NF-κB expression in mononuclear cells from patients with chronic heart failure observed in endotoxin tolerance

EWA ZACZYŃSKA[1], ANNA CZARNY[1], EWA A. JANKOWSKA[2], MACIEJ SOBCZYŃSKI[3], PIOTR PONIKOWSKI[2]

[1]Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland; [2]Cardiology Department, Military Hospital, Wrocław, Poland; [3]Institute of Genetics and Microbiology, Wrocław, Poland

Abstract

Inflammatory immune activation is observed in patients with chronic heart failure (CHF). LPS translocated from the intestine into circulation during episodes of decompensation can modulate the activation of immunocompetent cells in patients with CHF.

The expression of transcriptional nuclear factor kappa-B (NF-κB) in patients with CHF was significantly highest than control subject. Subsequently in vitro stimulation of peripheral blood mononuclear cells (PBMC) with lipopolysaccharide (LPS) did not induce NF-κB nuclear translocation in cells from patients with CHF with NYHA II-IV as compared to the cells from healthy patients with LPS induction. This phenomenon of "LPS- tolerance" or "deactivation" can explain the endotoxin hypothesis, which suggests that bacterial translocation through the oedematous gut wall with subsequent release of endotoxin may pose the relevant stimulus to trigger NF-κB system.

Key words: *nuclear factor kappa-B, chronic heart failure, LPS, peripheral mononuclear cells.*

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Introduction

Chronic heart failure (CHF) is a common and debilitating condition that becomes a major public healthy problem [1]. Despite recent developments in the management of CHF, the patients quality of life remains unacceptably poor and morality is comparable to that observed in many malignant diseases with the 5-year survival of less than 50% [1, 2].

Traditionally, the clinical syndrome of CHF was thought to be a consequence of impaired function of the heart and compromised haemodynamics [3]. In fact, the pathophysiology of CHF is far more complex with an involvement of several non-cardiac systems: musculoskeletal, renal, neuro-humoral, endocrine, immune. The peripherial mechanisms are important determinants of the clinical picture of CHF and play an important role in the progression of the diseases [3]. Current evidence now suggest that CHF is a state of inflammatory immune activation, with pro-inflammatory cytokines contributing to both the central and peripheral manifestation of this syndrome [4-6]. The overexpression of certain inflammatory cytokines is involved in contractile depression and development of heart muscle hypertrophy, reduces peripheral blood flow, and many trigger the process of muscle wasting [7-9]. It has been also demonstrated that elevated serum levels of tumor necrosis factor-alpha (TNF-α), soluble TNF receptors 1 and 2 (sTNFR-1,2) and interleukin-6 (IL-6) are strong prognostic markers in patients with CHF [10, 11]. In advanced heart failure, a wasting syndrome known as cardiac cachexia can develop, where circulating pro-inflammatory cytokine levels are especially high, with is associated with a particularly poor prognosis [9].

Recent studies investigating pathways responsible for an activation of pro-inflammatory cytokines system in CHF revealed the role of transcriptional nuclear factor kappa-B

Correspondence: Ewa Zaczyńska, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla 12, 53-114 Wrocław, Poland. Phone number: +48 71 337 11 72, fax number: +48 71 337 13 82, Email: ezacz@immuno.iitd.pan.wroc.pl

(NF-κB). The NF-κB family of pleiotropic transcriptional factors constitutes the universal and evolutionary conservative systems with controls the transcription of series of genes involved in innate and adaptive immune response in immunocompetent cells [12-14]. The NF-κB family consist of 5 members (p50, p52, p65, c-Rel and RelB) which form various homo- and heterodimers. The NF-κB dimmers are present in the cytoplasm in inactive forms bound to inhibitory subunits (IκBs). In response to differ stimuli IκB is phosphorylated, ubiquitinated and proteolytically degraded [12-14]. Then, the NF-κB dimmers without inhibitory protein can enter the nucleus, bind to DNA within the promoter/enhancer regions of target genes and activate their transcription [15]. It has been demonstrated that myocardial tissue from patients with CHF of various etiologies exhibits the activation of NF-κB and increased expression of genes it regulates such as inducible cyclo-oxygenase, TNF-α, inducible NO synthase, and leukocyte adhesion molecules [16].

Apart from the evaluation of the pattern of NF-κB activation, the signaling pathways involved in the activation of this transcription factor either in cardiomyocytes and in immunocompetent cell have also been investigated [16]. Among numerous stimuli responsible for downstream activation of NF-κB system, lipopolysaccharide (LPS, endotoxin cell-wall component Gram-negative bacteria) has attained particular interest. Only recently, it has been proposed that LPS might be an important stimulus for immune system in CHF through its action on circulating immunocompetent cell [17, 18]. During circulatory decompensation, venous pulmonary congestion and peripheral oedema are accompanied by the mesenteric venous congestion whit the bowel wall oedema. The increased permeability of oedematous bowel wall result in translocation of bacteria (and LPS) from the intestine lumen into the circulation [17, 18]. In this aspect, recurrent episodes of mild, subclinical haemodynamic deterioration may constitute a basis for recurring delivery of intestinal LPS into circulation in CHF. This theory is supported by the findings that decompensated CHF patients whit peripheral oedema have elevated plasma endotoxin levels that normalize after diuretic therapy administration [18]. Furthermore, LPS levels in the hepatic vein are higher than those in the left ventricle, suggesting that gut/liver may be a potential source of LPS [19].

The endotoxin hypothesis suggests that LPS (lipopolysaccharide) is an important trigger for cytokine production in CHF. In this context transcriptional factor NF-κB may be important as a regulator of the responses to LPS. The aim of this study was investigated of NF-κB activation, assessed by immunocytochemical localization and protein expression of NF-κB in PBMC from healthy and CHF patients, using the polyclonal rabbit IgG anti-p65-subunit antibody. Additionally, in series of *in vitro* experiments, the role of LPS as a stimulus for NF-κB system in PBMC was evaluated. The study demonstrated the activation of NF-κB systems in peripheral blood leukocytes from healthy subjects and patients with chronic heart failure (NYHA I-IV) showed no significant changes in normal or LPS-tolerant cells treated with LPS. Taking into consideration a seminal role of an overactive NF-κB system in the development of immune imbalance in CHF, it may become a potential therapeutic target.

Materials and Methods

Patients population

We studied: 46 patients (mean age 62 years) with a documented history of CHF at least 6 months duration, with impaired left ventricular systolic function (left ventricular ejection fraction (LVEF) <45%, NYHA (class – II/III/IV/IVd-6/10/8/9) patients were admitted to our Cardiology Department and 13 healthy young.

The study was approved by the respective Ethics Committees and written informed consent was obtained from all patients (nr RNN/164/04/KB – 18.05.20040).

Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated from heparinised peripheral blood (10 U/ml) by gradient centrifugation in Percoll with a density of 1.077 g/ml (Biochrom AG, Berlin, Germany). Five ml of blood were layered on three ml of Percoll and centrifuged for 30 min. at 400 × g. The cells were collected from the interphase, washed two times with Dulbecco medium supplemented with 2% of calf serum (c.s.) and suspended in this medium at a density of 2×10^6 cells/ml.

Stimulation of PBMC with LPS

The PBMC from patients with CHF and healthy subjects were plated at 1×10^6 cells per well in 24-well plates (Costar, MA, USA) in Dulbecco with 2% calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and 2 mM L-glutamine and cultured in a humidified atmosphere (37°C,5% CO_2) for 6 hours without the addition of LPS or with the addition of LPS (*E. coli* strain O111:B4) at the following concentrations: 0.01; 0.1; 1 and 10 ng/ml (Sigma-Aldrich, Fine Chemicals, Lu, USA). This range of concentration was selected to represent the low concentrations likely to mimic decompensated CHF up to concentrations resembling those found in sepsis.

Immunocytochemical staining of PBMC for NF-κB

PBMC were placed on poly-L-lysine-coated microscope slides using cytocentrifugation by 5 min at 500 rpm. Slides were fixed in 4% paraformaldehyde solution at room temperature for 15 min. After washing in distilled water, endogenous peroxidase activity was blocked by incubation slides in 3% hydrogen peroxide solution in methanol for 10 min, and washed in 10 mM phosphate buffered saline (PBS, pH=7.5). PBMC were treated with universal blocking serum for 20 min at room temperature. Next, the cells were

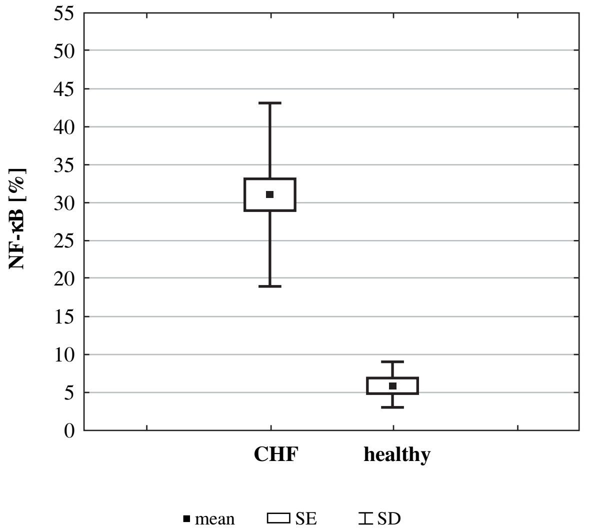

Fig. 1. Activation of the NF-κB system in peripheral blood mononuclear cells from CHF patients versus healthy controls

incubated at room temperature for 2 hours in a wet chamber with a polyclonal rabbit anti-NF-κB IgG antibody (p-65 subunit – Chemicon International Inc.,Ca, USA). After washing in PBS, preparations were incubated with a biotinylated secondary anti-rabbit antibody (Novocastra Laboratories Ltd., United Kingdom) at room temperature for 30 min. This was followed by washing in PBS and an application of peroxidase-conjugated avidin (Novocastra Laboratories Ltd., United Kingdom) at room temperature for 30 min. After washing in PBS, chromogen fast diaminobenzidine-DAB was used for 5-10 min (Liquid DAB Substrate Kit for Peroxidase, Novocastra Laboratories Ltd., United Kingdom). Preparations were counterstained in hematoxilin and finally washed with distilled water. PBMC expressed p-65 in the nucleus were labelled as NF-κB(+)cells. Activation of the NF-κB system in PBMC was expressed as the percentage of NF-κB(+)cells from all quantified PBMC.

Statistical analysis

The inter-group differences were tested using ANOVA with Scheffe's post hoc test and nonparametric Kruskal-Wallis test, as appropriate. Claims of ANOVA ware tested using Levene test and normal plots. P values<0.05 were considered.

Results

Activation of the NF-κB system in PBMC from CHF patients and healthy subjects

Patients with CHF had increased activation of the NF-κB system in PBMC compared to healthy controls. The mean percentage of NF-κB(+)PBMC in CHF patients 32% was significantly greater than in healthy controls 6% (p<0.000002).

The effects of in vitro LPS stimulation of PBMC from healthy and CHF patients on the activations of the NF-κB system

In PBMC from healthy, LPS stimulation all tested concentrations resulted in an increase in NF-κB activation in PBMC as evidenced by the translocation of p65 into nucleus (at 0.01 ng/ml: 6% vs. 10%; 0.1 ng/ml: 6% vs. 13%; 1 ng/ml: 6% vs. 22% and 10 ng/ml 6% vs. 24%; the percentage of NF-κB(+)PBMC after the control stimulation without LPS vs. after the stimulation with LPS at different concentrations (p=0.0027) (figure 2A). Following LPS stimulation of PBMC from healthy controls, the pattern of PBMC p-65 staining was similar to that detected in CHF patients. Our next series of experiments were performed to demonstrate the expression of NF-κB in PBMC from CHF patients. We examined the effect of LPS stimulation on NF-κB expression at 6 h after induction. Following LPS treatment at all concentrations (0.01; 0.1; 1 and 10 ng/ml) NF-κB levels were not changed at any of the concentrations examined following LPS stimulation (figure 2B). Patients with advanced CHF symptoms classified as being in New York Heart Association – (NYHA) class II-IV demonstrated enhanced activation at the NF-κB system in PBMC when compared with healthy controls (p=0.0001) but patients with CHF in NYHA class II-IV showed the similar level expression of p65 (p=0.3834) (figure 3).

Discussion

NF-κB regulation of transcriptional by the classical or canonical pathway can be divided into two phases [20, 13]. First is a cytosolic phase in witch there is IκB kinaseB-depended activation of IκBα followed by ubiquition – directed IκB degradation on that allows translocation of p50 and p65 into the nucleus. The second of nuclear phase of NF-κB regulation involves events that result in derepression NF-κB-dependent promoters, chromatin remodeling, and binding of transcriptionally active p65 and p50 as a heterodimer to cognate DNA on enhancer and promoter elements. This canonical mechanism for NF-κB activation is fundamental in transactivating genes expressed by innate immune cells, and these gene products are critically involved in host protection and host injury, including septicemia [21]. However, soon after septicemia is initiated, the NF-κB pathway is disrupted at the level of transcriptional [22-24] and this occurs coincident with repression of pro-inflammatory genes such as TNF-α and IL-1β. Thus, these and other proinflammatory genes are reprogrammed during septicemia to be LPS (endotoxin) tolerant. Endotoxin is a crucial component of the outer leaflet of the external membrane of Gram-negative bacteria

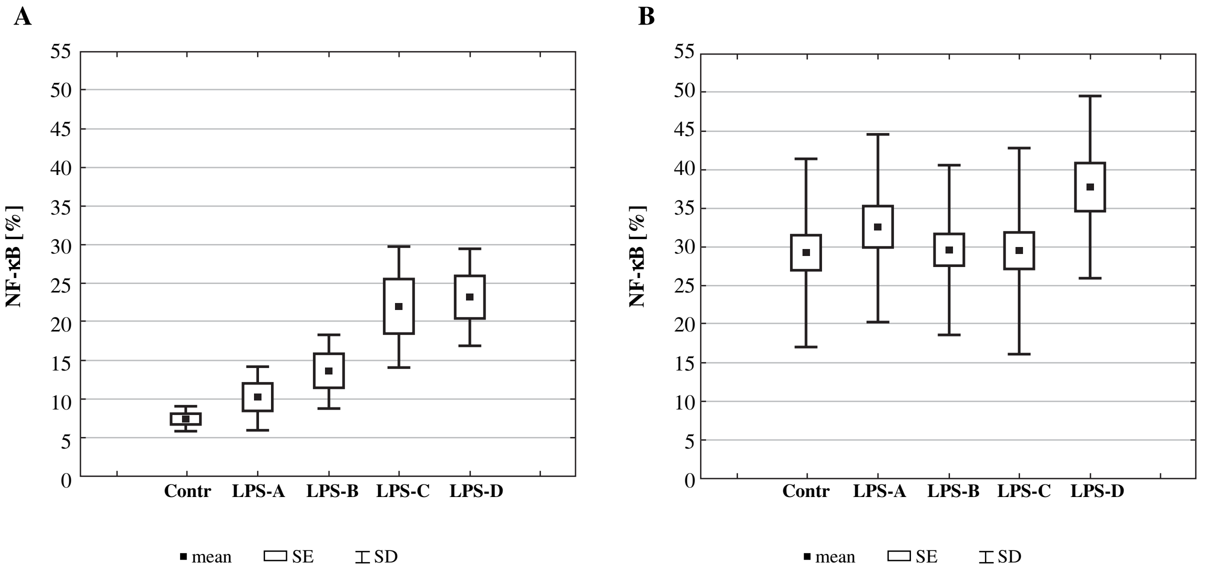

Fig. 2. The nuclear expression of NF-κB in PBMC from healthy controls (A) and CHF patients (B) exposed in vitro to LPS at different concentrations. (A) Changes in the NF-κB activation in PBMC from healthy controls exposed in vitro to LPS at different concentrations: LPS-A=0,01 ng/ml; LPS-B=0,1 ng/ml; LPS-C=1,0 ng/ml; LPS-D=10,0 ng/ml; (B) The NF-κB expression in PBMC from CHF patients is not affected by LPS treatment; LPS-A=0,01 ng/ml; LPS-B=0,1 ng/ml; LPS-C=1,0 ng/ml; LPS-D=10,0 ng/ml

[25, 26]. LPS possesses multitude of immune effects which are subsequent to binding to its specific receptor (CD14) coupled with Toll-Like Receptor (TLR) on monocytes and macrophages participating in innate immunity responses [25, 26]. The interaction of LPS with the membrane complet CD14-TLR leads to the rapid activation of intercellular signaling pathway including NF-κB system, which results in the production and releases of proinflammatory mediators: TNF-α, IL-1, IL-6, IL-8, IL-12, chemokines, interferon, reactive oxygen intermediates, etc. [25, 26].

In this study, we have demonstrated augmented activation of the NF-κB system in peripheral blood mononuclear cells in patients with CHF (figure 1). Our study demonstrated that *in vitro* exposition of PBMC from healthy subjects to low does of LPS (approximately 0.01-10 μg/ml-concentrations found *in vivo* in CHF patients, which are able to induce the TNF-α production in *ex vivo* whole blood from CHF patients [27] can result in the translocation of p-65 submit from cytoplasm into nucleus and therefore activate the NF-κB system in these cells (figure 2A) Thus, after exposures to LPS a pattern of NF-κB activatin in PBMC from healthy controls resembles a pattern observed in CHF patients [28]. In the other hand, the LPS-induced transient impaired inflammatory response to subsequent LPS challenge, has been described at the cellular and molecular level most extensively in monocytes and macrophages. Monocytes/macrophages rendered endotoxin tolerant are characterized by: 1) impaired activation of intercellular signaling pathway, including NF-κB and MAPKS [29], 2) decreased pro--inflammatory gene transcription and protein production, including TNF-α, IL-1, IL-6 and IFN-γ [23] and 3) down--regulated pro-inflammatory cellular function [30]. Ogawa et all demonstrated endotoxin tolerance in human microvascular endothelial cell from gut characterized by

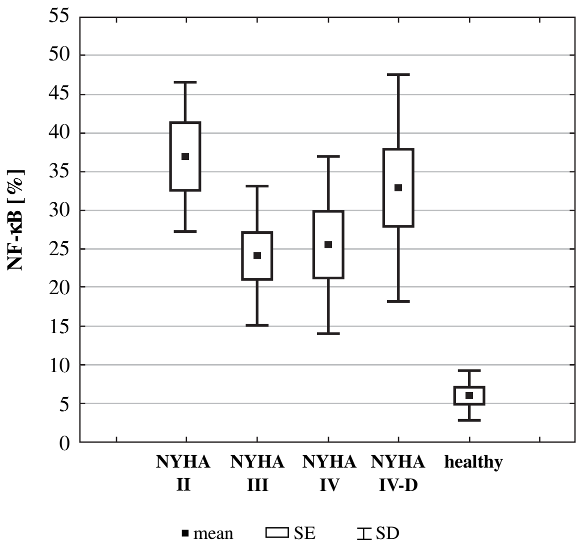

Fig. 3. Activation of the NF-κB in PBMC from CHF patients classified as NYHA class II-IV versus healthy control

reduced leukocyte – adhesion, selectively decreased or increased gene/protein expression impaired NF-κB activation and decreased superoxide production [31]. Circulating leukocyte from patients with sepsis, trauma hemorrhage, and thermal injury have a reduced capacity to produce cytokines in response to LPS stimulation [32-34]. This phenomenon known as "deactivation", "desensitization", "anergy" or "refractoriness" occurs in many inflammatory stress is very similar to another phenomenon described as "endotoxin tolerance" [35].

Among numerous stimuli responsible for downstream activation of NF-κB system LPS has attained particular interest. It was shown that patients with decompensated CHF have elevated levels of endotoxin, which normalize following diuretic therapy [18]. Furthermore, LPS levels in the hepatic vein are higher than in the left ventricle of CHF patients, implicating that the gut/liver may be a potential source [19]. To study the *in vitro* NF-κB expression, we chose to analyze the whole mononuclear cell population than isolated monocytes to minimize cell manipulation that could interfere and induce activation signals altering NF-κB expression. We found that all patients with chronic heart failure in NYHA class II, III and IV showed the similar level expression of p65 after *in vitro* LPS stimulation (figure 3). It is important the PBMC of these patients with CHF (HYHA II-IV) were not able to perform NF-κB translocation upon LPS activation (figure 2B), similar to endotoxin-tolerized cells. The survivors of severe sepsis and patients with trauma showed low expression of both active (p65p50) and inactive (p50p50) forms of NF-κB after LPS stimulation, resembling what is found in same endotoxin tolerance experiments, where tolerance was associated with depletion of both forms NF-κB [36-38], whereas nonsurvivors of severe sepsis showed a predominance of the inactive homodimer and a low p65p50/p50p50 ratio similar to the tolerized cells described by Ziegler-Heitbrock [39].

In summary, we conclude that mononuclear cells of patients with chronic heart failure in NYHA class II-IV acquired endotoxin tolerance toward repeated LPS stimulation, characterized by lack of change NF-κB activation. PBMC, such as monocytes/macrophages, have precisely defined immunoregulatory responses to repeated LPS exposure. LPS tolerance in mononuclear cells from CHF patients may represent important mechanism during immune homeostasis in health and diseases.

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