European Journal of Pharmacology 796 (2017) 149–157

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Full length article

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

Esther Peters[a,b,*,1], Tom Schirris[b,c,1], Alexander H van Asbeck[d], Jelle Gerretsen[a], Jennifer Eymael[a], Angel Ashikov[e], Merel J.W. Adjobo-Hermans[d], Frans Russel[b,c], Peter Pickkers[a,2], Rosalinde Masereeuw[f,2]

[a] Department of Intensive Care Medicine, Radboud University Medical Center, PO Box 9101, Internal Mailbox 710, 6500 HB Nijmegen, The Netherlands
[b] Department of Pharmacology and Toxicology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, PO Box 9101, Internal Mailbox 710, 6500 HB Nijmegen, The Netherlands
[c] Radboud Institute for Mitochondrial Medicine, Radboud University Medical Center, PO Box 9101, Internal Mailbox 710, 6500 HB Nijmegen, The Netherlands
[d] Department of Biochemistry, Radboud University Medical Center, PO Box 9101, Internal Mailbox 710, 6500 HB Nijmegen, The Netherlands
[e] Department of Neurology, Translational Metabolic Laboratory, Department of Laboratory Medicine, Radboud University Medical Center, PO Box 9101, Internal Mailbox 710, 6500 HB Nijmegen, The Netherlands
[f] Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, PO BOX 80082, 3508 TB Utrecht, The Netherlands

ARTICLE INFO

Chemical compounds studied in this article:
Antimycin A (PubChem CID: 16218979)
Lipopolysaccharide (PubChem CID: 11970143)
Myxothiazol (PubChem CID: 6437357)

Keywords:
Human recombinant alkaline phosphatase
Inflammation
Adenosine
Proximal tubule epithelial cells
Mitochondria
Respiratory inhibition

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

* Corresponding author at: Department of Intensive Care Medicine, Radboud University Medical Center, PO Box 9101, Internal Mailbox 710, 6500 HB Nijmegen, The Netherlands.
E-mail addresses: Esther.Peters@radboudumc.nl (E. Peters), Tom.Schirris@radboudumc.nl (T. Schirris), Sander.BaronvanAsbeck@radboudumc.nl (A.H. van Asbeck), Jelle.Gerretsen@radboudumc.nl (J. Gerretsen), Jenny.Eymael@radboudumc.nl (J. Eymael), Angel.Ashikov@radboudumc.nl (A. Ashikov), Merel.Adjobo-Hermans@radboudumc.nl (M.J.W. Adjobo-Hermans), Frans.Russel@radboudumc.nl (F. Russel), Peter.Pickkers@radboudumc.nl (P. Pickkers), R.Masereeuw@uu.nl (R. Masereeuw).
[1] Both authors contributed equally to this work.
[2] Both authors share senior authorship.

http://dx.doi.org/10.1016/j.ejphar.2016.12.034
Received 26 September 2016; Received in revised form 19 December 2016; Accepted 21 December 2016
Available online 22 December 2016
0014-2999/

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