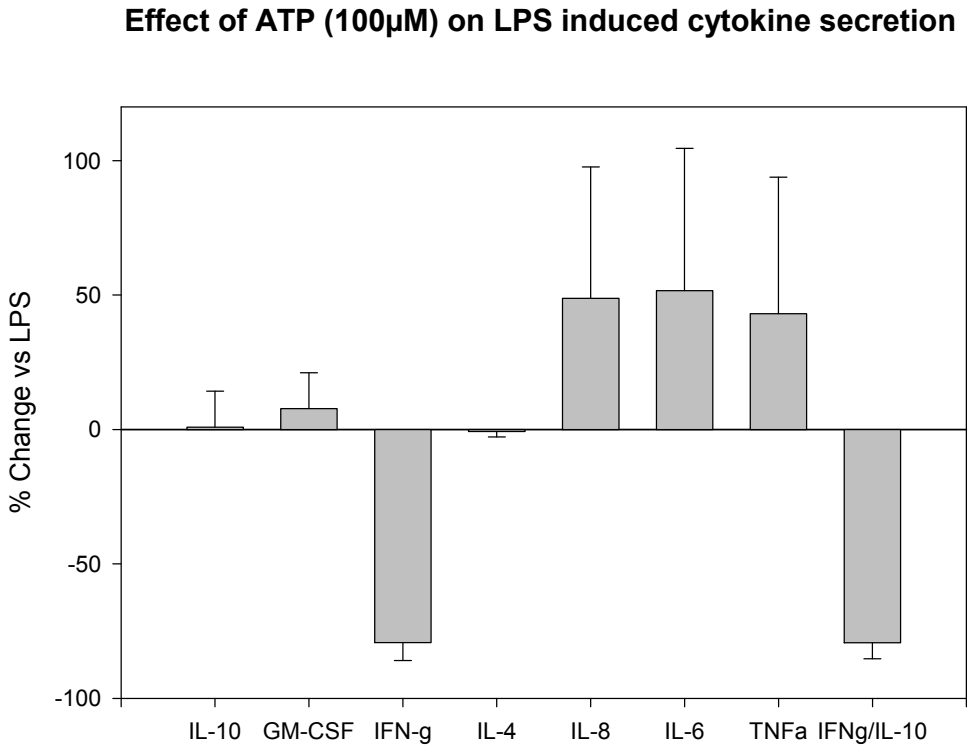
*h: Samples haemolysed.

WB 4—whole blood culture (WBC) incubated for four hours; WB 8—WBC incubated for eight hours; L 4—lipopolysaccharide (LPS) stimulated WBC incubated for four hours; L 8—LPS stimulated WBC incubated for eight hours; A 4—WBC incubated with adenosine for four hours; A 8—WBC incubated with adenosine for eight hours; L+A 4—LPS stimulated WBC with adenosine post-treatment for four hours; L+A 8—LPS stimulated WBC with adenosine post-treatment for eight hours; A+L 4—LPS stimulated WBC with adenosine pre-treatment for four hours; A+L 8—LPS stimulated WBC with adenosine pre-treatment for eight hours.

6.3.2 Effects of ATP on cytokine secretion in WBC

In whole blood from seven healthy human volunteers incubated for 24-hours in the absence of both LPS and exogenous ATP we measured multiple cytokine production using Luminex® 10-plex kit. The following cytokines were measured 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 except for minute concentrations of IL-5 and IL-10 in whole blood from one volunteer (0.09and 0.62 pg/mL respectively). 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 and thus could not be accurately quantified (data not shown).
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). Such a consistent response was not observed for other cytokines. The mean absolute levels of TNFα were lower with ATP treatment but in three out of seven individuals more TNFα was released. The apparently higher IL-8 levels in ATP treated cultures were due to an increased secretion in just two volunteers. Finally, the reduced IFNγ secretion following ATP incubation resulted in a decrease of the IFNγ/IL-10 ratio from 63 with LPS alone to 8 with LPS and ATP together (p=0.018).

Figure 8. Data are expressed as percentage, with zero line representing individual cytokines release under stimulation by LPS.



7.
DISCUSSION

7.1 Hemodynamic effects of ATP-$MgCl_2$ likely mediated by purinergic receptors are responsible for its effects in I-R and sepsis.

The aim of the animal studies was to confirm the previously reported, and above discussed, effects of ATP-$MgCl_2$ on systemic and hepatosplanchnic hemodynamics, oxygen exchange, and metabolism as well as markers of reperfusion injury in I-R and endotoxin induced injury. Titrating proximal (above clamp) 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 standard treatment with sodium nitroprusside (SNP). The reactive tachycardia was less pronounced, the cardiac inotropy enhanced and proximal MAP was easier controlled with ATP-$MgCl_2$. Nevertheless, the best hemodynamic profile during cross clamping was achieved by the combination of SNP and esmolol, confirming the well known beneficial effects of beta blockade on perioperative outcomes ([cc]). Both ATP-$MgCl_2$ and SNP increased oxygen delivery by increasing cardiac output, however, as overall oxygen consumption was not increased, it does dangerously stress the heart

without a beneficial metabolic effect. During clamping induced ischemia both arterial and portal blood flow to the liver were reduced. As opposed to combined SNP/esmolol treatment ATP-$MgCl_2$ led to 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. The beta-adrenergic receptor blockade proved to be useful in this model, particularly in the light of very high catecholamine plasma levels produced by the high thoracic aortic clamping.

In the long-term hyperdynamic model of porcine sepsis we demonstrated that ATP-$MgCl_2$ maintained and tended to further increase cardiac output above the effects of endotoxin alone but oxygen delivery remained the same possibly secondary due to intrapulmonary shunting. As expected from its vasodilator properties, ATP-$MgCl_2$ also decreased mean arterial pressure 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 ([cci,ccii]). This effect might be explained by ATP-$MgCl_2$-induced improvement in endothelium-dependent relaxation ([156]) 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, ([cciii,cciv]) leading to prostacyclin and NO production, arteriolar smooth muscle relaxation, ([ccv]) and increase in portal blood flow ([ccvi]) since both molecules are important endogenous regulators of hepatic arterial buffer response ([ccvii]). Moreover ATP-$MgCl_2$ allowed maintenance of hepatic lactate uptake, presumably by preserving the metabolic coupling between liver lactate influx and utilization. Similarly, ATP-$MgCl_2$ significantly increased splanchnic blood flow, which was associated with a normalization of the ileal mucosal-arterial pCO_2 gap despite the fact that microcirculatory derangements caused by endotoxin were not altered by ATP-$MgCl_2$.

Importantly, by measuring tissue adenine nucleotides concentrations we could not confirm that ATP-$MgCl_2$ treatment leads to improved tissue energy charge ([c]) 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.

7.2 Extracellular adenosine and ATP lead to immune suppressive cytokine milieu in LPS stimulated human WBC

Since purinergic receptor are implicated in some if not all of ATP-$MgCl_2$ effects ([clviii,clix]) we have further investigated the effects of adenosine and ATP on immune system by means of studying their respective role on cytokine secretion in whole human blood cultures (WBC) stimulated with endotoxin.

Stimulated WBC serves as a simple model of human sepsis allowing for the development of complex cellular and humoral network in the blood compartment. The main findings of the *ex vivo* studies are that adenosine enhanced early IL-10 and ATP reduced IFNγ secretion in endotoxin stimulated human whole blood compared to stimulated control cultures. 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 although very high concentration of some cytokines (IL-1β, IL-8, TNFα) in several individual' cultures precluded their measurement with the particular assay used an inherent feature of the multiplex beads method.

The early rise in IL-10 secretion upon exposure to adenosine (8 hours) seems to occur 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. ([lxvi]) Nevertheless, our results are concordant with others who demonstrated that adenosine leads to enhanced IL-10 production in human monocytes activated by LPS ([lxxxiv]) and that addition of the non-selective adenosine agonist (NECA) or adenosine A2a receptor agonist (CGS-21680) increases IL-10 production in LPS (1μg/mL) as well as in *Staphylococcus aureus* stimulated WBC after 18hrs ([ccviii]). 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 IL-10 production after 8 hours ([ccix]). Although we could prove that adenosine leads to an early rise in LPS stimulated IL-10 secretion we have also observed variations in individual responses. This 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 ([ccx,ccxi]). For example recent reports have shown association of allele G in the IL-10-1082 gene promotor region with propensity for increased IL-10 production in severe infections. Patients with this particular genotype have impaired bacterial clearance, are more likely to develop septic shock, and have increased morbidity and mortality ([ccxii]). Similarly to IL-10, the inter-individual TNFα secretion response to 100 μM ATP varied and we could not confirm consistent change in TNFα secretion as reported in the previous study ([lxvi]).

Comparatively, 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 ([ccxiii,ccxiv]).

Human studies have clearly shown that IFNγ production to different stimuli *in vitro* by monocytes and lymphocytes as well as *ex vivo* in whole blood cultures is severely depressed in critically ill patients ([ccxv,ccxvi]). Decreased synthesis of IFNγ by lymphocytes ([ccxvii]) as well as of IFNγ in whole blood after cardiac surgery has been reported ([ccxviii,ccxix]). 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 ([ccxx]). 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" ([ccxxi]). Firstly, deactivation of IFNγ producing leukocytes following an insult and secondly, the presence of serum suppressive factors different from IL-4, IL-10, or TGFβ$_1$. Based on our results we suggest that extracellular release of ATP may mediate, at least in part, the suppression of IFNγ secretion thus inducing immune suppression.

SUMMARY OF CONCLUSIONS

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

The previously reported beneficial effects of the intriguing substance ATP-$MgCl_2$ were tested in two clinically relevant large animal models. We observed mainly cardiac and vascular effects of ATP-$MgCl_2$ likely related to purinergic receptors stimulation. Using comparably higher concentrations of ATP and its degradation product adenosine we have further tested whether modulation of inflammation might be responsible for some of the ATP-$MgCl_2$ effects adding them to *ex vivo* LPS stimulated whole human blood cultures and measuring resulting cytokine secretion. The results can be summarized as follows:

- Infusing ATP-$MgCl_2$ intravenously in a porcine I-R injury model using high 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 superior hemodynamic control to ATP-$MgCl_2$.
- In the 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 on hepatosplanchnic hemodynamics and metabolic function we were unable to observe diminished reperfusion related structural injury with ATP-$MgCl_2$.
- In the same model we were unable to confirm increased tissue adenine nucleotides concentrations after several hours of ATP-$MgCl_2$ infusion suggesting that provision of energy and/or substrates for endogenous tissue nucleotides recovery is not the mechanism of ATP-$MgCl_2$ action at least not with the doses used in our experiment. Instead, the vascular effects observed were similar to those reported for purinergic receptor stimulation.
- Adding the retaliatory metabolite adenosine to LPS stimulated whole human blood lead to increased early release of IL-10. This suggests that extracellular adenosine at clinically relevant levels may contribute to earlier and higher secretion of IL-10 during endotoxemia thus potentially preventing host tissue damage but impairing immune defence against pathogens.
- Using similar more standardized whole human LPS stimulated 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 subsequent immune stimulation are associated with nosocomial infections, poor infection clearance and increased mortality.

9.
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