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

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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

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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-

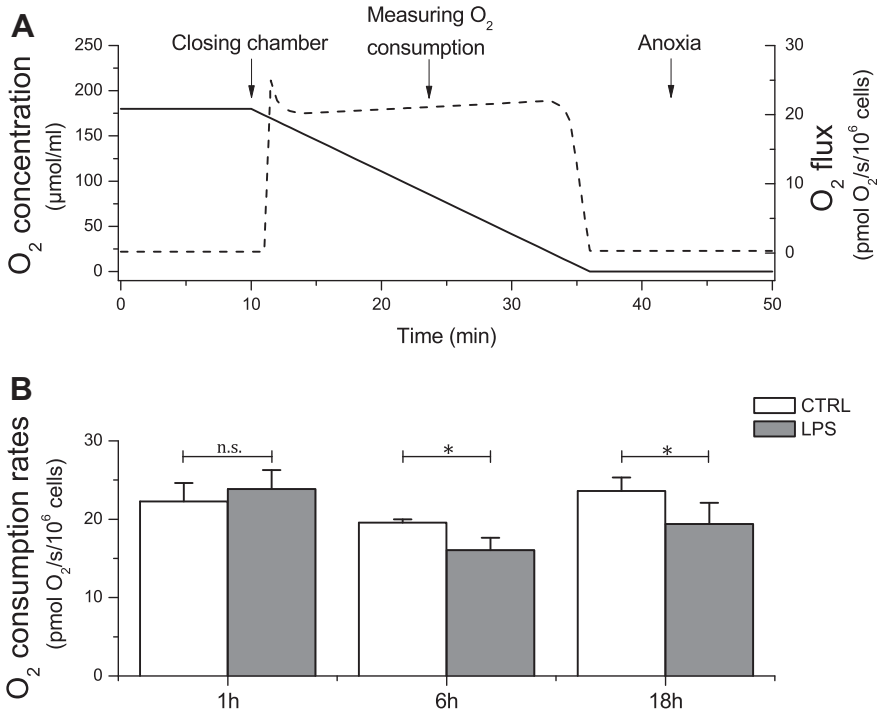

Fig. 1. Changes in O_2 consumption rates (slopes) of HK-2 cells (1.5×10^7 cells/assays) in DMEM. (A) Schematic O_2 concentration (solid line) and O_2 flux (dashed line) variation profiles of high-resolution respirometry during the oxygen consumption assay. (B) Influence of 1 h, 6 h and 18 h of LPS incubation on the O_2 consumption rates ($n = 3$). n.s., non-significant; *, $P < 0.05$; CTRL, control; LPS, lipopolysaccharide.

crease in $[O_2]$ over time and compared the oxygen consumption of HK-2 cells (7.5×10^6 cells/ml) between control cells and cells incubated with 1 µg/ml LPS. No modification of the slopes was observed after an incubation period of 1 h and 3 h, but the slopes decreased quite markedly when cells were treated with LPS for 6 h and 18 h (Fig. 2). When the results are expressed in terms of a mean oxygen consumption rate (OCR) (Fig. 3A), we observed that the mean OCR did not change when cells were incubated for a short period of time with LPS: -8.79 ± 2.07/min/7.5×10^6 cells in the control group ($n = 3$), 8.20 ± 0.43/min/7.5×10^6 cells in the 1 h incubation time group ($n = 3$) and 8.15 ± 2.54/min/7.5×10^6 cells in the 3 h incubation time group ($n = 4$). By contrast, when the cells were incubated for a long period of time with LPS, the mean OCR dropped markedly (~30%) to 6.64 ± 1.34/min/7.5×10^6 cells in the 6 h incubation time group ($P < 0.05$, n = 4) and to 5.99 ± 1.21/min/7.5×10^6 cells in the 18 h incubation time group ($P < 0.05$, $n = 3$).

3.3. Reversibility

The reversibility of this decrease in oxygen consumption rate of HK-2 cells was tested by washing several times cells incubated for 6 h with LPS and by incubating them for 24 h more in LPS-free medium prior to experiment. As shown in Fig. 3B, after the free-LPS incubation time, the oxygen consumption rate measured by ESR oximetry remains low -6.15 ± 2.21/min/7.5×10^6 cells compared to control group -9.13 ± 2.58/min/7.5×10^6 cells ($P < 0.05$, $n = 5$).

4. Discussion

Sepsis has a profound deleterious effect on kidney functions through complex mechanisms, which may involve hemodynamic instability, microcirculatory disorders, inflammatory pathways and intracellular dysfunction [10–13]. Those factors are difficult to discriminate *in vivo*. So we developed a simplified *in vitro* model of sepsis-induced AKI to get a better understanding of renal respiratory dysfunction. Using this cell model, we have tested the effect of an inflammatory stimulation on renal oxygen consumption rate by LPS challenge. In this way, renal cellular functions were directly targeted and hemodynamic and hypoxic effects were removed.

Both high resolution respirometry and ESR oximetry have shown that HK-2 cells incubation with LPS results in a decreased oxygen consumption rate (20% and 30% respectively) if the incubation period is at least 6 h. These results are in well-agreement with the study of James et al. [20] that highlighted that LPS can directly influence the rate of oxygen consumption by murine kidney cortex cell lines. This alteration in the renal respiratory function could be simply due to a decrease in the cell viability. However, cells showed no decrement in viability irrespective of whether the cells had been incubated with LPS or not (data not shown). Taking this into account, the decrease in oxygen consumption rate in HK-2 cells after LPS challenge must be predominantly a functional problem and may be in relation with a metabolic down-regulation [25,26]. This phenomenon has already been described at the cellular level in response to a diminished oxygen supply during hypoxic or ischemic events [26,27]. Cells enter in a hibernating state and maintain viability by down-regulating oxygen consumption, stopping non essential cellular functions and reducing energy demand. With respect to this literature data, we hypothesize that the LPS-targeted inflammatory response induces direct intracellular dysfunction. Cells have no longer the capacity to maintain their metabolic functions despite adequate oxygenated environment due to the impossibility of using the available oxygen for the respiratory chain. Our study supports the hypothesis that the inflammatory response inherent to sepsis must be considered as a direct mechanism of AKI. Renal energetics in sepsis is disrupted not just because O_2 delivery is impaired but also because the capacity of cells to utilize O_2 might be compromised.

In our cellular model of inflammation-induced AKI, the decreasing metabolic activity and O_2 demand during LPS challenge is not

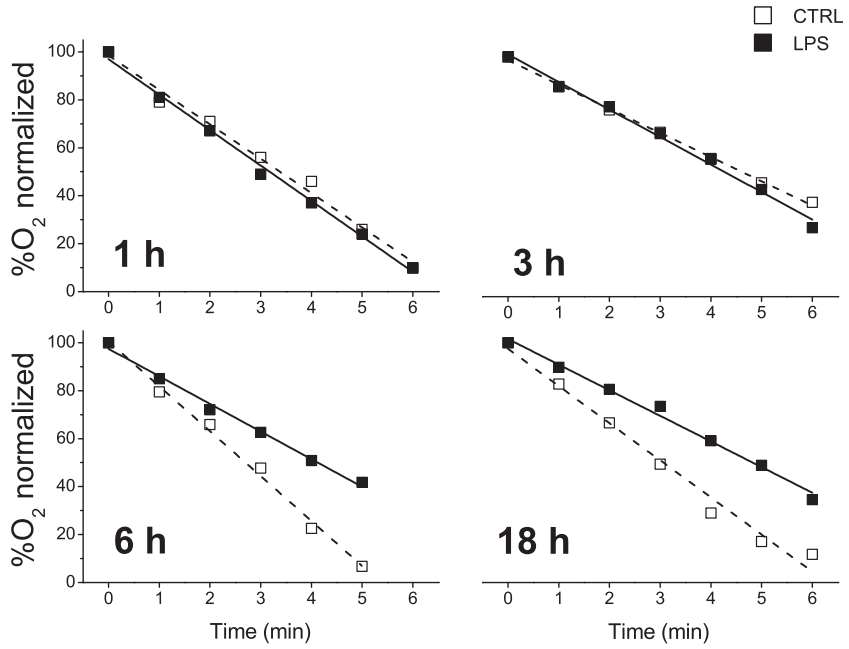

Fig. 2. Oxygen consumption rate measured by ESR oximetry of HK-2 cells (7.5×10^6 cells/ml). The graph presents the variations in the normalized percentage of oxygen as a function of time for different period of LPS incubation. When the incubation period of LPS is smaller than 3 h, LPS-treated cells have a non significant effect in oxygen consumption. When the incubation period of LPS is higher than 6 h, LPS-treated cells consumed oxygen significantly more slowly than control cells. CTRL, control; LPS, lipopolysaccharide.

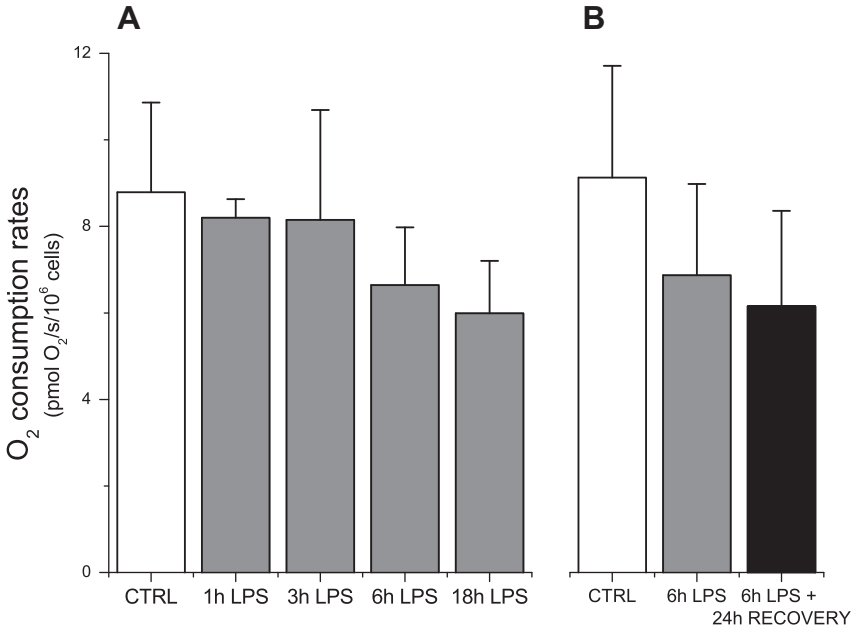

Fig. 3. A: The mean oxygen consumption rate (OCR) measured by ESR oximetry in the control cells were higher than those in LPS-treated cells with incubation time of 6 h ($P < 0.05$, $n = 4$) and 18 h ($P < 0.05$, $n = 3$) but not higher than those in LPS-treated cells treated with incubation time of 1 h ($P > 0.05$, $n = 3$) and 3 h ($P > 0.05$, $n = 4$). B: Study of the HK-2 cells ability to recover normal respiration when incubated in a LPS-free fresh medium for 24 h after a 6 h LPS challenge. After the free-LPS incubation time, the oxygen consumption rate remains low compared to control group ($P < 0.05$, $n = 5$). CTRL, control; LPS, lipopolysaccharide.

reversible. When HK-2 cells are incubated for 6 h with LPS then for 24 h in free-LPS fresh medium, they do not recover a normal basal respiration level. The cell damage caused by the LPS-targeted inflammatory response is permanent. The primitive adaptive response may turn into a pathologic process [26–28]. Extrapolating these results to the clinical pathology of acute kidney injury, one can propose that the kidney energy failure induced by sepsis will persist even if the hemodynamic factors are well re-established at the organ level. In the inflamed organ, cellular metabolic down-regulation likely manifests as a kidney dysfunction and may be seen at first sight as affording protection to promote renal cell viability. However, as the phenomenon is not reversible, what may begin as an organ survival adaptation in response to unfavorable environmental conditions may progress to become a pathologic process and conduct the patient to a sepsis-induced AKI.

Overall, this work supports the hypothesis that the inflammatory response inherent to sepsis must be considered as a direct mechanism of AKI. In our cellular model, we choose LPS as a caus-

ative agent to trigger inflammatory responses, generally associated with clinical condition of sepsis, in an *in vitro* model using HK-2 cells. Our high-resolution respirometry and ESR oximetry studies have demonstrated a strong irreversible decrease in the oxygen consumption rates in renal cells subjected to endotoxins. LPS-stressed HK-2 cells are no longer capable to use the available oxygen to maintain its metabolic functions. This basal respiration alteration of renal HK-2 cells is a sign of pathologic metabolism down-regulation.

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