Biochemical and Biophysical Research Communications 423 (2012) 350–354

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Endotoxin-induced basal respiration alterations of renal HK-2 cells: A sign of pathologic metabolism down-regulation

C. Quoilin [a,*], A. Mouithys-Mickalad [b], J. Duranteau [c], B. Gallez [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] Department of Anaesthesia and Surgical ICU, CHU Bicêtre, University Paris XI Sud, 94275 Le Kremlin Bicêtre, France
[d] Biomedical Magnetic Resonance Group, Louvain Drug Research Institute, Université catholique de Louvain, 1200 Brussels, Belgium

ARTICLE INFO

Article history:
Received 16 May 2012
Available online 31 May 2012

Keywords:
Sepsis
Inflammation
Acute kidney injury (AKI)
Endotoxin
Oxygen consumption
Metabolism

ABSTRACT

To study the mechanism of oxygen regulation in inflammation-induced acute kidney injury, we investigate the effects of a bacterial endotoxin (lipopolysaccharide, LPS) on the basal respiration of proximal tubular epithelial cells (HK-2) both by high-resolution respirometry and electron spin resonance spectroscopy. These two complementary methods have shown that HK-2 cells exhibit a decreased oxygen consumption rate when treated with LPS. Surprisingly, this cellular respiration alteration persists even after the stress factor was removed. We suggested that this irreversible decrease in renal oxygen consumption after LPS challenge is related to a pathologic metabolic down-regulation such as a lack of oxygen utilization by cells.

1. Introduction

Sepsis can be considered as a heterogeneous disease process generated by a complex interaction of pathogen and host inflammatory response [1,2]. The dysfunction of the immune response is initiated by the recognition of microorganisms and their pathogen-associated molecular patterns (PAMPs) as exogenous harmful signals by specific sensors of the host [3]. Half the cases of sepsis are caused by Gram-negative bacteria [4–6]. The main factor contributing to the pathogenesis of Gram-negative organisms appears to be lipopolysaccharide (LPS) [7]. This endotoxin is one of the major triggers of inflammatory responses in sepsis, as it is known to produce an early rise in pro-inflammatory cytokines that activate potent immune response through the activation of Toll-like receptor 4 [8].

Sepsis may lead to organ dysfunction distant from the primary site of infection and cause serious downstream effects such as multiple organ failure [9]. The kidney is one of the target organs of sepsis which is well-known to be a risk factor for the development of acute kidney injury (AKI) [10]. The mechanisms involved in the development of AKI in sepsis are extremely complex and still remain controversial [11]. The kidney is an organ highly sensitive to hypoxia due to its unique microvasculature architecture associated with high demand of oxygen from the tubular salt-water reabsorption. It was long thought that hypoperfusion or ischemic induced injuries were likely to alter renal function but recent studies revealed that hemodynamic mechanisms might not be relevant in the understanding of the pathophysiology of AKI [12]. So it appeared that non hemodynamic pathways, such as immunologic, toxic and inflammatory factors, are likely to be at work and may affect the microvasculature and the tubular cells [11–13].

The kidney is faced to an impairment of oxygen extraction during sepsis. Two main mechanisms are suggested to explain the inability of the injured kidney to extract oxygen: tissue hypoxia and cellular energetic metabolism dysfunction [14,15]. Our working hypothesis of the pathophysiology of AKI is based on cellular respiratory dysfunction due to the inflammatory response inherent to sepsis. Therefore, we chose lipopolysaccharide (LPS) as a causative agent triggering inflammatory responses in an *in vitro* model using human tubular proximal cell lines (HK-2) and we carried out the present investigation to characterize renal oxygen respiration in this inflammation-induced model for AKI. To reach this goal, we used two complementary oximetry techniques, high-resolution respirometry and electron spin resonance (ESR) spectroscopy, and we tried to demonstrate whether renal cells had different respiration rates when submitted to LPS challenge.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 was obtained from Sigma–Aldrich (Bornem, Belgium). The stock solution

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

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.05.128

was made up in sterile home-made phosphate buffered saline (PBS, pH = 7.2) at 1 mg/ml and stored at −20 °C until use. Salts (phosphate, potassium, etc.) and other reagents (e.g. ESR probe) were of high-grade quality.

2.2. Cell culture

HK-2 cells, an immortalized proximal tubular epithelial cell line from normal adult human kidney [16], were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's culture medium (DMEM, Gibco Laboratories) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco Laboratories), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Invitrogen, Merelbeke, Belgium). Cells were maintained at 37 °C in a humidified 5% CO_2 incubator. Cells were not used at passages higher than 25 and all experiments were carried out using exponentially growing HK-2 cells.

2.3. Treatment of HK-2 cells with lipopolysaccharide

To determine the effects of the endotoxin on the oxygen consumption of renal cells, HK-2 cells were incubated with fresh solution containing 1 μg/ml LPS for different incubation periods (1 h, 3 h, 6 h and 18 h) in a humidified 5% CO_2 incubator at 37 °C. Cells were then washed with PBS and collected by trypsination prior measurements.

To study the reversibility of the phenomenon, cells were rather incubated with fresh medium containing 1 μg/ml LPS for 6 h, washed several times with PBS and replaced in LPS-free medium for 24 h.

For each experiment, cells incubated with culture medium without any endotoxin and drugs were set up as control group.

2.4. Cell viability

Before and after each experiment, collected cells were centrifuged for 5 min at 1000 rpm and the pellet was resuspended in fresh DMEM. The cell viability was then checked using the Trypan blue dye exclusion assay and was found to be >90% at all times.

2.5. High-resolution respirometry

The principle of respirometry in a closed chamber is based on monitoring oxygen concentration, which declines as the biological sample consumes oxygen [17,18]. The O_2 consumption by 1.5×10^7 LPS-treated HK-2 cells was monitored in 2 ml of air-saturated DMEM with polarographic oxygen microelectrodes (Oroboros oxygraph, Paar Physica, Austria) at 37 °C [19]. The measurement started just after transferring the mixture in the respiration chamber and closing it. As control assays, 1.5×10^7 cells not treated with LPS were put in the second chamber of the oximeter and their normal respiration rate was monitored by closing the chamber. The oxygen concentration (μmol/ml) and the oxygen flux (pmol O_2/s/10^6 cells) were recorded online in the closed chambers using the Datlab software. The slopes of O_2 consumption were calculated with the Oroboros oxygraph included software. Each oximetry assay was done in triplicate.

2.6. ESR oximetry

ESR oximetry has been used extensively to measure oxygen concentrations ([O_2]) *in vitro* [20,21] and *in vivo* [22,23]. In our experiments, we used the neutral nitroxide ^{15}N 4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-1-oxyl (^{15}N-PDT, CDN Isotopes, Pointe-Claire, QC, Canada) whose line shape and width are very sensitive to oxygen [24]. When ^{15}N-PDT is added to the cell suspension, the probe freely diffused throughout the extracellular and intracellular space, and the resulting line width reports on [O_2]. The calibration curve giving ESR nitroxide spectrum changes as a function of oxygen concentration has been established by Diepart et al. [21].

Oxygen consumption rates were obtained by measuring the [O_2] in a closed tube over time and finding the slope of the resulting linear plot. LPS-treated HK-2 cells were suspended in DMEM in order to have a cellular concentration of 7.5×10^6 cells/ml. 20% dextran (Sigma–Aldrich, Belgium) by weight was also added to the medium to delay the settling of the cells. ^{15}N-PDT (0.2 mM) was finally added to 100 μl aliquots of renal cells that were then drawn into glass capillary tubes. The capillary tube was sealed at both ends avoiding the entrapment of any air bubbles. The sealed tubes containing samples were placed into quartz ESR tubes and maintained at 37 °C. The ESR line width was then scanned repeatedly at 1 min intervals for 6–10 min, allowing the calculation of the oxygen consumption rates. A control sample was run for each experiment.

All ESR spectra were recorded using a Bruker EMX ESR spectrometer equipped with a variable temperature controller accessory. The typical instrument settings were: 9.5 GHz microwave frequency; 3350 G center field strength with 1 G as sweep width; 100 kHz modulation frequency; 2 mW microwave power and 0.05 G modulation amplitude. The time constant and the conversion time were both fixed at 5.12 ms. Three kinetics were acquired for each measurement.

2.7. Statistical analysis

Data were represented as mean ± SD. The paired Student *t*-test was used to compare two conditions using the original data. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Measurement of oxygen consumption rate by high-resolution respirometry

Using high-resolution respirometry, we observed that 1.5×10^7 HK-2 cells completely consumed O_2 within 25 min with a respiration slope around 21 pmol O_2/s/10^6 cells as illustrated in Fig. 1A. As shown in Fig. 1B, the incubation of HK-2 cells with LPS for 1 h had no significant effect on the basal cellular respiration rate. However, when the incubation time with LPS became longer (at least 6 h), the HK-2 cells respiration rate was attenuated by ~20% (Fig. 1B): the slope decreased in treated cells from −19.57 ± 0.41 pmol O_2/s/10^6 cells in the control group to −16.07 ± 1.57 pmol O_2/s/10^6 cells in the 6 h incubation time group ($P < 0.05$, $n = 3$) and from −23.62 ± 1.71 pmol O_2/s/10^6 cells in the control group to −19.40 ± 2.69 pmol O_2/s/10^6 cells in the 18 h incubation time group ($P < 0.05$, $n = 3$). This variation in oxygen consumption rate observed by high-resolution respirometry was then compared using ESR oximetry. This latter method is known to be more sensitive for oxygen consumption measurement [21] and best suited to check if the LPS did not really act before 6 h of incubation on renal cells basal respiration.

3.2. Measurement of oxygen consumption rate by ESR oximetry

High-resolution respirometry measures directly the basal state of the cellular respiration but consumes oxygen by the electrode, while ESR oximetry uses an oxygen sensor, the ^{15}N-PDT spin probe, that distributes homogeneously throughout the sample and that does not consume oxygen. By ESR oximetry, we measured the de-