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ORIGINAL ARTICLE

Adrenergic Effect on Cytokine Release After *Ex Vivo* Healthy Volunteers' Whole Blood LPS Stimulation

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Abstract—Catecholamines are molecules with immunomodulatory properties in health and disease. Several studies showed the effect of catecholamines when administered to restore hemodynamic stability in septic patients. This study investigates the effect of norepinephrine and dobutamine on whole blood cytokine release after *ex vivo* lipopolysaccharide (LPS) stimulation. Whole blood collected from healthy individuals was stimulated with LPS, in the presence of norepinephrine or dobutamine at different concentrations, with or without metoprolol, a β_1 receptor antagonist. Cytokine measurement was performed in isolated cell culture supernatants with ELISA. Results are expressed as mean ± SEM and compared with Mann-Whitney rank-sum test. Both norepinephrine and dobutamine significantly reduced TNF-α and IL-6 production after *ex vivo* LPS stimulation of whole blood in a dose-dependent manner, and this effect was partially reversed by the presence of metoprolol. Norepinephrine and dobutamine reduce the LPS-induced production of pro-inflammatory cytokines, thus possibly contributing to altered balance between the inflammatory and anti-inflammatory responses, which are vital for a successful host response to severe disease, shock, and sepsis.

KEY WORDS: sepsis; inflammation; norepinephrine; dobutamine.

INTRODUCTION

Septic shock is a clinical condition characterized by impaired function of heart and vessel tone and excessive activation of pro-inflammatory cytokines. The mechanism is better described as a systemic inflammatory response syndrome (SIRS) and accounts for a mortality of 30–40 % in intensive care unit (ICU) patients [1]. Escalation from inflammation to severe SIRS may be initiated by cytokines, which are produced by various immune competent cells. Catecholamines are a family of hormones and neurotransmitters derived from the amino acid tyrosine and regulate the neuroendocrine-immune response to stress. Endogenous catecholamines are released in severe sepsis and septic shock, while exogenous catecholamines are frequently administered in case of prolonged hypotension, in order to stabilize cardiovascular parameters, as an indispensable part of the therapy [2, 3]. Depending on the chemical structure of the sympathomimetic drug, catecholamines exert their pharmacologic effects by activating directly or indirectly either α- or β-adrenergic receptors. Naturally occurring norepinephrine and the synthetic derivative dobutamine are often administered in critically ill patients for hemodynamic support, due to their vasotonic and inotropic properties [2, 3].

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Abbreviations: ELISA, Enzyme-linked immunosorbent assay; ICU, Intensive care unit; IL, Interleukin; LPS, Lipopolysaccharide; MODS, Multiple organ dysfunction syndrome; NF-κB, Nuclear factor-κB; PMA, Phorbal-myristate-acetate; ROS, Reactive oxygen species; SIRS, Systemic inflammatory response syndrome; sTNFR, Soluble TNF-α receptor; TNF-α, Tumor necrosis factor-α

Nonetheless, catecholamines are important immunomodulators in health and disease. Several studies have outlined their ability to modulate the release of lipopolysaccharide (LPS)-induced cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-12, and IL-10 [4–10].

In septic patients, pro-inflammatory TNF-α is produced by various immune cells as a response to the invading pathogen, leading to further release of pro-inflammatory cytokines, reactive oxygen species (ROS), and growth factors, finally causing multiple organ dysfunction syndrome (MODS) [11, 12]. High levels of soluble TNF-α receptors (sTNFRI and sTNFRII) are present in septic patients' serum, possibly binding to TNF-α, as a potential mechanism to suppress its activity [13, 14]. Furthermore, IL-6 release by various activated cell types follows shortly after TNF-α in experimental endotoxemia and is involved in modulating the acute phase response and T cell activation and differentiation [15, 16].

Catecholamine-induced modulation of immune response came around by the observation that endogenous catecholamines play an important role in the host defense during experimental sepsis [17]. Since catecholamines may alter the immune response, it is of great interest to understand their immunomodulatory effects in the setting of septic shock, where the immune system of the patients is already dysregulated.

The purpose of the present study is to investigate the effect of norepinephrine and dobutamine on the production of TNF-α, IL-6, and sTNFR, in a model of *ex vivo* whole blood stimulation with LPS. The chosen molecules are shown to play a major role in the pathogenesis of SIRS and sepsis and are suggested to be markers of the outcome for critically ill patients [15]. Furthermore, due to the presence mainly of β-adrenergic receptor on immune cells [18], we further examine the potential involvement of β_1 receptor pathway in the same setting.

METHODS

Subjects

Ten male healthy volunteers, residents of the urban area of Athens, were enrolled in this study. Medical history, physical examination, hematological and biochemical screening, and electrocardiogram were all normal. All participants were informed about the aim and the expected results of the study, and written consent was obtained from each one. None of the volunteers was smoking, taking medication, or having febrile illness in the month preceding the study.

Blood Collection

Peripheral blood (10 ml) was collected at 8.00 am and placed into tubes as follows: 6 ml in one tube without anticoagulant for serum (baseline) cytokine measurements before any intervention and 4 ml with EDTA as anticoagulant for total cell blood count, *ex vivo* LPS stimulation, and subsequent cytokine measurement. All blood samples were immediately transferred to the laboratory and were processed accordingly.

Whole Blood Assay-LPS Stimulation

Cytokine induction by LPS was performed as previously described by our group [19]. Briefly, whole blood was diluted 1:10 in RPMI 1640 culture medium (Invitrogen Life Technologies, Gaithersburg, MD, USA), to a final volume 1 ml and placed to plastic culture dishes with or without 500 pg/ml LPS (0111:B4, Sigma-Aldrich Chemical Co; St Louis, MO, USA). Dobutamine (10^{-6}, 10^{-5}, 10^{-4} M) or norepinephrine (10^{-6}, 10^{-5}, 10^{-4} M) was also added to the wells with or without metoprolol (β_1 receptor blocker) at a standard dose of 10^{-5} M. The selected range of concentrations of dobutamine and norepinephrine was based on previous similar studies, where a range between 10^{-7} and 10^{-3} M was used [5, 20–24]. Also, the dose of metoprolol was selected according to previous literature [25].

Samples were maintained at 37 °C in a 5 % CO_2 atmosphere for 4 h. After incubation, samples were centrifuged (1800 rpm, 5 min) and supernatants were collected and stored at −70 °C until measurements. Subsequently, cytokine level in the supernatant was evaluated by enzyme-linked immunosorbent assay (ELISA).

Cytokine Assays

Serum and supernatant levels of IL-6, TNF-α, sTNFRI, and sTNFRII were determined using commercially available human-specific enzyme-linked immunoassays kits (Cytoscreen™ ELISA kit, Biosource International; Camarillo, CA), with sensitivity of detecting levels for TNF-α >0.09 pg/ml, TNFRI >0.1 ng/ml, TNFRII >0.05 ng/ml, and IL-6 >2 pg/ml. Cytokine levels were measured at baseline before LPS stimulation.

Statistical Analysis

Data were evaluated by the use of nonparametric Mann-Whitney rank-sum test and are given as mean

values ± SEM. Differences with $p < 0.05$ were considered statistically significant.

RESULTS

Demographics

Ten male healthy volunteers 33.6 ± 1.5 years old were included in the study. Total white blood cells and differentials were recorded (Suppl. Fig. 1).

Baseline Cytokines

Incubation of whole blood without LPS did not result in detectable levels of TNF-α or IL-6 in most samples, while the upper limit values were at a range of 0.1–27.7 pg/ml (data not shown). Similarly, these cytokines remained undetected when cells were incubated with dobutamine or norepinephrine alone. Moreover, the levels of TNFRI and TNFRII were similar in the above conditions (data not shown).

Effect of Norepinephrine on LPS-Induced Cytokine Release and Partial Inhibition by β_1-Adrenergic Antagonist Metoprolol

Ex vivo stimulation of whole blood with 500 pg/ml LPS induced both TNF-α and IL-6 production (Fig. 1). Norepinephrine caused a dose-dependent decrease of LPS-induced cytokine release (Fig. 1), with significant reduction of TNF-α release at 10^{-5} and 10^{-4} M and significant reduction of IL-6 release at the highest adrenergic drug concentration (10^{-4} M). No difference was detected in the levels of TNFR subunits upon LPS and norepinephrine stimulation (Suppl. Fig. 2). Since immune cells are suggested to possess mainly β adrenergic receptors, we hypothesized that the norepinephrine-induced suppression of TNF-α and IL-6 production could be mediated by an effect on β_1-adrenergic receptor. For this purpose, metoprolol was added in the culture of whole blood with LPS and norepinephrine. Metoprolol partly reversed the effect of norepinephrine on TNF-α and IL-6 release, although a statistically significant prevention by metoprolol was observed regarding the inhibitory effect of 10^{-5} M norepinephrine on TNF-α production ($p < 0.05$) (Fig. 1).

Effect of Dobutamine on LPS-Induced Cytokine Release and Partial Inhibition by β_1-Adrenergic Antagonist Metoprolol

Similar to norepinephrine, dobutamine caused a dose-dependent decrease of LPS-induced cytokine release (Fig. 2), with significant reduction of TNF-α release at 10^{-5} and 10^{-4} M and significant reduction of IL-6 release at the highest adrenergic drug concentration (10^{-4} M). No

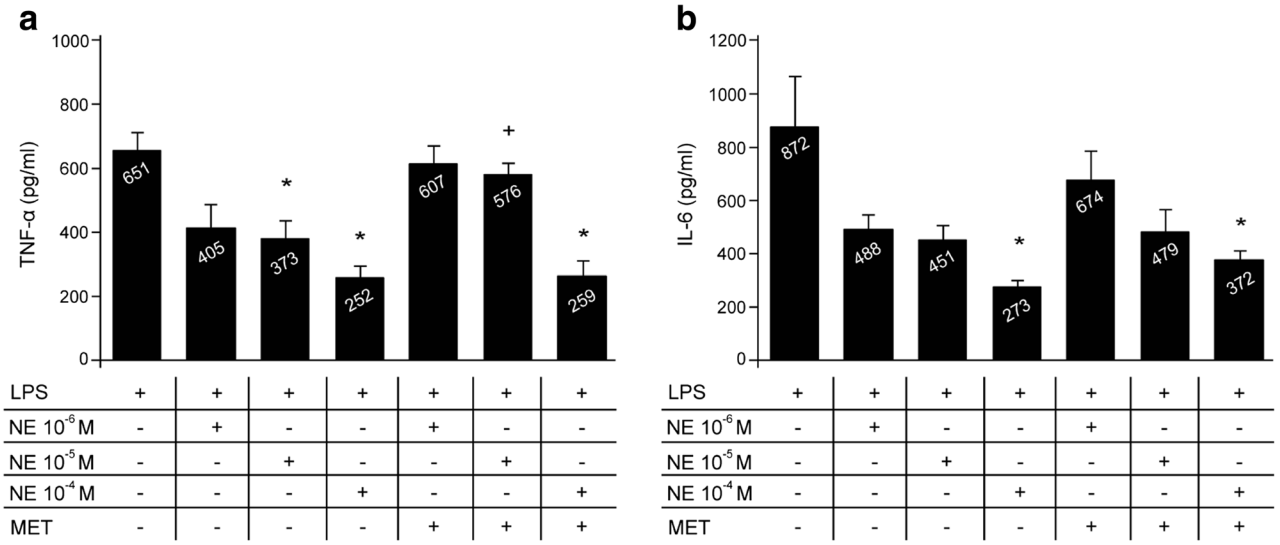

Fig. 1. Effect of norepinephrine on cytokine production. Levels of TNF-α (**a**) and IL-6 (**b**) in the supernatants after *ex vivo* whole blood stimulation with LPS (500 pg/ml), in the presence of indicated doses of norepinephrine (*NE*), with or without metoprolol (*MET*; 10^{-5} M). The mean ± SEM from ten subjects is shown and the mean absolute values are indicated with *white numbers*. *$p < 0.05$ compared with LPS alone; $^{+}p < 0.05$ for comparison between NE 10^{-5} M with or without MET.

difference was detected in the levels of TNFR subunits upon LPS and dobutamine stimulation (Suppl. Fig. 3). Since dobutamine is a synthetic β_1 mimetic derivative, we hypothesized that the norepinephrine-induced suppression of TNF-α and IL-6 production could be mediated by an effect on β_1-adrenergic receptor. For this purpose, metoprolol was added in the culture of whole blood with LPS and dobutamine. Metoprolol partly reversed the effect of dobutamine on TNF-α and IL-6 release, although a statistically significant prevention by metoprolol was observed regarding the inhibitory effect of 10^{-5} M dobutamine on TNF-α production ($p < 0.05$) and the effect of 10^{-4} M dobutamine on IL-6 production ($p < 0.05$) (Fig. 2).

DISCUSSION

In this study, we addressed how norepinephrine and dobutamine may modulate the immune response in *ex vivo* whole blood stimulation with LPS endotoxin. Our observations suggest that, in the treatment of LPS-stimulated whole blood, both norepinephrine and dobutamine decrease TNF-α and IL-6 production in a dose-dependent manner. Moreover, the partial reversal of this adrenergic effect by metoprolol (β_1-adrenergic antagonist) indicates that the immunomodulatory action of the chosen drugs could be associated with β_1-adrenergic mechanism. Of course, the involvement of α-adrenergic pathway in this phenomenon cannot be excluded and needs to be further examined.

Norepinephrine and dobutamine are currently used in the treatment of septic shock as vasotonic and inotropic drugs, to restore adequate blood pressure and cardiac function. Along with these properties, catecholamines have been suggested to regulate immunity with contradictory results, depending on several factors (setting, drug, *etc.*) [26, 27]. Critically ill patients are characterized by a dysregulated immune system [28], and although the immune response to sepsis is under investigation for the past decades, the complexity of underlying mechanisms and the ambivalent immune state of septic patients reflects the difficulty of finding therapeutic targets for immunomodulation [29, 30].

The effect of catecholamines on cytokine release has been previously studied in healthy volunteers and septic patients, using whole blood or isolated monocytes. Farmer *et al.* reported that β receptor agonists inhibit the release of TNF-α and IL-8, via cAMP production and protein kinase A (PKA) activation, possibly contributing to the suppression of inflammatory responses [31]. Using isoproterenol, another β receptor agonist, *in vitro*, Suberville *et al.* found

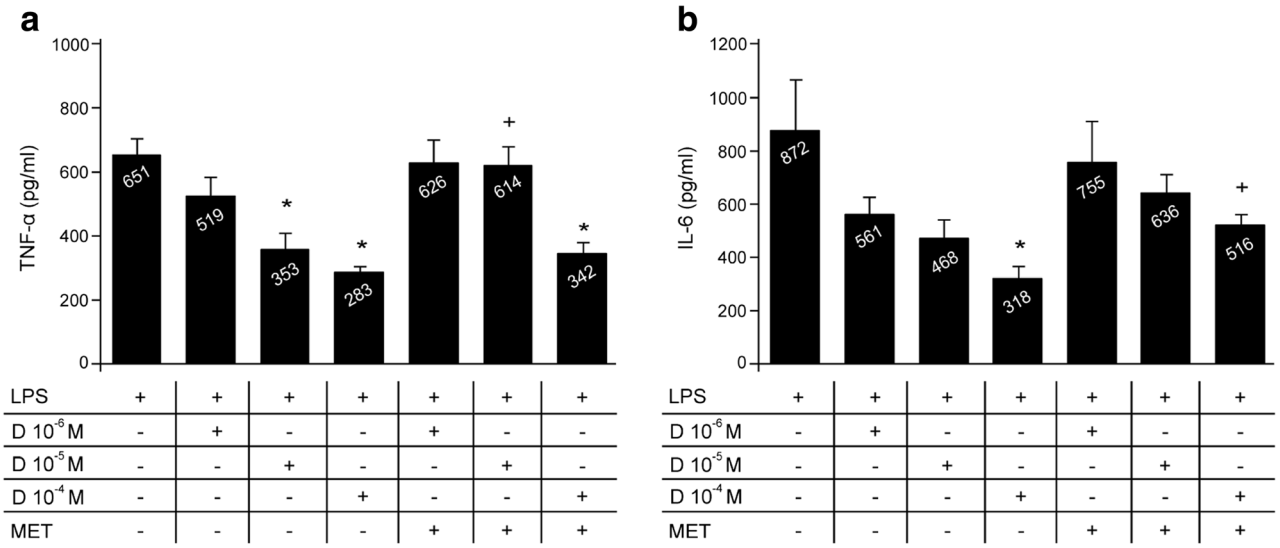

Fig. 2. Effect of dobutamine on cytokine production. Levels of TNF-α (**a**) and IL-6 (**b**) in the supernatants after *ex vivo* whole blood stimulation with LPS (500 pg/ml), in the presence of indicated doses of dobutamine (*D*), with or without metoprolol (*MET*; 10^{-5} M). The mean ± SEM from ten subjects is shown and the mean absolute values are indicated with *white numbers*. *$p < 0.05$ compared with LPS alone; $^{+}p < 0.05$ for comparison between D 10^{-5} M with or without MET (**a**) and between D 10^{-4} M with or without MET (**b**).

reduced production of TNF-α in isolated peritoneal macrophages from LPS-stimulated mice [32].

Our findings regarding norepinephrine are in accordance with previous *ex vivo* human whole blood studies [21, 22]. However, we measured cytokine release in supernatants, while Roentgen *et al.* performed intracellular staining to detect cytokine production specifically by monocytes [22]. Moreover, we propose a possible β_1 receptor-mediated effect of norepinephrine, although an α_2 receptor mechanism is also suggested [21].

Van der Poll *et al.* is the only study examining the effect of norepinephrine on whole blood *ex vivo* that found reduced TNF-α and IL-6 production after LPS stimulation using ELISA, which is in accordance with our finding [20]. However, in that study, LPS was used in higher concentration (1–10 ng/ml) as compared to our study (500 pg/ml).

Notably, in our settings, dobutamine and norepinephrine possess equivalent inhibitory capacity, since they reduce TNF-α levels when used at the same concentration. Similarly, these drugs are equally capable of reducing IL-6 levels, although at higher concentrations compared to that needed for TNF-α reduction.

In vitro studies have shown that treatment of human monocytic cell line THP-1 with dobutamine results in decreased production of IL-8 and MIP-1α. Thus, macrophage chemotaxis was found decreased, indicating a dampened inflammatory response [24].

Here, we demonstrate a negative correlation between dobutamine and pro-inflammatory cytokines upon *ex vivo* whole blood stimulation with LPS. This finding is in accordance with the effect of the drug on phorbal-myristate-acetate (PMA)-stimulated human T lymphocytes *in vitro* [23], by inhibiting the activation of nuclear factor-κB (NF-κB). NF-κB is a transcription factor, responsible for the transcription and expression of hundreds of genes, including TNF-a and IL-6 [33]. Therefore, β_1 agonists, such as dobutamine, increase intracellular cAMP, which in turn activates PKA, leading to CREB phosphorylation and direct inhibition of NF-κB [34, 35]. Another *in vivo* study using dobutamine in a rat endotoxemia model showed a decrease in TNF-a plasma levels [36], which is in accordance with our findings. Nonetheless, the reported results may not agree with the lack of effect of the drug in the human endotoxemia model [4] and the positive relation between TNF-a and increasing doses of dobutamine in septic patients [26]. Such discrepancies may be related to differences of the methodological approach, such as the concentration of LPS and drugs, and different model and adrenergic receptors involved.

In this study, we investigated the effect of LPS at 500 pg/ml, and this choice was based on previous studies, reporting that maximum production of TNF-α and IL-6 was recorded at this dose [19].

Although the present study does not reflect an *in vivo* clinical situation, *ex vivo* whole blood studies have advantages over stimulation of isolated monocytes [37, 38]. Whole blood culture contains normal cell-cell interactions, and the monocytes are provided with the regulatory factors from the environment, which are essential for their viability. Thus, this method has been suggested to be the most appropriate to investigate cytokine production in an environment that resembles natural conditions. Additionally, whole blood stimulation assay is a simple, quick, and less expensive method.

Nonetheless, our study has several limitations. First of all, this study does not analyze the underlying mechanism of the described observation. Secondly, the *ex vivo* model of pro-inflammatory cytokine production in response to endotoxin stimuli cannot reflect *in vivo* conditions, since it lacks the complex physiological effects catecholamines on other systems. A basic methodological disadvantage of this study is that the number and the viability of each cell type remain unknown. Although the used concentration of norepinephrine, dobutamine, and metoprolol was selected according to previous studies, one important limit of this study is the lack of concentration range curves for these substances. Moreover, this study lacks the use of another β_1 receptor antagonist, apart from metoprolol, in order that justified conclusions are drawn. Further studies would shed light on the effect of other catecholamines used in sepsis too, such as adrenaline, and the underlying mechanisms could be examined using selective α- and β-adrenergic receptor antagonists.

In summary, *ex* vivo whole blood LPS stimulation results in high levels of pro-inflammatory cytokines, which are though suppressed by both norepinephrine and dobutamine in healthy volunteers. Moreover, the inhibitory adrenergic effect may be partially exerted *via* β_1-adrenergic receptor, although other receptor pathways cannot be excluded. Conclusively, apart from the well-described effect of adrenergic drugs in hemodynamic support, their potential anti-inflammatory properties during sepsis remain to be fully elucidated.

COMPLIANCE WITH ETHICAL STANDARDS

This *ex vivo* study included healthy volunteers, who were not treated with any factor.

Informed Consent. All participants were informed about the aim and the expected results of this study, and written informed consent was obtained from all individual participants included in the study.

Conflict of Interest. The authors declare that they have no conflict of interest.

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