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Mitochondrial dysfunction in peripheral blood mononuclear cells in early experimental and clinical acute pancreatitis

Mandira Chakraborty [a, *, 1], Anthony J.R. Hickey [b, c, 1], Maxim S. Petrov [a], Julia R. Macdonald [a], Nichola Thompson [a], Lynette Newby [d], Dalice Sim [e], John A. Windsor [a], Anthony R.J. Phillips [a, b, c]

[a] Department of Surgery, Faculty of Medical and Health Sciences, University of Auckland, New Zealand
[b] School of Biological Sciences, Faculty of Science, University of Auckland, New Zealand
[c] Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, New Zealand
[d] Department of Critical Care Medicine, Auckland City Hospital, Auckland, New Zealand
[e] School of Mathematics, Statistics and Operations Research, Victoria University of Wellington, Wellington, New Zealand

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ABSTRACT

Background/objectives: Mitochondrial dysfunction occurs in vital organs in experimental acute pancreatitis (AP) and may play an important role in determining severity of AP. However, obtaining vital organ biopsies to measure mitochondrial function (MtF) in patients with AP poses considerable risk of harm. Being able to measure MtF from peripheral blood will bypass this problem. Furthermore, whether mitochondrial dysfunction is detectable in peripheral blood in mild AP is unknown. Therefore, the objective was to evaluate peripheral blood MtF in experimental and clinical AP.

Method: Mitochondrial respiration was measured using high resolution oxygraphy in an experimental study in caerulein induced AP and in a separate study, in patients with mild AP. Superoxide, cytochrome *c*, mitochondrial membrane potential ($\Delta\Psi$) and adenine triphosphate (ATP) were also measured as other markers of MtF.

Results: Even though some states of mitochondrial respiration were increased in both experimental and clinical AP, this did not lead to an increase in net ATP in patients with AP. The increased leak respiration in both studies was further proof of dyscoupled mitochondria. In the clinical study there were also features of mitochondrial dysfunction with increased leak flux control ratio, superoxide, $\Delta\Psi$ and decreased cytochrome *c*.

Conclusion: There is evidence of mitochondrial dysfunction with dyscoupled mitochondria, increased superoxide and decreased cytochrome *c* in patients with mild acute pancreatitis. Further studies should now determine whether mitochondrial function alters with severity in AP and whether mitochondrial dysfunction responds to treatments.

1. Introduction

Acute pancreatitis (AP) remains a challenging disease to manage, primarily because predicting severity of AP and monitoring responses to interventions are imprecise [1]. This reflects the need to better understand pathophysiology of AP. Patients die with AP due to multiple organ failure (MOF) [2], and one of the features of MOF is mitochondrial dysfunction [3]. The main function of mitochondria is to efficiently generate adenine triphosphate (ATP) by oxidative phosphorylation [4]. In addition, mitochondria are also involved in the generation and detoxification of free radicals, apoptosis, regulation of calcium, synthesis and catabolism of metabolites and transport of organelles to correct locations within the cell [4]. Abnormalities in any of these functions can result in mitochondrial and cellular dysfunction [4].

We have demonstrated significant dysfunction of mitochondrial electron transport system (ETS) in the pancreas and in jejunum early in caerulein induced AP [5]. Others have also reported mitochondrial dysfunction with inhibition of Complex I in the pancreas,

* Corresponding author. Department of Surgery, University of Auckland, Private Bag 92019, New Zealand.
E-mail address: mandira.chakraborty@gmail.com (M. Chakraborty).
[1] These authors contributed equally to this study.

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kidney and lung in a taurocholate experimental model of AP [6]. Further, supramaximal stimulation by caerulein decreased the capacity of pancreatic mitochondria to produce ATP [7]. However, what happens to mitochondrial function in mild AP without development of MOF is unknown. If mitochondrial dysfunction is detectable in mild AP, it might be relevant in determining severity of disease and help to track MOF in AP.

Despite advances in measuring mitochondrial function in experimental models of AP, mitochondrial function has never been measured in patients with AP. An obvious problem is that organ biopsies are required to measure organ MtF and obtaining organ biopsies will never be a practical or an ethical approach in AP. Therefore, measuring MtF from peripheral blood could provide a less invasive approach to determine a global measure of the bioenergetic state of the patient. In particular, peripheral blood mononuclear cells (PBMC) are known to be involved in the pathogenesis of AP. It has been reported that a decrease in $CD4^+$ and $CD8^+$ subsets of lymphocytes is inversely proportional to the severity of AP [8] and increased monocyte secretion of cytokines is associated with systemic complications of AP [9]. In AP, it is not known whether MtF in PBMC is altered early in the disease course. In this study, we aimed to design a protocol to study PBMC MtF in an experimental model and translated this protocol to patients with mild AP early in the disease course to test the hypothesis that PBMC MtF is altered early in mild AP.

2. Materials and methods

MtF was measured in PBMC obtained from a caerulein rodent model of AP [10] and also from patients with mild to moderate AP who were in the control group of a randomised clinical trial [11].

2.1. Animals

All experiments were approved by the Animal Ethics Committee of the University of Auckland and fulfilled requirements of the National Institutes of Health Guide [12] for the Care and Use of Laboratory Animals. Male Wistar rats were housed in pairs and kept under a constant 12 h light:dark cycle with 50–70% humidity at a temperature of 19–21 °C, Rats were fed standard Harlan Tekland 2018 rodent diet and were allowed free access to food and water throughout the experiments.

2.2. Induction of experimental AP

Eighteen male Wistar rats were randomised to a control group (n = 9) and caerulein pancreatitis group (n = 9). They received either five, hourly subcutaneous injections of 0.5 ml saline (control) or 50 μg/kg of caerulein on the back of the conscious animal's neck. Six hours after the initial injection of caerulein/saline, anaesthesia was induced by isoflurane (5%; 2 L/min O_2) and maintained with an isoflurane nose cone (40% O_2: air). Two minutes after an intravenous injection of heparin (100 IU/kg) through a tail vein, blood was obtained through a cardiac puncture. The pancreas wet weight was measured and divided by total body weight as a measure of the pancreatic oedema index [13].

2.3. Patients with mild AP

This study was approved by the New Zealand regional ethics committee (NorthernX), NTX/08/11/107 [NCT01128478] and NTX/10/11/119 [ACTRN12612000047897]. Patients with mild to moderate AP were recruited from the Emergency Department of the Auckland City Hospital, NZ. Written informed consent was obtained from the patient or next of kin. A diagnosis of AP required at least two of the following: 1) abdominal pain typical of AP, 2) amylase and/or lipase more than three times the upper limit of normal, and/or 3) findings consistent with AP on abdominal imaging.

Mild AP was defined as the absence of systemic inflammatory response syndrome and/or the absence of organ failure. Systemic inflammatory response syndrome was defined by standard criteria and organ failure was defined in accordance with the Marshall scoring system as a score ≥2 for at least one of three organ systems: respiratory, renal without pre-existing renal disease, and cardiovascular. Patients were excluded if they had chronic pancreatitis, post endoscopic retrograde cholangiopancreatography pancreatitis, intraoperative diagnosis of AP, pregnancy or malignancy and when they failed to present to hospital within 96 h from the onset of symptoms.

20 ml of intravenous blood was collected from the arm within 24 h of presentation to the hospital. The control group consisted of healthy volunteers (HV) from a population within the community who were gender and age matched within five years of study patients. For the AP group, 3 ml of blood was send to Auckland City Hospital Laboratory for measurement of WBC, lymphocyte and monocyte counts.

2.4. Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were retrieved from whole blood using the density gradient separator Ficoll-Paque™ Plus (GE Lifesciences). After washing with phosphate-buffered saline, cells were re-suspended in assay medium containing (in mmol/l (final)) 0.5 EGTA, 3 $MgCl_2$, 60 K-lactobionate, 20 taurine, 10 KH_2PO_4, 110 sucrose, and 1 mg/ml Bovine Serum Albumin in 20 mmol/l HEPES. A cell count was performed and respiration assays normalized to per million live cells. Mononuclear cells were characterised with specific monoclonal antibodies using flow cytometry (see Supplementary Material).

2.5. Assays of the mitochondrial electron transport system

A multiple substrate-inhibitor titration protocol was employed (Fig. 1, Table 1s) because it is known to better reflect respiration flux *in vivo* and to explore the relative capacity of the electron transport system (ETS) and phosphorylation system (OXPHOS) components [14]. An OROBOROS Oxygraph-2K (Anton Paar, Graz, Austria) was used to measure mitochondrial respiration. Assays were performed at 37 °C in 2 ml incubation assay medium, and the oxygen concentration at air saturation of the medium was 215 nmol O_2/ml at 95 kPa barometric pressure. Oxygen flux was calculated as the time derivative of oxygen concentration using the DatLab 4 Analysis Software, OROBOROS (Innsbruck, Austria).

Approximately 10 million mononuclear cells were added per chamber and endogenous respiration was measured. The endogenous cellular respiration is the sum of oxidative phosphorylation, respiration due to proton leak across mitochondrial inner membranes (which does not contribute to ATP synthesis), and non mitochondrial oxygen consumption by numerous oxidases. Digitonin (25 μg/ml) was then added to permeabilise the cholesterol rich plasma membrane, thereby leaving mitochondrial membranes intact and permitting the diffusion of mitochondrial substrates. After waiting for oxygen flux to decline to a low steady state (Fig. 1), Complex I mediated leak (CI Leak) was stimulated by the addition of Complex I substrates (10 mmol/l glutamate, 2 mmol/l malate). Addition of excess ADP (1.25 mmol/l for PBMC) then stimulated oxidative phosphorylation (CI OXPHOS). Addition of cytochrome *c* (10 μmol/l) was used to test the functional integrity of mitochondrial inner membranes, and pyruvate (5 mmol/l) was then added to ensure saturation of CI

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