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# Effects of a human recombinant alkaline phosphatase during impaired mitochondrial function in human renal proximal tubule epithelial cells

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## ABSTRACT

Sepsis-associated acute kidney injury is a multifactorial syndrome in which inflammation and renal microcirculatory dysfunction play a profound role. Subsequently, renal tubule mitochondria reprioritize cellular functions to prevent further damage. Here, we investigated the putative protective effects of human recombinant alkaline phosphatase (recAP) during inhibition of mitochondrial respiration in conditionally immortalized human proximal tubule epithelial cells (ciPTEC). Full inhibition of mitochondrial oxygen consumption was obtained after 24 h antimycin A treatment, which did not affect cell viability. While recAP did not affect the antimycin A-induced decreased oxygen consumption and increased hypoxia-inducible factor-1α or adrenomedullin gene expression levels, the antimycin A-induced increase of pro-inflammatory cytokines IL-6 and IL-8 was attenuated. Antimycin A tended to induce the release of detrimental purines ATP and ADP, which reached statistical significance when antimycin A was co-incubated with lipopolysaccharide, and were completely converted into cytoprotective adenosine by recAP. As the adenosine $A_{2A}$ receptor was up-regulated after antimycin A exposure, an adenosine $A_{2A}$ receptor knockout ciPTEC cell line was generated in which recAP still provided protection. Together, recAP did not affect oxygen consumption but attenuated the inflammatory response during impaired mitochondrial function, an effect suggested to be mediated by dephosphorylating ATP and ADP into adenosine.

## 1. Introduction

Acute kidney injury (AKI) is a serious complication in critically ill patients that is independently associated with poorer outcomes. Consequently, AKI results in a tremendous burden for both patient and society (Lameire et al., 2013). In patients in the Intensive Care Unit (ICU), the incidence of AKI is 55–60% with an associated mortality of approximately 30% (Hoste et al., 2015). AKI may develop following cardiovascular surgery, administration of nephrotoxic drugs, trauma, and sepsis, with the latter representing the most prevalent cause (Case et al., 2013). Sepsis-associated AKI increases mortality up to 70% and survivors have a higher risk to develop end-stage renal

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disease (Bagshaw et al., 2007; Chawla et al., 2011). Despite the conduct of numerous clinical trials (Faubel et al., 2012), pharmacological therapeutic options are still unavailable and treatment is limited to supportive measures like renal replacement therapy.

Sepsis-associated AKI is a complex interplay of inflammation, microvascular dysfunction, and unbalanced renal bioenergetics (Gomez et al., 2014). Sepsis is accompanied by the systemic release of inflammatory mediators, endothelial leukocyte adhesion and activation of coagulation pathways, thereby promoting inflammation (Gustot, 2011). Within the kidney, the vascular endothelium and tubular cells are directly exposed to pathogens and danger-associated molecular patterns (PAMPS and DAMPS) inducing a local inflammatory response. The release of mediators like cytokines, reactive oxygen species and reactive nitrogen species characterize this response which, together with endothelial swelling and arteriolar vasoconstriction, leads to a compromised renal microcirculation (Ince, 2005). In response to this inflammatory and hypoxic insult, tubular cells reprioritize and down-regulate cellular functions in order to prevent further damage. This adaptive response is mainly orchestrated by the mitochondria, organelles primarily responsible for providing energy, in the form of ATP, through oxidative phosphorylation (McBride et al., 2006; Osellame et al., 2012). When exposed to stress, mitochondria trigger several protective cellular processes: reprioritization of energy consumption, removal and digestion of dysfunctional organelles (autophagy and mitophagy) and initiation of cell cycle arrest (Gomez et al., 2014). In case of overwhelming or sustained cellular stress, as observed during sepsis, the protective capacity of mitochondria might be exhausted, thereby limiting their ability to prevent further renal impairment (Parikh et al., 2015). In addition, this may induce the release of mitochondrial constituents (*e.g.* mitochondrial DNA, reactive oxygen species and ATP), which are indicated as potent DAMPs and activate cells of the innate immune system (Beckman and Ames, 1998; Krysko et al., 2011).

It appears plausible that a pharmaceutical compound eliciting a multimodal mode of action might be needed to treat a multifaceted disease like sepsis-associated AKI. One of the limited number of candidate drugs facilitating such an approach, may be the enzyme alkaline phosphatase (AP). Two small patient studies demonstrated that treatment with bovine intestinal AP was associated with renal protective effects during sepsis-associated AKI (Heemskerk et al., 2009; Pickkers et al., 2012). To follow-up on these results, a human recombinant AP (recAP) was developed, which is highly stable as well as biologically active (Kiffer-Moreira et al., 2014). Previous data demonstrated that recAP provides protection during LPS-induced inflammation in human renal proximal tubule cells (PTEC) through dephosphorylation of endotoxin (LPS, lipopolysaccharide), involved in sepsis pathogenesis, and detrimental purines adenosine triphosphate (ATP) and adenosine diphosphate (ADP), released during inflammation and hypoxia (Peters et al., 2015). In addition, *in vivo*, recAP could not modulate LPS-induced changes in systemic hemodynamics and renal oxygenation, but recAP did exert a clear renal protective anti-inflammatory effect (Peters et al., 2016a, 2015). Considering the diverse processes involved in the pathogenesis of sepsis-associated AKI, the aim of this study was to investigate the putative renal protective effects of recAP upon impaired mitochondrial function. Mitochondrial dysfunction is effectively induced by inhibition of the mitochondrial oxidative phosphorylation (OXPHOS) system, as it is the major mitochondrial energy generating system (Hüttemann et al., Biochimica et Biophysica Acta, 2007). We achieved such a block of the respiratory chain using antimycin A, a bona fide inhibitor of the third OXPHOS complex in a unique human conditional immortalized PTEC model, ciPTEC (Wilmer et al., 2010).

## 2. Material and methods

### 2.1. Cell culture

Routinely, ciPTEC were cultured at 33 °C as described previously (Wilmer et al., 2010). Preceding each experiment, cells were seeded at a 55,000 cells/cm$^2$ density, incubated for 1 d at 33 °C followed by a 7-d maturation period at 37 °C. On the day of the experiment, cells were either directly harvested (titration experiments) or incubated for 24 h with 10 nM antimycin A from *streptomyces* sp. (5 mM in ethanol; Sigma-Aldrich, Zwijndrecht, The Netherlands) or 50 nM myxothiazol (5 mM in ethanol; Sigma-Aldrich, Zwijndrecht, The Netherlands), to completely inhibit oxygen consumption as described below (see high-resolution respirometry section). The latter incubations were performed with or without recAP (10 U/ml (Peters et al., 2015); 19 μg/ml; kind gift from AM-Pharma, Bunnik, The Netherlands), also referred to as chimeric AP (ChimAP) (Kiffer-Moreira et al., 2014). Control cells were incubated with culture medium solely. Inactive recAP (19 μg/ml, kind gift from AM-Pharma) was used as a negative control. In a different set of experiments, cells were co-incubated with 10 μg/ml LPS (*E. coli* 0127:B8; Sigma-Aldrich, Zwijndrecht, The Netherlands) dissolved in 10 mM HEPES (Roche Diagnostics, Almere, The Netherlands) buffered HBSS (Gibco, Thermo Fisher Scientific, Bleiswijk, The Netherlands), pH7.4, a dose which does not affect cell viability (Peters et al., 2015).

### 2.2. Adenosine $A_{2A}$ receptor knockout cell line

An adenosine $A_{2A}$ receptor ciPTEC knockout cell line was developed using the CRISPR-Cas9 gene-editing system. SgRNA directed against a 5′ prime target site in the ADORA2A coding sequence was developed using the optimized algorithms of the Zhang-lab (available at www.crispr.mit.edu). Template and non-template sequences of the target site, with additional overhanging sequences (5′-accgGGCGGCGGCCGACATCGCAG-3′ and 3′-aaacCTGCGATGTCGGCCGCCGCCc-5′) were cloned into the plasmid pSpCas9(BB)–2A-GFR (PX458), which was a gift from Feng Zhang (Addgene plasmid #48138) (Ran et al., 2013). First, the oligos (10 μM each) were phosphorylated for 30 min at 37 °C with 5 units 3′-phosphatase minus PNK (NEB) and subsequently annealed by denaturation at 95 °C for 5 min followed by cooling to 25 °C at 5 °C/min. The annealed oligos were then diluted 1:250 and ligated using the golden-gate assembly method using digestion enzyme *Bbs*I to digest the plasmid, followed by transformation into MachI competent cells (Thermo Fisher Scientific, Bleiswijk, The Netherlands) using the heat shock method (35 s at 42 °C). Subsequently, ciPTEC cells were seeded in a 6-well plate 24 h before transfection at 33 °C and were 70–80% confluent at transfection. Cells were incubated in serum free culture medium with pre-mixed 2.5 μg pSpCas9(BB)-2A-GFR-sgRNA and 7.5 μl Lipofectamine® 3000 Reagent per well for 3.5 h, according to manufacturer's protocol. After 24 h, GFP-positive, life cells were FACS sorted using the Aria flow-cytometer and plated as single cells in 96-well plates. After approximately three weeks, colonies formed from which genomic DNA was extracted with the QIAamp DNA mini kit (Qiagen, Venlo, The Netherlands), the sequence surrounding the intended mutation was pre-amplified using PCR (fwd primer: agcctgcctgtcgtctgt; rvs primer: gccaggagactgaagatgga; at 67 °C for 35 cycles with Q5 high-fidelity polymerase) and sequenced using either the forward or the reverse primer of aforementioned PCR. Finally, one clone was obtained with a frame-shift mutation introducing a pre-mature stop-codon at amino acid 115 (Supplemental Table 1).

### 2.3. High-resolution respirometry

Cellular respiration was measured at 37 °C using a two-chamber Oxygraph equipped with Datlab 5 recording and analysis software (Oroboros Instruments, Innsbruck, Austria), as described previously (Liemburg-Apers et al., 2015). For antimycin A and myxothiazol

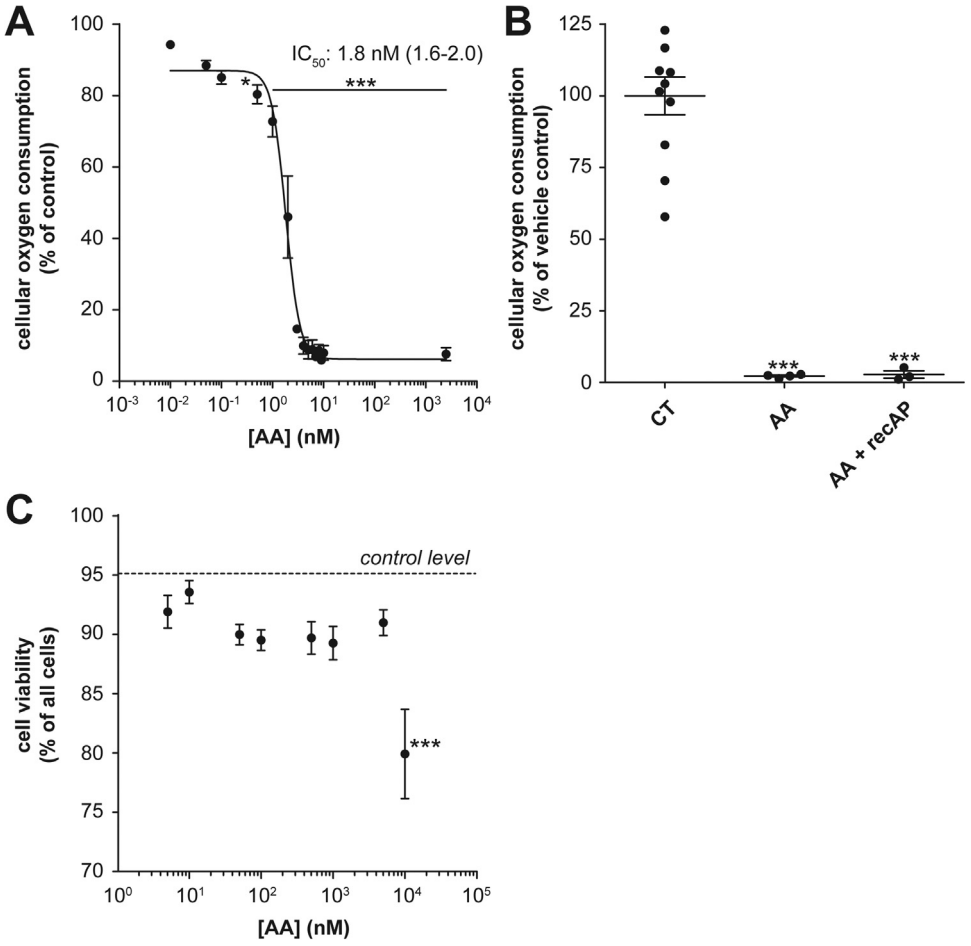


**Fig. 1.** Concentration-dependent inhibition of $O_2$ consumption by antimycin A in ciPTEC and effects of 24 h exposure on cellular respiration and viability. (A) $O_2$ consumption rates of ciPTEC in culture medium upon titration with antimycin A (AA). Respiration rates were normalized to basal respiration in the absence of inhibitor (25 ± 3 pmol/s/$10^6$ cells). (B) Routine $O_2$ consumption rates of ciPTEC in culture medium after 24 h exposure to 10 nM AA with or without recAP (10 U/ml). Respiration rates were normalized to basal respiration in the absence of inhibitor (CT, 15 ± 1 pmol/s/$10^6$ cells). (C) Cell viability after 24 h AA exposure of ciPTEC, expressed as the number of viable cells as a percentage of the total number of cells. Statistical analysis: one-way ANOVA with Bonferroni's (Panel B) or Dunnet's (Panels A and C) post hoc test, * $P < 0.05$, *** $P < 0.001$, mean ± S.E.M., n=3.

titrations, cells were harvested and approximately $3.0 \cdot 10^6$ cells were resuspended in their own culture medium, collected prior to harvesting. After addition of the cells to the chambers of the respirometer the oxygen consumption rates were allowed to reach a stable (routine) level. Next, increasing antimycin A or myxothiazol concentrations were added until the oxygen consumption rates could not be further reduced. To determine cellular respiration after 24 h exposures, approximately $1.5 \cdot 10^6$ cells were resuspended in their own culture medium, collected prior to harvesting. After routine oxygen consumption rates were reached a high antimycin A (2.5 μM) or myxothiazol (1 μM) concentration was added to determine non-mitochondrial respiration. Oxygen consumption rates were corrected for non-mitochondrial respiration by subtraction of these antimycin A or myxothiazol-inhibited rates.

### 2.4. Cell viability analysis

The effects of antimycin A on cell viability were evaluated as described before (Schirris et al., 2015). Briefly, cells were seeded in 96-wells black/clear imaging plates (Greiner Bio-one B.V., Alphen aan de Rijn, The Netherlands) and cultured as described above (see cell culture section). Cells were exposed to a serial-dilution of antimycin A (1 nM to 10 μM). After 24 h incubation, all nuclei were stained using Hoechst 33342 (20 μg/ml, Thermo Fisher Scientific, Bleiswijk, The Netherlands), and additionally Yo-Pro-1-iodide (2 μM, Thermo Fisher Scientific, Bleiswijk, The Netherlands) was used to stain nuclei for early apoptosis and necrosis. After 20 min of staining at 37 °C, fluorescence was imaged on a BD Pathway 855 high-throughput microscope (Becton Dickinson (BD) Bioscience, Breda, The Netherlands). Next, the total number of nuclei was analyzed using Image J (Schneider et al., 2012) and Hoechst-stained nuclei were also used to create a mask. This mask was subsequently used to identify Yo-Pro-1-iodide-positive nuclei. Finally, cell viability was calculated as the percentage of Yo-Pro-1-iodide-negative nuclei.

### 2.5. Cytokine and purine release

IL-6 and IL-8 concentrations were quantified in supernatant using human ELISA kits (Duosets; R & D Systems, Abingdon, UK) according to manufacturer's instructions. Supernatant was collected 20 min after antimycin A or LPS administration, with or without recAP co-incubation, in Krebs-Henseleit buffer containing 10 mM HEPES (pH 7.4), followed by measurement of total purine content by HPCL as described previously (Peters et al., 2015).

### 2.6. Real-time PCR analyses

To determine gene expression levels, cell pellets were collected after 6 or 24 h of incubation and stored at −20 °C until processed. RNA was extracted from frozen cell pellet by Trizol reagent. RNA was reverse-transcribed into cDNA using Moloney Murine Leukemia Virus (*m*-MLV) Reverse Transcriptase (Invitrogen, Breda, The Netherlands).

Real-time quantitative PCR (RQ-PCR) was performed using Taqman® (Applied Biosystems, Bleiswijk, The Netherlands). Genes were amplified and normalized to the expression of RPL27 (Ct: 23.1 ± 0.4; n=5). Differences between groups were calculated by the comparative ΔΔCt method. Primers/probe sets are summarized in Supplemental Table 2.

### 2.7. Statistical analysis

Normality of the data was assessed by Kolmogorov-Smirnov test and expressed accordingly as mean ± S.E.M. or median [25th percentile, 75th percentile]. Unless indicated otherwise, differences between groups were compared by ANOVA with post-hoc comparisons using Bonferroni's or Dunnet's multiple comparison test. A two-sided P-value less than 0.05 was considered statistically significant. All tests were performed with Graphpad Prism 5.03 for Windows (Graphpad Software Inc., California, USA).

## 3. Results

### 3.1. Antimycin A-inhibited respiration is not influenced by recAP treatment

Respiratory inhibition in ciPTEC was achieved using antimycin A, an inhibitor of the $Q_i$-binding site of the third multi-protein complex of the mitochondrial OXPHOS system (Brandt et al., 1988). Antimycin A administration has, however, been associated with high levels of cytotoxicity in primary mouse renal epithelial cells upon long-term (≥24 h) treatment (Breggia and Himmelfarb, 2008). To exclude the influence of such rather non-specific cytotoxic effects, we first determined the lowest antimycin A concentration giving full respiratory inhibition. Titration demonstrated that 10 nM is sufficient to inhibit the cellular oxygen consumption directly after administration (Fig. 1A). Moreover the full respiratory complex inhibition was still present after 24 h treatment (Fig. 1B), without a significant effect on cell viability (Fig. 1C). In the presence of sufficient extracellular glucose this may indicate a shift towards anaerobic metabolism, according to the Warburg effect (Upadhyay et al., 2013). Inhibition of mitochondrial oxygen consumption was confirmed by the antimycin A-induced gene expression levels of hypoxia-dependent genes, such as adrenomedullin and hypoxia-inducible factor (HIF)-1α (Table 1). RecAP treatment did not affect the oxygen consumption after 24 h (Fig. 1B), or the increased adrenomedullin and HIF-1α expression levels (Table 1).

### 3.2. RecAP attenuates the antimycin A-induced inflammatory response

Impaired mitochondrial function is a known trigger of inflammation (Valcarcel-Ares et al., 2014), as confirmed in our model by the antimycin A-induced production and increased gene expression levels of pro-inflammatory mediators IL-6 and IL-8 (Fig. 2A-B). The cytokine production after 24 h was attenuated when cells were pre-incubated with recAP (10 U/ml) for 2 h (Fig. 2A). Similar results were obtained when recAP was added simultaneously with antimycin A. In addition, enzymatically inactive recAP had no effect, indicating that the protective effects are related to the dephosphorylating nature of recAP. After 6 h of antimycin A treatment, when adrenomedullin and HIF-1α were not yet significantly up-regulated (Table 1), recAP reduced the pro-inflammatory gene expression levels (Fig. 2B), whereas cytokine gene expression levels were not attenuated by recAP co-incubation after 24 h of antimycin A treatment (Fig. 2C). Vehicle (ethanol) was used as a negative control and had no effect (not shown). Alternatively, impaired mitochondrial function can also be induced by myxothiazol, another complex III inhibitor acting via the second substrate pocket of complex III ($Q_o$-site) (Supplemental Fig. 1A). After 24 h of full respiratory inhibition (Supplemental Fig. 1B), recAP treatment had similar effects on the IL-8 cytokine production (Supplemental Fig. 1C), indicating that recAP does not exert its effects via a direct interaction with antimycin A. IL-6 was not induced by myxothiazol (not shown). Subsequently, considering the role of renal inflammation in the pathogenesis of sepsis-associated AKI (Peters et al., 2014), the effects of recAP were investigated during a co-incubation of LPS and antimycin A during which recAP still attenuated the inflammatory response (Fig. 3).

### 3.3. Effects of recAP are mediated by dephosphorylation of ATP and ADP

Another hallmark of cellular stress is the release of the endogenous pro-inflammatory molecules ATP and ADP (Carta et al., 2015; Chatterjee and Sparks, 2014). Whereas antimycin A alone did not significantly increase the extracellular release of ATP and ADP compared to control after 20 min incubation, upon co-incubation with LPS, the release of these molecules was significantly increased (Fig. 4A-B). This was similar for AMP (Fig. 4C). Co-incubation with recAP resulted in a significant increase in adenosine compared to antimycin A treatment only (control: 100%; recAP+antimycin A: 222 ± 29% vs. antimycin A: 109 ± 9%, $P < 0.05$). RecAP combined with antimycin A and LPS resulted in a 3.2-fold increase compared to antimycin A and

**Table 1**
Gene expression levels following antimycin A exposure in ciPTEC.

| | *Ct values* | | | | *Fold increase (2^ΔΔCt)* | | | |
|---|---|---|---|---|---|---|---|---|
| | *Control* | *recAP* | *Antimycin A* | *recAP and Antimycin A* | *Control* | *recAP* | *Antimycin A* | *recAP and Antimycin A* |
| *6 h* | | | | | | | | |
| ADM | 24.7 ± 0.2 | 24.2 ± 0.4 | 23.6 ± 0.3 | 23.8 ± 0.4 | 1 ± 0 | 1.3 ± 0.1 | 1.3 ± 0.3 | 1.6 ± 0.3 |
| HIF-1α | 21.2 ± 0.3 | 21.0 ± 0.4 | 20.2 ± 0.3 | 20.6 ± 0.5 | 1 ± 0 | 1.4 ± 0.4 | 1.4 ± 0.2 | 1.2 ± 0.2 |
| $A_1$ | 27.0 ± 0.2 | 26.8 ± 0.3 | 27.5 ± 0.3 | 27.0 ± 0.4 | 1 ± 0 | 1.6 ± 0.3 | 0.5 ± 0.1 | 1.0 ± 0.1 |
| $A_{2A}$ | 29.4 ± 0.1 | 31.1 ± 0.3 | 27.1 ± 0.5 | 29.5 ± 0.3 | 1 ± 0 | 0.4 ± 0.1 | 3.8 ± 0.5[a] | 0.8 ± 0.1[b] |
| $A_{2B}$ | 26.2 ± 0.4 | 26.7 ± 0.3 | 25.9 ± 0.6 | 26.8 ± 0.2 | 1 ± 0 | 0.9 ± 0.1 | 0.9 ± 0.1 | 0.7 ± 0.1 |
| $A_3$ | 35.7 ± 0.3 | 35.2 ± 0.3 | 35.7 ± 0.4 | 35.5 ± 0.4 | 1 ± 0 | 2.0 ± 0.5 | 0.8 ± 0.2 | 1.2 ± 0.2 |
| *24 h* | | | | | | | | |
| ADM | 23.9 ± 0.2 | 24.1 ± 0.3 | 22.5 ± 0.5 | 22.3 ± 0.3 | 1 ± 0 | 1.1 ± 0.1 | 5.2 ± 0.3[a] | 6.1 ± 0.7[a] |
| HIF-1α | 21.1 ± 0.3 | 21.4 ± 0.2 | 21.2 ± 0.5 | 21.1 ± 0.3 | 1 ± 0 | 0.9 ± 0.1 | 1.6 ± 0.2[a] | 1.8 ± 0.2[a] |
| $A_1$ | 28.0 ± 0.3 | 28.1 ± 0.3 | 30.6 ± 0.6 | 30.1 ± 0.4 | 1 ± 0 | 1.3 ± 0.1 | 0.3 ± 0.0[a] | 0.3 ± 0.1[a] |
| $A_{2A}$ | 30.1 ± 0.2 | 30.6 ± 0.3 | 27.9 ± 0.6 | 26.9 ± 0.6 | 1 ± 0 | 0.8 ± 0.1 | 9.0 ± 0.8[a] | 14.7 ± 1.9[a,b] |
| $A_{2B}$ | 27.7 ± 0.2 | 28.0 ± 0.2 | 29.7 ± 0.5 | 29.0 ± 0.3 | 1 ± 0 | 1.0 ± 0.9 | 0.9 ± 0.9 | 0.8 ± 0.1 |
| $A_3$ | 36.7 ± 0.2 | 37.5 ± 0.6 | 38.1 ± 0.6 | 37.6 ± 0.5 | 1 ± 0 | 1.0 ± 0.4 | 0.7 ± 0.2 | 1.0 ± 0.3 |

Data is expressed mean ± S.E.M. Significant differences of the fold increase were calculated using one-way ANOVA with Bonferroni post-test. n=5, n=4 for $A_{2A}$ 24 h due to a significant outlying control sample. [a] $P < 0.05$ compared to control. [b] $P < 0.05$ compared to antimycin A. RecAP, human recombinant Alkaline Phosphatase; ADM, adrenomedullin; HIF-1α, hypoxia-inducible factor 1, alpha subunit; $A_1$, adenosine $A_1$ receptor; $A_{2A}$ adenosine $A_{2A}$ receptor; $A_{2B}$, adenosine $A_{2B}$ receptor; $A_3$, adenosine $A_3$ receptor.

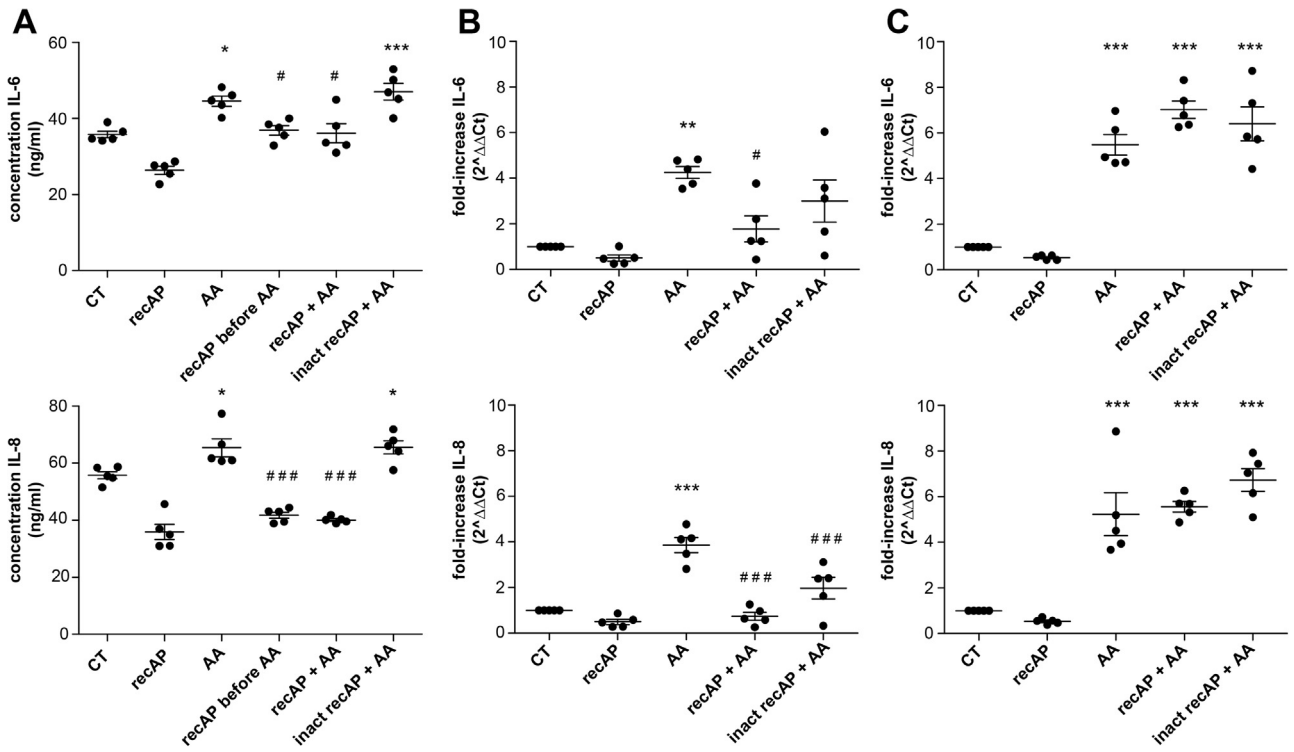


**Fig. 2.** Effects of recAP on antimycin A-induced production and gene expression of cytokines in ciPTEC. Cells were treated with 10 nM antimycin A (AA), with or without recAP (10 U/ml, 19 μg/ml), administered simultaneously with or 2 h preceding AA, or with inactive AP (0 U/ml, 19 μg/ml), lacking hydrolyzing properties. After 24 h, IL-6 (top panel) and IL-8 (bottom panel) were measured on protein level in supernatant by ELISA after 24 h of AA exposure (A) or on gene level in cells by qPCR following 6 h (B) or 24 h (**C**) of AA exposure. Control cells were incubated with culture media. Statistical analysis: one-way ANOVA with Bonferroni's post hoc test, * $P < 0.05$ compared to CT (control), ** $P < 0.01$ compared to CT, *** $P < 0.001$ compared to CT, # $P < 0.05$ compared to AA, ### $P < 0.001$ compared to AA, mean ± S.E.M., n=5.

LPS (Fig. 4D). Adenosine exerts its protective effects through binding to one of the four adenosine receptor $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ (Eltzschig, 2009), of which only the adenosine $A_{2A}$ receptor was up-regulated after 6 h antimycin A exposure, and reduced when recAP was co-administered, but not after 24 h co-exposure (Table 1). Here, recAP co-administered with antimycin A increased the adenosine $A_{2A}$ receptor expression levels. To further explore the role of the adenosine $A_{2A}$ receptor in the renal protective effect of recAP, an adenosine $A_{2A}$ receptor knockout ciPTEC cell line was generated using CRISPR-Cas technology (Ran et al., 2013). A frameshift deletion (c161del, counted from ATG) was introduced, resulting in a premature stop-codon at amino acid 115 (Supplemental Table 1) and consequently, to a expression of a truncated receptor lacking all predicted agonist binding sites. In the adenosine $A_{2A}$ receptor knockout cells, recAP treatment still attenuated the inflammatory response induced by antimycin A and LPS, alone and when combined (Fig. 5), suggesting that adenosine $A_{2A}$ receptor signalling solely may not have a key role in the anti-inflammatory effect of recAP in ciPTEC.

## 4. Discussion

Sepsis-associated AKI is a multifactorial syndrome in which several processes, including inflammation and renal microcirculatory dysfunction play a profound role (Gomez et al., 2014; Ince, 2005). Tubular cells respond to these stressors by down-regulation and reprioritization of cellular functions to prevent further injury, processes which are orchestrated by mitochondria (McBride et al., 2006; Osellame et al., 2012; Singer, 2014). Here, we investigated the effects of recAP, a

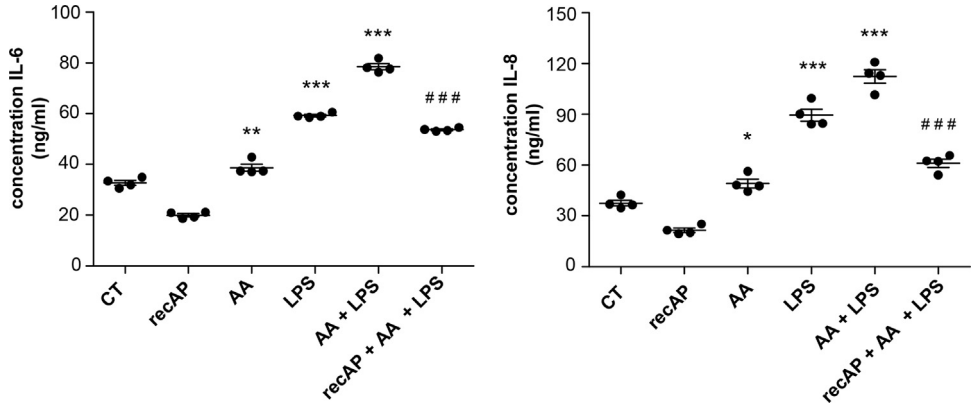


**Fig. 3.** Effects of recAP on antimycin A and LPS-induced cytokine production in ciPTEC antimycin A- and LPS-induced. Protein level in supernatant of IL-6 and IL-8 following 24 h exposure of 10 nM antimycin A (AA), LPS (10 μg/ml) or AA with LPS, with or without recAP (10 U/ml) was measured by ELISA. Control cells were incubated with culture media. Statistical analysis: one-way ANOVA with Bonferroni's post hoc test, * $P < 0.05$ compared to CT (control), ** $P < 0.01$ compared to CT, *** $P < 0.001$ compared to CT, ### $P < 0.001$ compared to AA+LPS, mean ± S.E.M., n=4.

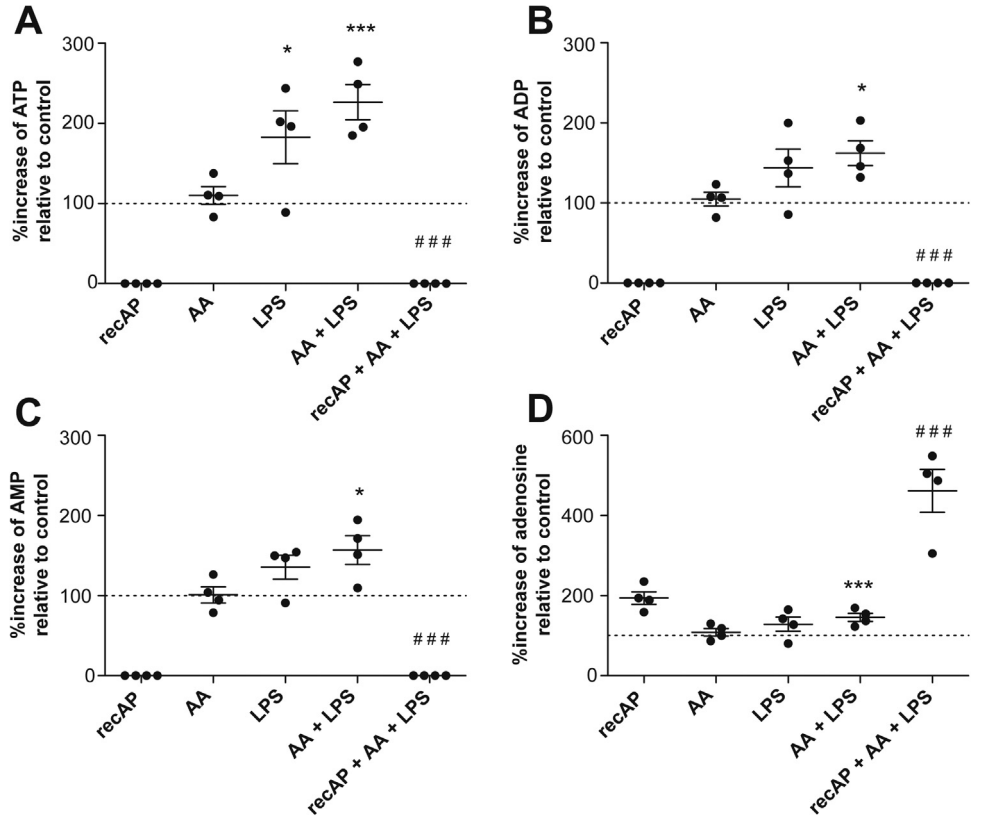


**Fig. 4.** Effect of recAP on antimycin A- and LPS-induced ATP and ADP release in ciPTEC. CiPTEC were incubated with 10 nM antimycin A (AA), LPS (10 μg/ml) or AA with LPS, with or without recAP (10 U/ml) in Krebs-Henseleit buffer containing 10 mM HEPES (pH 7.4). After 20 min, supernatant was collected to determine ATP (A), ADP (B), AMP (C) and adenosine (D) content by HPLC. Control cells were incubated with buffer. Statistical analysis: one-way ANOVA with Bonferroni's post hoc test, ** $P < 0.01$, *** $P < 0.001$, mean ± S.E.M., n=4.

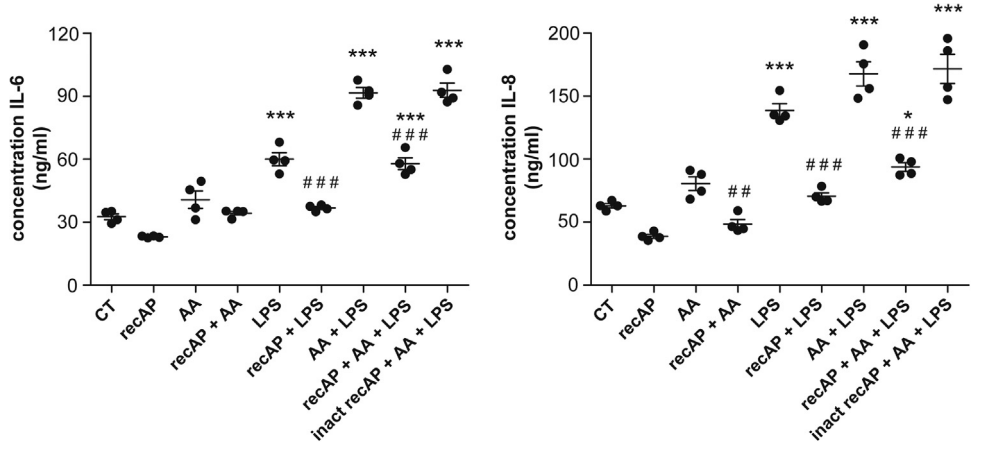


**Fig. 5.** Effect of recAP on antimycin A- and LPS-induced cytokine production in adenosine $A_{2A}$ receptor knockout ciPTEC. An adenosine $A_{2A}$ receptor knockout ciPTEC cell line was generated using the CRISPR-Cas technology, by which a frameshift deletion (c161del) was introduced, resulting in a premature stopcodon at amino acid 115. Protein level in supernatant of IL-6 and IL-8 following 24 h exposure of 10 nM antimycin A (AA), LPS (10 μg/ml) or AA with LPS, with or without recAP (10 U/ml, 19 μg/ml) was measured by ELISA. Cells exposed to AA and LPS were also co-incubated with inactive AP (inact recAP) (0 U/ml, 19 μg/ml), lacking hydrolyzing properties. Control cells were incubated with culture media. Statistical analysis: one-way ANOVA with Bonferroni's post hoc test, * $P < 0.05$ compared to CT (control), *** $P < 0.001$ compared to CT, ## $P < 0.01$ compared to AA, ### $P < 0.001$ compared to corresponding stimuli without recAP, mean ± S.E.M., n=4.

potential new treatment option for sepsis-associated AKI, during impaired mitochondrial function and demonstrate that recAP attenuates the inflammatory response, without affecting oxygen consumption.

Despite a marked decline in renal function and the occurrence of tubular dysfunction, histological signs of apoptosis or necrosis are barely present in patients with sepsis-associated AKI (Hotchkiss and Karl, 2003). Considering the severity of injury, cells are likely to have a mechanism to limit activation of cell death pathways, which might be arranged by mitochondria (Gomez et al., 2014). Mitochondria are most abundantly expressed in the heart, followed by the renal proximal tubule cells, where there is a high need for ATP in order to facilitate solute exchange (Parikh et al., 2015). During stress, mitochondria reduce the metabolic activity of cells by reducing energy requirements. To this end, mitochondria remove and digest dysfunctional organelles and initiate cell cycle arrest to prevent further damage (Gomez et al., 2014). During this state, akin to hibernation, mitochondria do not function normally, but do produce enough ATP to prevent activation of cell death pathways (Singer, 2014). Exactly how mitochondrial dysfunction contributes to the development of AKI is poorly understood, but during overwhelming cellular stress, the protective capacity of

mitochondria might be exhausted (Parikh et al., 2015).

To mimic impaired mitochondrial function, respiration was inhibited in human proximal tubule cells by antimycin A. This compound inhibits the $Q_i$-binding site of mitochondrial complex III of the OXPHOS system, thereby completely inhibiting cellular oxygen consumption as confirmed by respiration experiments (Brandt et al., 1988). Impaired mitochondrial function promotes the inflammatory response (Valcarcel-Ares et al., 2014) as observed by an increased production of the pro-inflammatory cytokines IL-6 and IL-8 in our experiments, which was even more pronounced upon co-incubation with LPS. Alternatively, as 24 h of oxygen consumption inhibition did not affect cell viability, cells may have shifted to anaerobic metabolism according to the Warburg effect, where cells rely on glycolysis for energy production, even in the presence of oxygen (Upadhyay et al., 2013). This process by itself could also induce inflammation via accumulation of succinate, an intermediate metabolite of the citric acid cycle, thereby stabilizing the transcription factor HIF-1α, resulting in the production of pro-inflammatory cytokines (Wen and Ting, 2013). We report that recAP treatment attenuates the inflammatory response induced by antimycin A, which corroborates previous results when inflammation was induced by LPS administration (Peters et al., 2015). These effects were explained by dephosphorylation of LPS (Poelstra et al., 1997) and the conversion of extracellular detrimental purines ATP and ADP into the cytoprotective molecule adenosine (Peters et al., 2015). Also, inhibition of oxygen consumption induces cellular purine release (Dutta et al., 2004), as confirmed here by significant antimycin A-induced increase of ATP and ADP release when combined with inflammation, which were completely converted into adenosine by recAP. RecAP attenuated pro-inflammatory cytokine gene expression levels after 6 h of antimycin A treatment, whereas recAP had no effects when cells were incubated for 24 h with antimycin A. Since purines are rapidly released upon cellular stress (Lim et al., 2015) and a sustained period of oxygen consumption inhibition induces intracellular ATP depletion (Steinbach et al., 2003) (thereby limiting extracellular release of ATP), it appears plausible that recAP is only able to provide protection during the initial phase. Removal of these purines prevents further injury, potentially provoked by purinergic P2X and P2Y receptor signalling, thereby accelerating inflammation and tissue injury by phagocyte recruitment and NLRP3 inflammasome activation (Eltzschig et al., 2012).

Another effect of antimycin A administration was a profound up-regulation of the adenosine $A_{2A}$ receptor. Adenosine exerts renal protective and anti-inflammatory effects upon binding to the adenosine $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ receptors (Yap and Lee, 2012). As the adenosine $A_{2A}$ receptor is known to be induced by inflammation (Eltzschig et al., 2012), and adenosine $A_{2A}$ receptor expression levels were attenuated by recAP after 6 h and not 24 h, comparable to cytokine gene expression level, this is likely to reflect a cellular response on inflammation (Peters et al., 2015). To further explore whether or not the adenosine $A_{2A}$ receptor is involved in the renal protective effects of recAP, an adenosine $A_{2A}$ receptor knockout cell line was generated with CRISPR-CAS technology. This recently developed genome editing technology is superior to other techniques, like zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and small interfering RNA (siRNA), as it is permanent, easier to design and highly specific (Gaj et al., 2013; Ran et al., 2013). RecAP still attenuated the inflammatory response in this model, suggesting that adenosine $A_{2A}$ receptor signalling solely may have no key role in the anti-inflammatory effect of recAP in ciPTEC. This confirms earlier findings where blocking the adenosine $A_{2A}$ receptor by the specific antagonist ZM-241385 did not prevent the recAP-mediated protection on the LPS-induced inflammatory response in these cells (Peters et al., 2015). Yet, based on these results, we cannot exclude that adenosine exerts protective effects via signalling through one of the other three adenosine receptors, possibly combined with adenosine $A_{2A}$ receptor signalling (Yap and Lee, 2012). Moreover, the protective effects observed *in vivo* could also be due to the effect of adenosine on other cell types. For instance, bone marrow-derived cell signalling through the adenosine $A_{2A}$ receptor has been implicated in kidney protection upon ischemia through their anti-inflammatory effects (Day et al., 2003). However, it was beyond the scope of the current studies to investigate these possibilities.

Despite a renal anti-inflammatory effect, recAP did not affect the antimycin A-induced decreased oxygen consumption and subsequent increase in hypoxia-inducible gene expression levels of HIF-1α or adrenomedullin. During a prolonged period of reduced oxygen consumption, cells activate adaptive mechanisms for survival during which HIFs are key. The most commonly studied factor HIF-1 consists of an α and β subunit, of which HIF-1α is oxygen-sensitive and heterodimerizes with HIF-1β to bind DNA (Solaini et al., 2010). During hypoxia, HIF-1 breakdown is prevented resulting in stabilization of HIF-1, which actively represses oxygen consumption by preventing entrance of pyruvate into the citric acid cycle, thereby increasing oxygen availability and preventing cell death (Papandreou et al., 2006). Furthermore, HIF-1 induces an increase of adrenomedullin gene expression levels (Garayoa et al., 2000; Nguyen and Claycomb, 1999), another survival factor against hypoxic cell death of which the exact mechanism of action remains to be elucidated (Kim et al., 2010). Whether or not recAP may improve oxygen consumption in patients with sepsis-associated AKI is difficult to extrapolate from our *in vitro* model. Here, mitochondrial function is inhibited in all cells simultaneously, whereas in the *in vivo* situation, AKI presents as patchy areas of injured cells, with some cells being affected whereas others are not (Hotchkiss and Karl, 2003).

Moreover, one may argue that a more representative model of the *in vivo* situation would be a condition of reduced oxygen delivery, rather than reduced cellular oxygen consumption whereas oxygen is still available. While mitochondrial inhibition can also be achieved during oxygen deprived settings (Snyder and Chandel, 2009), hypoxia is an inflammatory stimulus by itself. As such, exposing cells to low oxygen conditions induces a pro-inflammatory response, as well as the activation of several counter-regulatory mechanisms (e.g. anti-inflammatory pathways) (Kiers et al., 2016). As we only aimed to study the effects of recAP on mitochondrial inhibition, exclusive inhibition of the mitochondrial respiratory chain provides the simplest and most direct model.

To conclude, mitochondrial dysfunction plays an important role in sepsis patients suffering from organ dysfunction (Singer et al., 2004). We report that recAP attenuates the initial inflammatory response provoked by mitochondrial dysfunction, thereby confirming the previous observed renal protective anti-inflammatory effect of recAP *in vitro* and *in vivo* and of bovine intestinal AP in patients with sepsis and associated AKI (Peters et al., 2015; Pickkers et al., 2012). These effects are presumably mediated by dephosphorylating ATP and ADP into adenosine. The potential clinical value of recAP to treat critically ill patients with sepsis-associated AKI is currently being investigated in a large phase IIa/b clinical trial with outcomes expected in the second half of 2017 (Peters et al., 2016b).

## Statement of competing financial interests

P Pickkers has received speaking and consultation fees from AM-Pharma, which developed the bovine intestinal AP and recAP therapeutics. The remaining authors declare no competing financial interests. AM-Pharma, the manufacturer of recAP, was not involvement in the conception, design, conduct, analysis and interpretation of data of this study.

## Author contributions

EP, TS, PP and RM contributed to the conception or design of the work. EP, TS, SvA, JG, JE, AA, MA, FR, PP and RM contributed to the

acquisition, analysis, or interpretation of data for the work. EP and TS drafted the manuscript. SvA, JG, JK, AA, MA, FR, PP and RM revised the manuscript. All authors have read and approved submission of this manuscript and take full responsibility for the manuscript.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejphar.2016.12.034.

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