Biochimica et Biophysica Acta 1837 (2014) 1790–1800

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Evidence of oxidative stress and mitochondrial respiratory chain dysfunction in an *in vitro* model of sepsis-induced kidney injury

C. Quoilin [a,*], A. Mouithys-Mickalad [b], S. Lécart [c], M.-P. Fontaine-Aupart [c,d], M. Hoebeke [a]

[a] Laboratory of Biomedical Spectroscopy, Department of Physics, University of Liège, 4000 Liège, Belgium
[b] Center of Oxygen Research and Development, Department of Chemistry, University of Liège, 4000 Liège, Belgium
[c] Centre de Photonique Biomédicale, CPBM/CLUPS, Fédération LUMAT, University Paris Sud, 91405 Orsay, France
[d] Institut des Sciences Moléculaires d'Orsay, CNRS and University Paris Sud, 91405 Orsay, France

ARTICLE INFO

Article history:
Received 18 April 2014
Received in revised form 29 June 2014
Accepted 5 July 2014
Available online 11 July 2014

Keywords:
Sepsis
Acute kidney injury
Mitochondrial dysfunction
Cytopathic hypoxia
Oxidative stress
Nitric oxide

ABSTRACT

To investigate the role of oxidative stress and/or mitochondrial impairment in the occurrence of acute kidney injury (AKI) during sepsis, we developed a sepsis-induced *in vitro* model using proximal tubular epithelial cells exposed to a bacterial endotoxin (lipopolysaccharide, LPS). This investigation has provided key features on the relationship between oxidative stress and mitochondrial respiratory chain activity defects.

LPS treatment resulted in an increase in the expression of inducible nitric oxide synthase (iNOS) and NADPH oxidase 4 (NOX-4), suggesting the cytosolic overexpression of nitric oxide and superoxide anion, the primary reactive nitrogen species (RNS) and reactive oxygen species (ROS). This oxidant state seemed to interrupt mitochondrial oxidative phosphorylation by reducing cytochrome c oxidase activity. As a consequence, disruptions in the electron transport and the proton pumping across the mitochondrial inner membrane occurred, leading to a decrease of the mitochondrial membrane potential, a release of apoptotic-inducing factors and a depletion of adenosine triphosphate. Interestingly, after being targeted by RNS and ROS, mitochondria became in turn producer of ROS, thus contributing to increase the mitochondrial dysfunction.

The role of oxidants in mitochondrial dysfunction was further confirmed by the use of iNOS inhibitors or antioxidants that preserve cytochrome c oxidase activity and prevent mitochondrial membrane potential dissipation. These results suggest that sepsis-induced AKI should not only be regarded as failure of energy status but also as an integrated response, including transcriptional events, ROS signaling, mitochondrial activity and metabolic orientation such as apoptosis.

1. Introduction

Sepsis is a very complex clinical condition characterized by stimulation of a systemic inflammatory response related to an infection [1,2]. This process often leads to widespread tissue injury and multiple organ dysfunction [3]. In particular, the development of acute kidney injury (AKI) is a risk factor for mortality in septic patients [4,5].

The mechanisms leading to AKI are extremely complex and still remain controversial. It was long thought that microvascular dysfunction whose net effect is a failure of oxygen delivery was likely to alter renal function at the cellular level [6]. This tissue hypoxia paradigm has been challenged by recent studies revealing that the hemodynamic mechanism might not be relevant in the pathophysiology of AKI [7,8]. It seems that the kidney is more challenged by impairment of cellular oxygen utilization rather than inadequate oxygen delivery [8–10]. This hypothesis has been corroborated in our previous work in which we have shown the incapacity of renal tubular epithelial (HK-2) cells to use adequately the available oxygen under bacterial endotoxin simulation [11].

Renal tubular epithelium seems to be no longer a passive victim of hypoxic injury [12]. Increasing number of studies suggest that it is a major site of oxidative stress, and that overproduction of reactive nitrogen species (RNS) and reactive oxygen species (ROS) may be important contributors in the mechanism of kidney injury [13–15]. These oxidants can react with cellular components, such as DNA, proteins and lipids, leading to their degradation and thereby accelerating the loss of tubular epithelium function [16,17]. However, as these studies are based on

Abbreviations: 1400W, N-(3-(aminomethyl)benzyl)acetamidine; ADP, adenosine diphosphate; AKI, acute kidney injury; ATP, adenosine triphosphate; CTRL, control; DCF, 2′-7′ dichlorofluorescin; DNA, deoxyribonucleic acid; DPI, diphenylene iodonium; EBS, ebselen (2-phenyl-1, 2-benzisoselenazol-3(2H)-one); GSH, L-glutathione; HK-2, human kidney cell lines; L-NMMA, L-N_G-monomethylarginine acetate salt; LPS, lipopolysaccharide; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); NBT, nitroblue tetrazolium; ·NO, nitric oxide radical; NO_3^-, nitrate; NO_2^-, nitrite; iNOS, inducible nitric oxide synthase; NOX-4, NADPH oxidase 4; $O_2^{\bullet -}$, superoxide anion radical; $ONOO^-$, peroxynitrite anion; RNA, ribonucleic acid; RNS, reactive nitrogen species; ROS, reactive oxygen species; RT-PCR, real time polymerase chain reaction; TMRE, tetramethylrhodamine ethyl ester

* Corresponding author at: Laboratory of Biomedical Spectroscopy, Department of Physics B5a, Allée du 6 Août 17, 4000 Liège, Belgium. Tel.: +32 4 366 9568; fax: +32 4 366 3629.
E-mail address: cquoilin@ulg.ac.be (C. Quoilin).

http://dx.doi.org/10.1016/j.bbabio.2014.07.005
0005-2728/

animal models, it is difficult to dissociate direct toxicity to renal epithelial cells from side effects of renal vasoconstriction and peritubular capillary hypoperfusion.

In addition to oxidative stress, mitochondrial abnormalities have been recognized within *in vivo* and *in vitro* models. Animal models of sepsis, using either the bacterial endotoxin lipopolysaccharide (LPS) or cecal ligation and puncture as stress inducers, mainly focused on metabolic changes occurring at the level of oxidative phosphorylation. In LPS-treated rats, cytochrome c oxidase and adenosine triphosphate (ATP) synthase were down-regulated both at the transcript and protein level within 48 h after treatment [18]. This inhibition of cytochrome c oxidase was also observed in severe sepsis and had the characteristic of being irreversible [19,20]. In another case of endotoxic shock, the LPS treatment resulted in a 70% decrease of ATP levels after 8 h, a critical time for which about 30% of the animals were dead [21]. Moreover, several studies have pointed out that antioxidant treatment reversed cell mitochondrial dysfunction in sepsis animal models but there is still uncertainty concerning the involvement of ROS and RNS [22,23].

Despite this increasing evidence of oxidative stress and mitochondrial injury being important in the development of sepsis-induced AKI, it remains unclear if mitochondrial dysfunction is the primary event that leads to oxidative stress and further mitochondrial impairment, or if the oxidative stress initiates mitochondrial dysfunction with a subsequent RNS and ROS release [24,25]. In addition, the cellular source of ROS and RNS generation is still unclear. Indeed, radical species were once thought to originate almost entirely from mitochondrial metabolism but recent data focused on the crucial role of cellular enzymes, such as NADPH oxidases, as important sources of ROS [26]. This emphasizes that the mechanisms underlying ROS, RNS production and mitochondrial dysfunction have not been yet fully elucidated. To highlight this phenomenon, we developed a sepsis-induced *in vitro* model using human proximal tubular epithelial (HK-2) cells exposed to LPS. This investigation has provided key features on the relationship between oxidative stress and mitochondrial respiratory chain activity defects.

2. Materials and methods

2.1. Cell culture and treatment

HK-2 cells, an immortalized proximal tubular epithelial cell line from normal adult human kidney [27], were cultured in DMEM (Lonza, Switzerland) supplemented with 10% heat-inactivated FBS (Biochrom, Germany), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Belgium). Cells were grown to approximately 70% confluence at 37 °C in a humidified 5% CO_2 incubator.

To determine the toxic effects of LPS on mitochondrial functionality of HK-2 cells, they were incubated with fresh medium containing 1 μg/ml LPS from *Escherichia coli* 055:B5 (Sigma Aldrich, Belgium) for different incubation times. The role of oxidants was investigated by incubating cells for 6 h with fresh medium containing 1 μg/ml LPS and different types of antioxidants (all from Enzo Life Science, Belgium): 10 μM L-NMMA, 10 μM 1400W, 1 μM diphenylene iodonium (DPI), 100 μM L-glutathione (GSH), or 10 μM ebselen (EBS). For each experiment, cells were incubated without any endotoxin or drugs and taken as control groups.

2.2. Measurement of NO_2^-/NO_3^- in culture media

To investigate the implication of nitric oxide radical (•NO) in the mechanism by which LPS induced HK-2 cell stress, its production was controlled. At the end of the LPS treatment of HK-2 cells, the culture supernatants (without red phenol) were collected and the concentrations of NO_2^-/NO_3^- were quantified by using the Total Nitric Oxide Detection Kit (Enzo Life Sciences, Belgium). This assay is based on the enzymatic reduction of NO_3^- to NO_2^- by nitrate reductase. The reaction was followed by the colorimetric detection of NO_2^- as a colored azo dye, product of the Griess reaction that absorbs visible light at 540 nm. The total •NO released by cells was determined by subtracting NO_2^-/NO_3^- levels initially present in the media from the levels determined after incubation.

2.3. RT-PCR for iNOS induction

Inducible nitric oxide synthase (iNOS) is one of the major sources of •NO by catalyzing L-arginine. Its messenger RNA (mRNA) levels was estimate by RT-PCR experiments. Total RNA was purified from HK-2 cells using RNeasy Mini Kit (Quiagen, Canada) as described by the manufacturer and quantified by its UV absorbance. RNA was then reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Canada). Specific primers from TaqMan Gene Expression Assays (Applied Biosystems, Canada) for iNOS and actin were used for amplification of cDNA using TaqMan Universal Master Mix II (Applied Biosystems, Canada). Amplification was performed by successive 40 cycles of temperature (95 °C, 15 s; 60 °C, 60 s) in a temperature cycler (DNA Thermal Cycler, Perkin-Elmer). The primers used were human β-actin (ACTB, Hs99999903_m1) and human iNOS (NOS2, Hs01075529_m1). The LPS-stimulated iNOS expression relative actin was reported as the fold increase compared to that of non-stimulated.

2.4. Quantification of $O_2^{\bullet -}$ production

As ROS are possible mediators of cell alterations, we considered the possible implication of superoxide anion radicals ($O_2^{\bullet -}$). After LPS treatment, the concentration of $O_2^{\bullet -}$ in HK-2 cells was determined by the spectrophotometric method based on the reduction of nitroblue tetrazolium (NBT) to formazan in the presence of $O_2^{\bullet -}$ [28,29]. In a 24-well plate containing HK-2 cells treated with LPS, 200 μl of 0.2% NBT solution was added for 1 h at 37 °C. After incubation, cells were washed twice with prewarmed PBS, then once with methanol in order to completely remove the extracellular formazan. NBT deposits inside cells were then dissolved with 2 M KOH and DMSO and the resulting color reaction was measured spectrophotometrically on microplate reader at 650 nm (Multiskan Microplate Reader, Thermo scientific, Belgium).

2.5. NOX-4 western blot analysis

NADPH oxidase 4 (NOX-4) is a potent source of $O_2^{\bullet -}$ in kidney [30]. Its expression in HK-2 cells during LPS treatment was detected by western blot experiments. Cellular preparations were homogenized in lysis buffer containing a mixture of protease and phosphatase inhibitors, 150 mM NaCl, 20 mM Tris, 10% glycerol, 5 mM EGTA, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate (all chemicals from Sigma-Aldrich, Canada). Samples were centrifuged at 14000 rpm, 4 °C for 10 min and the supernatant protein concentrations were determined using the BCA protein assay kit (Pierce, Canada) following the manufacturer's instructions. Samples were subjected to DTT and glycerol, boiled for 5 min and stored at −20 °C until analysis.

20 μg of proteins per well was separated on 7.5% SDS-PAGE gel and transferred to nitrocellulose membranes (materials were from Bio-Rad Laboratories — Clinical Diagnostics, Canada). After 1 h of blocking in TBS containing 0.1% Tween and 5% BSA, membranes were incubated overnight at 4 °C with primary antibodies: 0.4 μg/ml of goat polyclonal anti-NOX-4 antibody (Santa Cruz Biotechnology, sc-55142) and 1/20000 dilution of mouse anti-actin (Millipore, MAB1501). After washes, membranes were incubated for 1 h with the appropriate HRP-conjugated secondary antibody. The immunoreactive bands were detected by the enhanced chemiluminescence method and intensities were quantified using the ImageJ program.