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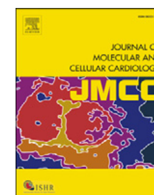

Original article

Lipopolysaccharide responsiveness is an independent predictor of death in patients with chronic heart failure

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ABSTRACT

Background: The origin of pro-inflammatory activation in chronic heart failure (HF) remains a matter of debate. Lipopolysaccharide (LPS) may enter the blood stream through the morphologically altered and leaky gut barrier. We hypothesized that lower LPS reactivity would be associated with worse survival as compared to normal or higher LPS reactivity.
Methods: LPS responsiveness was studied in 122 patients with chronic HF (mean ± SD: age 67.3 ± 10.3 years, 24 female, New York Heart Association class [NYHA] class: 2.5 ± 0.8, left ventricular ejection fraction [LVEF]: 33.5 ± 12.5%) and 27 control subjects of similar age (63.7 ± 7.7 years, $p > 0.05$). Reference LPS was added at increasing doses to ex vivo whole blood samples and necrosis factor-α (TNFα) was measured. Patients were subgrouped into good- and poor-responder status according to their potential to react to increasing doses of LPS (delta TNFα secretion). The optimal cut-off value was calculated by receiver–operator characteristic curve (ROC) analysis.
Results: A total of 56 patients with chronic HF died from any cause during follow-up. At 24 months, cumulative mortality was 16.4% (95% confidence interval 16.0–16.7%). The delta TNFα value representing the optimal cut-off for the prediction of mortality was 1522 pg/mL (24 months) with a sensitivity of 49.3% (95% confidence interval 37.2–61.4%) and specificity of 81.5% (95% confidence interval 61.9–93.6%). LPS responder status remained an independent predictor of death after multivariable adjustment (hazard ratio 0.09 for good- vs. poor-responders, 95% confidence interval 0.01–0.67, $p < 0.05$).
Conclusions: LPS responsiveness in patients with chronic HF is an independent predictor of death.
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1. Introduction

Chronic heart failure (HF) is a major public health challenge. Overactivity of neuroendocrine pathways is crucially involved in poor outcomes in this disease, although the introduction of beta-blockers, angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers, and aldosterone antagonists into current HF treatment guidelines [1], has helped improving the patients' prognosis. Novel avenues embrace repletion of iron deficiency and heart rate reduction with ivabradine. Overactivity of the immune system may be another worthwhile therapeutic target for implementing prognostic improvements [2].

An important initial step in this regard is the development of an understanding of the interplay between neuroendocrine and inflammatory players. Preclinical data suggest that elevated serum levels of pro-inflammatory cytokines become detectable earlier in the course of the disease than, for example, elevated levels of angiotensin II [3]. Pro-inflammatory players embrace predominantly tumor necrosis factor α (TNFα), interleukin (IL) 1 and IL-6, all of which have been shown to carry prognostic value in patients with chronic HF [4,5]. One of the strongest pathophysiological inducers of pro-inflammatory mediators is lipopolysaccharide (LPS), a cell wall constituent of Gram-negative bacteria. Our group has previously shown that minute concentrations of LPS deemed pathophysiologically relevant are able to effectively induce TNFα release in an ex vivo model of whole blood from patients with chronic HF [6]. Following the concept that LPS may enter the

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blood stream through the morphologically altered and leaky gut barrier (and possibly also through the pulmonary circulation) [7–9], we used LPS for our stimulation experiments.

Patients with longer duration of clinical HF may experience repeated LPS challenges, particularly during episodes of acute edematous decompensation, and immune cells such as monocytes or lymphocytes may not be able to respond adequately to such repeated LPS exposures. In patients with sepsis, the term LPS desensitization has been proposed to describe this phenomenon, and it is a frequent finding in critically ill patients [10]. Similarly, our group has shown that LPS-desensitization may play a significant role in patients with HF [11]. Therefore, understanding the impact of LPS desensitization on survival may help in the development of novel therapies. An understanding of the pathophysiology of the trace element selenium may complement such approaches, as recent data suggest that inflammatory responses are selenium-dependent [12]. Several selenoproteins have anti-oxidant properties that take part in pivotal pathways inside leukocytes [13]. Additionally it has been shown that selenium supplementation suppresses pro-inflammatory gene expression in LPS-treated macrophages [14], as well as in a murine LPS-induced model for septic shock [15]. Selenium and selenoprotein levels are strongly decreased in patients with severe inflammatory pathologies [16–18], and are in discussion as prognostic markers for survival in septic [19,20], and renal cancer patients [21].

We hypothesized that lower LPS reactivity would be associated with worse survival as compared to normal or higher LPS reactivity in patients with chronic HF. In addition, we sought to elucidate the role of the selenium status in this context.

2. Methods

2.1. Study population and follow-up

Patients were recruited from the Royal Brompton Hospital specialist HF clinic (London, UK) as part of different studies into the responsiveness of ex vivo whole blood to LPS. Control subjects were recruited from patients' relatives and hospital staff. Patients participated in projects designed to investigate novel biochemical markers and provided written informed consent. Patients were diagnosed according to guidelines issued by the European Society of Cardiology [1], together with documented objective evidence of left ventricular impairment (left ventricular ejection fraction [LVEF] <45%). Patients taking non-steroidal anti-inflammatory drugs (except low dose aspirin) or other immunomodulatory agents (e.g. steroids) were excluded. Subjects with clinical signs of infection, severe neuro-muscular disease, rheumatoid arthritis, significant renal dysfunction (serum creatinine >250 μmol/L), or cancer were also excluded, as were patients younger than 18 years of age and those with a history of unstable angina, myocardial infarction, or stroke within three months prior to the study.

A total of 122 patients with chronic HF and 27 control subjects of similar age who participated in different previously published prospective studies of immune function were pooled [11,22–24]. Only subjects with available LPS stimulation data and a serum sample for the analysis of selenium were eligible for the present study. The clinical characteristics of these subjects are listed in Table 1. Patients and controls were followed up for survival until March 2010 when the follow-up was censored. All patients were stable on medication for at least four weeks prior to being studied. Ninety-four percent of patients received diuretics, 97% angiotensin converting enzyme inhibitors or angiotensin receptor blockers, 59% beta-blockers, 53% spironolactone, and 60% statins.

2.2. Whole blood stimulation experiments

Venous blood was collected from an antecubital vein after 15 minutes semi-supine rest early in the morning into LPS-free tubes (Chromogenix AB, Sweden). Whole blood samples were diluted 1:1 with RPMI 1640 (Life Technologies Ltd., Paisley, UK) supplemented with 10 U/mL heparin (Leo Laboratories Ltd., Bucks, UK) to elicit a more pronounced TNFα production than that of undiluted samples. E. coli-derived LPS (serotype 0111:B4, Sigma-Aldrich Co. Ltd., Irvine, UK) was added to achieve different final concentrations of 0.1, 1, 10 and 100 ng/mL. LPS was diluted in Hanks' balanced salt solution. The addition of Hanks' balanced salt solution alone served as control. Dilutions, aliquoting and stimulations were carried out under sterile conditions. A non-stimulated sample served as control. For stimulation, whole blood samples were incubated for 6 hours in a humidified atmosphere (37 °C, 5% carbon dioxide), unless otherwise indicated. Following incubation, samples were centrifuged at 1,500 rpm for 5 min. The supernatants were harvested and stored at −80 °C until final assessment. To assess LPS stimulatory capacity, we calculated the largest difference between the available LPS-stimulated TNFα values. The largest difference was usually present between the unstimulated sample and the sample stimulated with LPS at a dose of 10 or 100 ng/mL.

Cell viability was in all cases >90% as assessed using trypan blue exclusion. Trypan blue exclusion was performed using 100 μL of cell-rich suspension to which an equal volume of 0.4% trypan blue was added. The percentage of viable cells was calculated.

Table 1
Baseline data of control subjects and patients with chronic HF.

	Control subjects	Chronic HF patients	p-Value
Number (female)	27 (13)	122 (24)	<**0.01**
NYHA class		2.5 ± 0.8	
Aetiology (ischaemic/non-ischaemic)		75/47	
Ejection fraction (%)		33.5 ± 12.5	
Age (years)	63.7 ± 7.7	67.3 ± 10.3	0.09
Body mass index [kg/m^2]	26.9 ± 2.8	27.8 ± 5.3	0.58
Haemoglobin (g/dL)	14.3 ± 1.1	13.4 ± 1.6	<**0.01**
Haematocrit (%)	42.6 ± 3.0	40.1 ± 4.6	<**0.05**
Sodium (mmol/L)	139 ± 2.1	136 ± 3.4	<**0.01**
Potassium (mmol/L)	4.2 ± 0.3	4.3 ± 0.5	0.10
Creatinine (mmol/L)	81 ± 14	121 ± 43	<**0.0001**
Uric acid (mmol/L)	313 ± 78	442 ± 128	<**0.0001**
Alkaline phosphatase (U/L)	96 ± 67	99 ± 71	NS
Aspartate transaminase (U/L)	28 ± 16	24 ± 10	0.15
g Glutamyl transferase (U/L)	46.2 ± 58.6	54.6 ± 48.3	NS
Cholesterol (mmol/L)	5.9 ± 0.8	4.7 ± 1.2	<**0.0001**
HDL cholesterol (mmol/L)	1.5 ± 0.4	1.2 ± 0.4	<**0.01**

Bold values indicate significance at p < 0.05.

2.3. Determination of cytokines

Serum levels of IL-6, TNFα, soluble tumor necrosis factor receptor-1 (sTNFR-1) and sTNFR-2 were determined using high-sensitivity enzyme-linked immunosorbent assay (ELISA) kits (Quantine HS, R&D systems, Minneapolis, USA). The respective limits of detection were 0.039 pg/mL, 0.106 pg/mL, 7.8 pg/mL and 7.8 pg/mL. Levels of TNFα from cell culture supernatant were measured using a commercially available standard ELISA kit (R&D systems, Minneapolis, USA).

2.4. Selenium determination by total reflection X-ray fluorescence analysis

Serum samples of 12 μL were diluted with 12 μL of distilled water spiked with an internal gallium standard (2 g/L, Sigma-Aldrich). Samples were applied to quartz glass carriers and left to dry. Total reflection X-ray fluorescence analyses were performed using a Picofox S2 instrument (Bruker AXS Inc. Madison, Wisconsin, USA). The method was validated with a human serum Seronorm standard (Sero AS, Billingstad, Norway), and results were linear over a range of 1:2, 1:5 or 1:10 dilutions. Mean selenium concentrations determined were in accordance with the certified content (168.7 ± 8.8 μg/L).

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