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EXPERIMENTAL

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Endotoxin reduces maximal oxygen consumption in hepatocytes independent of any hypoxic insult

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Abstract *Objective:* The cause of the metabolic disturbances in sepsis remains uncertain, but there is increasing evidence suggesting that haemodynamic changes are not solely responsible. We addressed the question of whether endotoxin has a significant effect on cellular oxygen metabolism, independent of confounding haemodynamic defects.
Design: Prospective, controlled experimental study.
Setting: University Laboratory.
Model: Human hepatocyte cell line.
Methods: The oxygen consumption rate (OCR) was calculated from the fall in oxygen tension in a sealed cuvette containing Hep G2 cells in suspension. The oxygen tension was measured by porphyrin phosphorescence half-life analysis. Resting OCR was measured in control cells and after 1, 6 and 24 h of endotoxin exposure. In a second series of experiments, resting and maximal OCR was measured after 6 and 24 h of endotoxin exposure and in control cells using the addition of a mitochondrial uncoupler (FCCP); this uncouples the respiratory chain from ATP synthesis, thereby removing negative feedback and allowing the respiratory chain to work at maximal rate.
Results: Endotoxin caused a rise in resting OCR at 1 h which was significant by 6 h but had returned to control values by 24 h. Maximal OCR also increased at 6 h, however exposure to endotoxin for 24 h significantly reduced maximal OCR compared to the control cells.
Conclusions: Endotoxin has complex effects on cellular energy metabolism causing an initial rise in the oxygen consumption rate and a significant limitation in oxygen consumption capacity at 24 h. These complex effects would be in keeping with the varied responses seen in patients.

Key words Sepsis · Oxygen consumption · Mitochondria · Hepatocytes · Hypoxia · Endotoxin

Introduction

Septic shock [1] differs from all other causes of shock in that the delivery of oxygen to the tissues is increased and oxygen extraction by the tissues reduced. Despite this, there are features of anaerobic metabolism: the development of a metabolic acidosis and hyperlactataemia. The cause of the apparent anaerobic metabolism seen in sepsis remains unclear despite considerable research over many years.

There are two fundamental theories to explain this conundrum. Firstly that the haemodynamic disturbance causes a disordering of the microcirculation leading to tissue hypoxia, either globally or locally. Secondly that there is a defect in cellular energy metabolism with a resulting impairment in the ability to utilise oxygen. In both these areas the literature is conflicting. There are a number of reasons for this; there is no reliable marker of the severity of the septic response and the dose response of the whole animal to endotoxin is non-linear.

In fact, the dose response pattern varies between different variables even within the same model [2]. There is considerable inter-species variation in the response to endotoxin and also between endotoxins from different bacteria within the same species of animal [3, 4]. The degree of resuscitation of the model, particularly the fluid management, significantly influences the response [5]. The inherent complexity of the septic response means that many variables are changing simultaneously, making it very difficult to identify a causal link between any two events.

Despite these interpretation difficulties, a number of things are clear from the literature. There is no direct evidence to support the concept that areas of tissue are hypoxic in resuscitated sepsis models. Despite clear evidence of a disordering of the microvasculature there is no strong evidence to support shunting of oxygenated blood away from consuming tissues. Indeed, there is accumulating evidence that tissue oxygen availability is maintained or increased in both the fluid resuscitated model [2, 5–7] and the clinical situation [8–10].

In order to identify any direct defect in cellular oxygen metabolism caused by endotoxin, we have measured baseline and maximal oxygen consumption rates in a human hepatocyte cell line (Hep G2), thus excluding the effect of any microvascular changes. This cell line was chosen as hepatocytes have a central role in sepsis, being responsible for clearing endotoxin from the portal circulation, and liver dysfunction is common in sepsis although the precise mechanisms are unclear.

Materials and methods

Cell preparation

Hep G2 cells were maintained in log phase growth under standard conditions (37 °C, 5 % CO_2) in 15 ml of Eagles' MEM (Sigma chemicals, Poole, UK) with penicillin, streptomycin, amphoteracin, L-glutamine and 15 % foetal calf serum. Immediately before oxygen consumptiom studies were performed, the cells were lifted off the culture flask using 0.125 % trypsin EDTA (Sigma), washed and re-suspended in 2.25 ml of fresh medium with 35 mg/ml of bovine albumin (Sigma).

Experimental protocol

In the first set of experiments, endotoxin (*E. Coli* 0127:B8 trichloroacetic acid extraction, Sigma) was added to the culture flasks to achieve a final concentration of 10 μg/ml 1, 6 and 24 h before the oxygen consumption studies were performed. A control group, not exposed to endotoxin, was also studied. Oxygen consumption was measured as described below. A sample of the cell suspension was analysed immediately after completion of the oxygen consumption studies with a blood gas laboratory (ABL4, Radiometer, Copenhagen, Denmark), allowing the measurement of PO_2, PCO_2, pH and potassium. The cell concentration was then counted by haemocytometer (counting > 1200 cells/cuvette) and viability assessed with trypan blue exclusion.

For the second set of experiments three groups were studied, one exposed to endotoxin for 6 h, one for 24 h and a control group not exposed to endotoxin. Oxygen consumption measurements were made in the same way, except that carbonyl cyanide methoxy phenylhydrasone (FCCP, Sigma), a mitochondrial uncoupler used to maximise oxygen consumption, was injected when the oxygen tension fell to 35 torr. The FCCP was injected in 50 μl of 50 % ethanol in 0.9 % saline to achieve a final concentration of 50 μg/ml. In this way both basal (pre FCCP) and maximal (post FCCP) oxygen consumption were obtained from the same sample of cells.

Oxygen consumption studies

The cell suspension was placed in a 2 cm^3 disposable cuvette with a tight fitting lid to prevent the entry of atmospheric oxygen, containing 37.5 μg of palladium mesotetra porphyrin (Medical systems, via Digitimer, Welwyn Garden City, UK). The cuvette was placed in a circulating water bath maintained at 37 °C adjacent to the tip of the fibreoptic cable from the Oxyspot system (Medical systems). Continuous uniform mixing was achieved with a magnetic stirrer. Oxygen tension was measured by the oxygen dependent quenching of porphyrin phosphorescence [11] using the Oxyspot. In these experiments the phosphorescence half-life of five flashes were averaged. Measurements of oxygen tension were recorded every 0.5 s once oxygen tension had fallen to 60 torr, the upper limit of accuracy using this porphyrin, and were continued until oxygen consumption had ceased. Oxygen consumption was calculated from the rate of fall of oxygen tension in the sealed cuvette.

This basic design was altered for the second set of experiments in order to allow the injection of FCCP, saline or pentobarbitone. Prior to the start of the experiment, the lids were pierced by two lengths of 1.2 mm diameter polythene tubing which were sealed in place with a silicone sealant. One length of tubing was primed with FCCP, the other was a vent tube which filled with medium as the lid was secured, to form an effective diffusion barrier. Recordings of oxygen tension were made over the same range.

Data analysis

Oxygen consumption was calculated from the slope of the line given by plotting oxygen tension against time, the correlation of this to a perfect straight line was also calculated (Microsoft Excel). For the first experiments, data from 60 torr to 10 torr was used for line fitting. In the second set of experiments, data from 60 torr to 40 torr was used for baseline oxygen consumption and from 30 torr to 10 torr, i.e. after FCCP injection, for maximal oxygen consumption. Oxygen consumption is thus expressed as torr/s. In the first set of experiments this value was corrected for cell concentration by dividing by the number of cells/ml expressed in billions (10^9). The second set of experiments were not adjusted for cell concentration, as each acted as its own control for variation in cell number.

Oxygen consumption data from the first set of experiments were analysed by factorial analysis of variance with post hoc testing by Scheffe's test. In the second set of experiments the ratios of pre:post FCCP oxygen consumption were analysed, also by ANOVA. Statistical significance was taken as p less than 0.05.

Results

There were no significant differences between groups in PCO_2, pH, potassium or cell numbers. Cell viability was greater than 99% in all cases as assessed by trypan blue exclusion at the end of each experiment. In all cases the correlation of the oxygen tension versus time plot to a perfect straight line was excellent ($r^2 > 0.99$).

In the first set of experiments there was a non-significant rise in oxygen consumption rate between control ($n = 6$) and the cells to which endotoxin was added 1 h before ($n = 6$) and a significant rise in the cells to which endotoxin was added 6 h before ($n = 6$, $p < 0.05$). In the cells to which endotoxin was added 24 h before ($n = 6$) the oxygen consumption rate had fallen back to control levels (Fig. 1).

In the second set of experiments there was a similar trend in baseline oxygen consumption with a rise at 6 h followed by a return to baseline at 24 h. Maximal oxygen consumption also rose at 6 h but fell below baseline at 24 h. This is reflected in the significant fall in the ratio of baseline to maximal oxygen consumption at 24 h ($p < 0.05$) (Fig. 2).

Control experiments, injecting saline and saline plus ethanol into the cuvette at the same volume and time as the FCCP, showed no change in oxygen consumption rate after the injection. Pentobarbitone, which blocks NADH dehydrogenase, completely abolished oxygen consumption (Fig. 3). In addition, media at a range of oxygen tensions with no cells added showed no change in oxygen tension over time, indicating the very low oxygen consumption of the system and confirming that the cuvette was isolated from atmospheric oxygen.

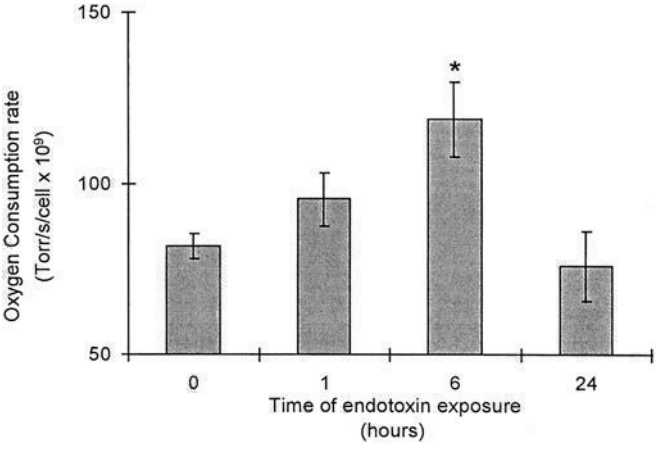

Fig. 1 The effect of endotoxin on cellular oxygen consumption with time. The *Y axis* shows oxygen consumption rate in torr/s per billion cells and the *X axis* shows the time of endotoxin exposure before measurements were made. Values plotted are means and *error bars* represent SEM (* represents $p < 0.05$; control [0 hours] $n = 15$, others $n = 6$)

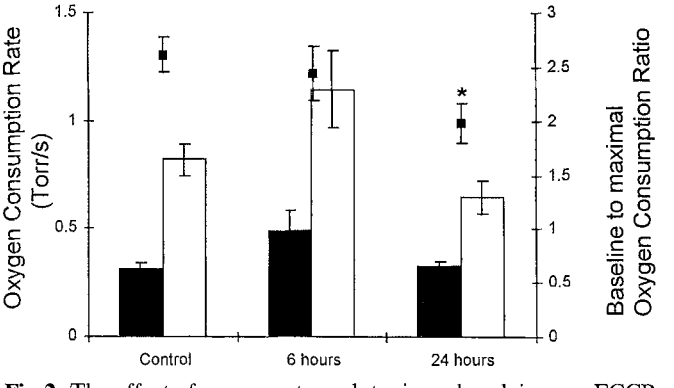

Fig. 2 The effect of exposure to endotoxin on basal, i.e. pre FCCP *(black columns)*, and maximal, i.e. post FCCP *(white columns)*, cellular oxygen consumption rate on the left hand axis. The ratio of baseline to maximal oxygen consumption is represented by *black squares* on the right hand axis. Data points are means and the *error bars* show SEM. * represents $p < 0.05$

Discussion

These data demonstrate that endotoxin has direct effects on cellular oxygen consumption in the absence of microvascular defects. Basal oxygen consumption is raised significantly by 6 h before falling back to baseline levels at 24 h. Although the baseline oxygen consumption rates of controls and endotoxin-treated cells are identical by 24 h, on maximising the oxygen consumption rate at this stage a hidden defect in cellular energy metabolism is unmasked. Reduced tissue oxygen supply due to a loss of microvascular control has been suggested to explain the oxygen consumption defects seen in sepsis. Here we show for the first time that in the absence of cellular hypoxia, as would be caused by circulatory abnormalities, endotoxin alone limits the maximal cellular oxygen consumption capacity of the mitochondrion.

The technique used for measuring oxygen consumption is based on the work of Robliolio et al. [12]. In brief, the sealed container prevents the entry of oxygen from the environment, therefore the rate of fall of oxygen tension is dependent only on the rate of oxygen consumption by the cells in suspension. That the oxygen was by consumed the respiratory chain was confirmed by the injection of pentobarbitone to the system.

The porphyrin phosphorescence method of measuring oxygen tension was chosen over an electrode-based technique as the absence of a physical object within the cuvette avoids stirring artefacts and vortex effects. Thus even distribution of oxygen within the cuvette is ensured so long as the medium is stirred. Cell adherence to electrodes may also alter their responsiveness and produce local consumption artefacts. The speed of re-

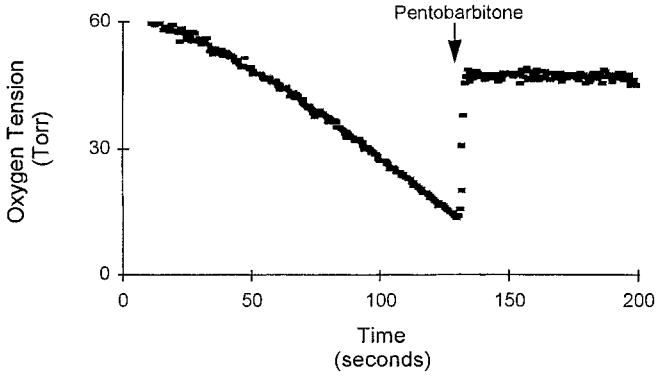

Fig. 3 The effect of the introduction of pentobarbitone, a respiratory chain blocker, to the cuvette. The injection point is indicated by the *arrow*. After equilibration there is no further oxygen consumption

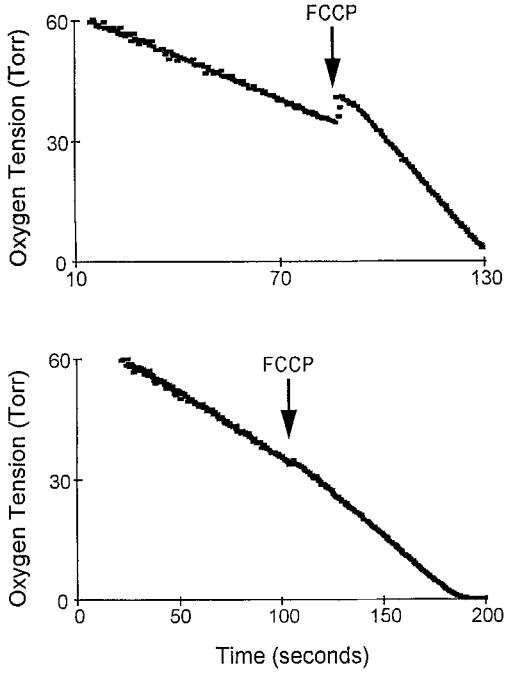

Fig. 4 Examples of plots of oxygen tension against time from which baseline and maximal oxygen consumption rates were calculated. In both cases the *arrow* represents the point at which FCCP is introduced. The *upper panel* shows a control (non-endotoxin-exposed) cell sample when FCCP induced a large rise in oxygen consumption and the *lower panel* a sample exposed to endotoxin for 24 h where a minimal rise in oxygen consumption was seen

sponse of the porphyrin phosphorescence system, its very low oxygen consumption and its accuracy at low oxygen tensions are superior to standard electrodes.

A number of disturbances of energy metabolism are seen in sepsis, although these are not consistent either between patients or models. Glycolysis is enhanced, with an increase in glucose turnover [13, 14] while the fatty acid oxidation cycle appears unaltered [15]. Within the mitochondrion there is an impairment of the pyruvate dehydrogenase complex in sepsis [16], this large enzyme complex converts pyruvate, the end product of glycolysis, to acetyl CO-enzyme A, the substrate of the citric acid cycle. This dysfunction has been suggested as the cause of the hyperlactataemia which is common in sepsis, however it appears to be a normal negative feedback response to an increase in acetyl CO-enzyme A [17], indicating that the the cause of the acidosis and hyperlactataemia lies within the citric acid cycle or the respiratory chain. Little is known specifically about the function of the citric acid cycle or the respiratory chain in sepsis.

Work from as far back as 1975 has suggested that endotoxin is capable of producing a profound effect on basal cellular oxygen consumption [18]. It has also been recently shown, with a rather more reliable methodology, that endotoxin reduces the basal oxygen consumption rate of a number of cell types, kidney cortex, macrophages and ovarian cells [19]. This confirms that endotoxin is capable of producing a profound alteration in energy metabolism. A reduction of cytochrome aa3, independent of hypoxia, has been demonstrated in sepsis [20], which supports the view that there is a direct impairment of energy metabolism. There is also evidence in the literature suggesting that mitochondrial function is enhanced in sepsis [21]. However, these studies were performed in isolated mitochondria which may not be representative of the intact cell. The data presented here suggest that the response of the cellular energetics to endotoxin vary with time. As none of the studies described above look at different times of endotoxin exposure, differences in their findings may simply be explained by this time response.

The results of our first series of experiments raises the question of why the oxygen consumption rate returns to normal after 24 h of endotoxin exposure. This could be taken to indicate either that the endotoxin effect has ended or that there is a second process acting to depress the oxygen consumption rate. In order to investigate this, the ability of the cells to increase their oxygen consumption was measured. The injection of the FCCP in the second set of experiments allowed the comparison of maximal to baseline oxygen consumption in the same group of cells without the further manipulation of the data. FCCP causes the hydrogen ion gradient across the inner mitochondrial membrane to be dissipated by allowing hydrogen ions to pass through the ATP synthase complex without creating ATP from ADP. This effectively uncouples the respiratory chain from ATP production, removing negative feedback and thus maximising oxygen consumption. FCCP is lipid-soluble, therefore enters cells freely and has a very rapid effect which allows it to be used as described. There was inevitably a small quantity of oxygen introduced to the cu-

vette at this point as the PO_2 of the injectate was atmospheric and a temporary loss of the linear fall in oxygen tension was seen as the FCCP mixed and took effect (Fig. 4). For this reason the data between 40 torr and 30 torr was not analysed. The significant impairment of the endotoxin treated cells' ability to increase their maximal oxygen consumption strongly implies that the return to baseline of resting oxygen consumption is due to a secondary effect of endotoxin rather than the resolution of the initial effect.

The data presented in the paper demonstrate, for the first time, an impairment of the maximal oxygen consumption capacity of endotoxin-treated cells. This is despite the cells studied in these experiments having at no time been hypoxic. This suggests that endotoxin causes a cellular metabolic defect and further calls into question the concept that the metabolic problems of sepsis relate solely to tissue hypoperfusion. All of the metabolic problems identified in sepsis could equally well be explained by a defect in respiratory chain function (whether direct or as a result of citric acid cycle dysfunction) as by cellular hypoxia. These data then support the hypothesis that the failure of energy metabolism seen in clinical sepsis is, at least in part, related to a defect in cellular energy metabolism rather than simply a perfusion defect.

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