

Noradrenaline Inhibits Lipopolysaccharide-Induced Tumor Necrosis Factor and Interleukin 6 Production in Human Whole Blood

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Sepsis and lipopolysaccharide (LPS) trigger the systemic release of both cytokines and catecholamines. Cytokines are known to be capable of eliciting a stress hormone response in vivo. The present study sought insight into the effect of noradrenaline on LPS-induced release of tumor necrosis factor alpha (TNF) and interleukin 6 (IL-6) in human whole blood. Whole blood was incubated with LPS for 4 h at 37°C in the presence and absence of noradrenaline and/or specific α and β antagonists and agonists. Noradrenaline caused a dose-dependent inhibition of LPS-induced TNF and IL-6 production. This effect could be completely prevented by addition of the specific β_1 antagonist metoprolol, while it was not affected by the α antagonist phentolamine. Specific β -adrenergic stimulation by isoprenaline mimicked the inhibiting effect of noradrenaline on LPS-evoked cytokine production, whereas α -adrenergic stimulation by phenylephrine had no effect. Fluorescence-activated cell sorter analysis demonstrated that β -adrenergic stimulation had no effect on LPS binding to and internalization into mononuclear cells or on the expression of CD14, the major receptor for LPS on mononuclear cells. In acute sepsis, enhanced release of noradrenaline may be part of a negative feedback mechanism meant to inhibit ongoing TNF and IL-6 production.

The lipopolysaccharide (LPS) component of the outer membrane of gram-negative bacteria is considered responsible for the pathological sequelae of gram-negative sepsis (10). LPS triggers the production of cytokines, which in turn mediate most of the biological effects of LPS. Ample evidence that tumor necrosis factor alpha (TNF) is the first cytokine in a cascade of these proteins that is induced by LPS exists (6). The essential role of TNF in the toxicity evoked by bacteremia has been carefully documented in animal studies showing that neutralization of TNF activity prevents mortality in otherwise lethal sepsis (25). Interleukin 6 (IL-6) release follows shortly after the appearance of TNF in experimental endotoxemia and sepsis (7, 27) and is likely to be the major stimulus for acute-phase protein synthesis in systemic infection (16).

In sepsis, high concentrations of TNF and IL-6 in serum are paralleled by markedly elevated levels of stress hormones in the circulation (8–11). Reciprocal interactions between cytokines and the endocrine system have been documented, i.e., cytokines can induce a stress hormone response when they are given systemically to various species, including humans (22, 26), and stress hormones can affect the production of cytokines (2, 20, 21). Mononuclear cells express both α - and β -adrenergic receptors (14, 15, 20, 21, 29). Adrenaline has been reported to diminish TNF production by mononuclear cells and human whole blood stimulated with LPS via an effect on β -adrenergic receptors (20). Noradrenaline, however, has been demonstrated to augment LPS-induced TNF production by murine macrophages via stimulation of α -adrenergic receptors (21).

In the present study, we sought further insight into the effect of noradrenaline on the production of TNF and IL-6 elicited

by LPS, as well as into the mechanisms that contribute to this effect. For this purpose, we used the previously validated system of endotoxin-stimulated cytokine production in human whole blood, which has been introduced as an ex vivo model to study cytokine synthesis in inflammatory conditions in which many of the physiologically present cellular interactions remain intact (3, 4).

MATERIALS AND METHODS

Materials. LPS (*Escherichia coli* O111:B4) was obtained from Difco Laboratories (Detroit, Mich.). Dilutions were made in Hanks' balanced salt solution. Adrenergic agents were obtained from the following manufacturers: noradrenaline (bitartrate) from OPG, Utrecht, The Netherlands; metoprolol (tartrate) (Seloken) from Astra, Rÿswÿk, The Netherlands; phentolamine (mesilate) (Regitine) from CIBA-GEIGY, Arnhem, The Netherlands; and isoprenaline (sulfate) and phenylephrine (hydrochloride) from OPG. Dilutions were made in isotonic saline. LPS-fluorescein isothiocyanate (FITC) (trichloroacetic acid) preparation from *E. coli* O111:B4 (5.7 μ g of FITC per mg of LPS) was obtained from Sigma Chemical Co., St. Louis, Mo. Phycoerythrin (PE)-conjugated monoclonal anti-CD14 antibody (anti-CD14-PE) (CD14 epitope, Leu-M3) was obtained from Becton Dickinson Immunocytometry Systems, San Jose, Calif.

Whole-blood stimulation. Venous blood samples were obtained from healthy male subjects between the ages of 20 and 40 years. Blood was collected aseptically with a pyrogen-free collecting system consisting of a 19-gauge butterfly needle connected to a sterile syringe (Becton Dickinson, Dublin, Ireland). Anticoagulation was obtained with pyrogen-free heparin (Organon Teknika BV, Boxtel, The Netherlands) (10 U/ml of blood, final concentration). Whole blood of individual subjects was incubated in aliquots of 2 ml for 4 h at 37°C in

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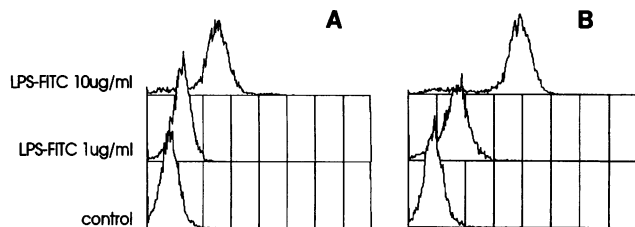


FIG. 1. Binding and internalization of LPS-FITC (in) to mononuclear cells. Whole blood was incubated for 1 h at 4°C (A) and 37°C (B), erythrocytes were lysed, mononuclear cells were gated, and LPS-FITC binding (A) and binding plus internalization (B) were measured by FACS analysis. A significant FITC signal was detected at both LPS-FITC concentrations used (1 and 10 µg/ml). x axis, fluorescence intensity; y axis, number of cells.

sterile tubes (Beckton Dickinson and Company, Lincoln Park, N.J.) which were preloaded with either LPS, adrenergic agonists or antagonists, or equivalent volumes of the respective media in which these substances were dissolved. In each experiment, LPS was added at a total volume of 100 µl; noradrenaline and adrenergic agonists and antagonists were added at a volume of 50 µl. It should be noted that noradrenaline is possibly metabolized during the 4-h incubation period. The levels of noradrenaline were not measured during the incubation. After incubation, plasma was prepared by centrifugation at $1,500 \times g$ for 20 min at 4°C. Plasma was stored at -70°C until assays were performed batchwise.

Fluorescence-activated cell sorter (FACS) analysis. To determine LPS-FITC binding and internalization, whole blood was incubated for 1 h at either 4 or 37°C with LPS-FITC (1 or 10 µg/ml), in the presence and absence of noradrenaline, isoprenaline, and phenylephrine (all at concentrations of 10^{-6} M). Nonspecific binding of LPS-FITC was excluded in experiments, demonstrating that anti-CD14 monoclonal antibody (clone 77; Central Laboratory of Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) prevented the binding of LPS-FITC (data not shown). After incubation, erythrocytes were lysed in ice-cold lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA in water [pH 7.4]) for 20 min and leukocytes were washed with ice-cold wash buffer (phosphate-buffered saline, 0.1% bovine serum albumin, 0.01% NaN₃) prior to flow cytometric analysis. To determine CD14 expression, whole blood was incubated for 1 h at either 4 or 37°C with and without noradrenaline (10^{-6} M). After incubation, erythrocytes were lysed and leukocytes were washed as described above, after which anti-CD14-PE (10 µg/ml) was added. LPS-FITC binding and CD14 expression were analyzed with a FAC-Scan cell sorter (Beckton Dickinson Immunocytometry Systems) equipped with a 15-mW argon ion laser, operating at 488 nm. The fluorescence signals were measured through 515- to 545-nm (FITC) filters and 563- to 607-nm (PE) filters. The fluorescence intensity was recorded on a logarithmic scale expressed in fluorescence units, and the data were analyzed with the Consort-32 and LYSYS II software packages (Beckton Dickinson). Mononuclear cells were gated by using a side scatter-by-forward scatter dot plot display. Significant binding to (incubation at 4°C) and binding to plus internalization into (incubation at 37°C) mononuclear cells of LPS-FITC could be detected at the concentrations used (1 and 10 µg/ml) to study the influence of adrenergic agonists on these processes (Fig. 1). As expected, incubation with anti-CD14-PE produced a strong PE signal in the mononuclear cell population at both 4 and 37°C (data not shown).

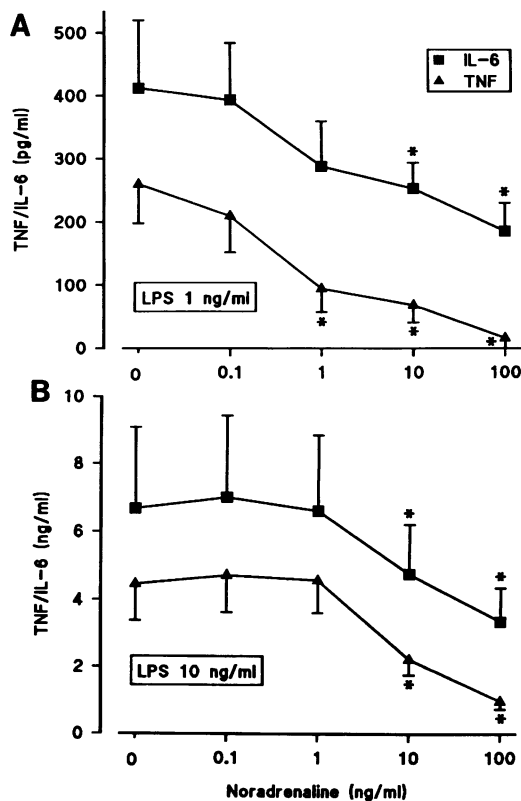


FIG. 2. Effect of noradrenaline on LPS-induced TNF and IL-6 production. Whole blood was incubated for 4 h at 37°C with LPS (1 or 10 ng/ml) in the presence and absence of noradrenaline. The concentrations of noradrenaline listed correspond with concentrations from 0.6×10^{-9} M (0.1 ng/ml) to 0.6×10^{-6} M (100 ng/ml). Results of incubations of whole blood from eight subjects are shown. Asterisks indicate $P < 0.05$ versus the TNF and IL-6 levels after incubation with LPS alone. Each vertical bar indicates the SEM.

Cytokine assays. Plasma TNF and IL-6 concentrations were measured with previously described enzyme-linked immunosorbent assays (5, 13).

Statistical analysis. All results are given as the mean \pm the standard error of the mean (SEM). The TNF and IL-6 levels of various treatment groups were compared by using the Wilcoxon test for matched samples. Statistical significance was set at $P < 0.05$.

RESULTS

Effect of noradrenaline on LPS-induced TNF and IL-6 production. Incubation of whole blood for 4 h at 37°C in the absence of LPS did not result in detectable levels of TNF or IL-6. In addition, noradrenaline did not induce cytokine production in the absence of LPS (data not shown). LPS at concentrations of 1 and 10 ng/ml induced both TNF and IL-6 production in the whole-blood samples of all eight subjects tested in these experiments (Fig. 2). Noradrenaline caused a dose-dependent inhibition of LPS-induced TNF and IL-6 release (Fig. 2). After incubation with 1 ng of LPS per ml, this inhibition was significant at a noradrenaline concentration of 1 ng/ml, whereas after incubation with 10 ng of LPS per ml, higher noradrenaline concentrations (10 ng/ml) were needed to inhibit cytokine production. Maximal inhibition was seen with the highest noradrenaline concentration (100 ng/ml),

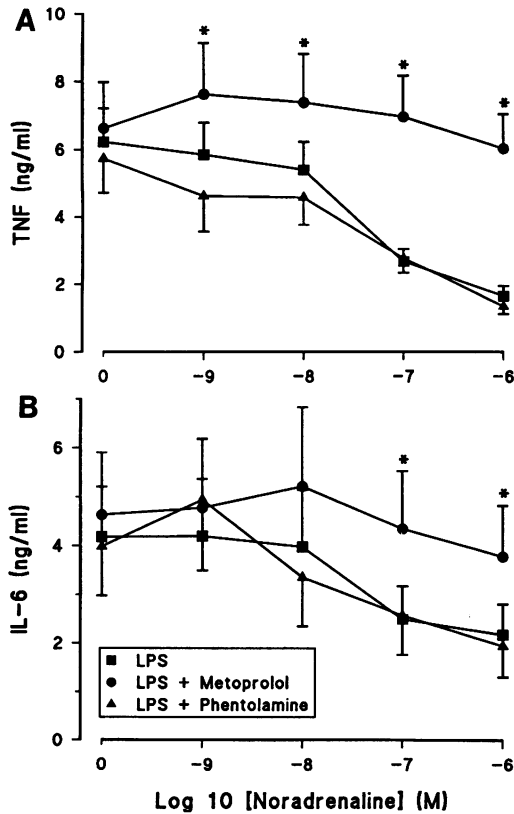


FIG. 3. Effect of α and β antagonists on the inhibition on LPS-induced TNF (A) and IL-6 (B) production by noradrenaline. Whole blood was incubated for 4 h at 37°C with LPS (10 ng/ml) and increasing doses of noradrenaline (10^{-9} to 10^{-6} M) in the absence and presence of the specific α antagonist phentolamine (10^{-5} M) or the specific β antagonist metoprolol (10^{-5} M). Results of incubations of whole blood from six subjects are shown. The addition of metoprolol completely restored TNF and IL-6 production to the levels found after incubation with LPS alone. Asterisks indicate $P < 0.05$ versus the TNF and IL-6 levels after incubation with only LPS and the corresponding noradrenaline concentration. Each vertical bar indicates the SEM.

which reduced TNF levels to $8.3\% \pm 8.3\%$ and $32.7\% \pm 8.8\%$ of those found after incubation with LPS (1 and 10 ng/ml, respectively) alone and IL-6 concentrations to $49.8\% \pm 4.8\%$ and $78.0\% \pm 8.0\%$.

Effect of α - and β -adrenergic antagonists on noradrenaline-induced inhibition of TNF and IL-6 production. To assess whether the inhibition of cytokine production by noradrenaline was mediated by an effect on α - or β -adrenergic receptors, whole blood from six subjects was incubated for 4 h at 37°C with LPS (10 ng/ml) and noradrenaline (10^{-9} to 10^{-6} M), with and without the specific α antagonist phentolamine (10^{-5} M) and/or the specific β antagonist metoprolol (10^{-5} M) (28) (Fig. 3). In the absence of noradrenaline, neither phentolamine nor metoprolol affected LPS-induced TNF and IL-6 production. In accordance with the experiments described above, noradrenaline caused a dose-dependent inhibition of LPS-induced TNF and IL-6 release. Phentolamine did not affect this inhibitory effect of noradrenaline. In contrast, metoprolol completely prevented it, i.e., TNF and IL-6 levels after incubation with LPS in the presence of noradrenaline and metoprolol were similar to those after incubation with LPS alone ($P > 0.2$ for the difference between LPS and noradren-

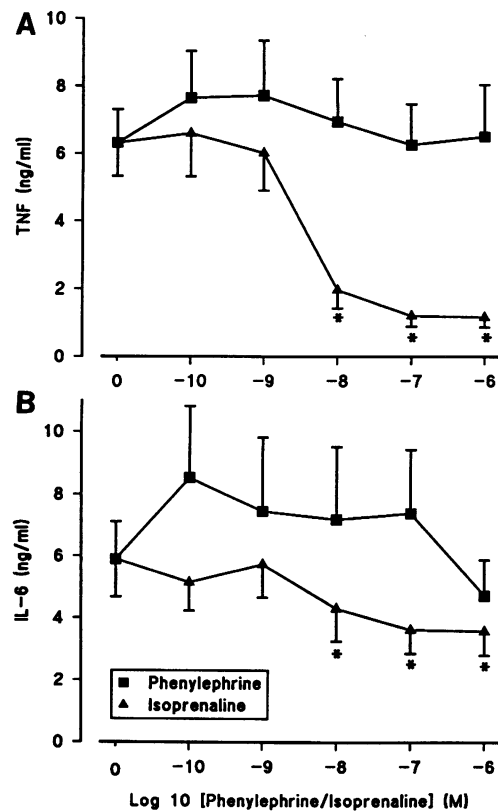


FIG. 4. Effect of α and β agonists on LPS-induced TNF (A) and IL-6 (B) production. Whole blood was incubated for 4 h at 37°C with LPS (10 ng/ml) in the presence and absence of the specific α agonist phenylephrine or the specific β agonist isoprenaline. Results of incubations of whole blood from six subjects are shown. Asterisks indicate $P < 0.05$ versus TNF and IL-6 levels after incubation with LPS alone. Each vertical bar indicates the SEM.

aline plus metoprolol versus LPS alone for both TNF and IL-6 at all noradrenaline concentrations tested). The addition of metoprolol together with phentolamine (both 10^{-5} M) to whole blood incubated with LPS and noradrenaline resulted in TNF and IL-6 concentrations similar to those found after the addition of metoprolol alone (data not shown). Hence, these results suggested that noradrenaline inhibited LPS-induced cytokine production exclusively by an effect on the β_1 -adrenergic receptor.

Effect of specific α or specific β agonist on LPS-induced TNF and IL-6 production. To gain further evidence that the inhibition of cytokine production by noradrenaline was achieved by β -adrenergic stimulation, whole blood from six subjects was incubated for 4 h at 37°C with LPS (10 ng/ml) in the presence and absence of the specific α -adrenergic agonist phenylephrine (10^{-10} to 10^{-6} M) or the specific β_1 and β_2 agonist isoprenaline (10^{-10} to 10^{-6} M) (28) (Fig. 4). Phenylephrine did not affect LPS-induced TNF or IL-6 release. Isoprenaline, however, caused a dose-dependent reduction in LPS-induced TNF and IL-6 production, an effect that was significant at concentrations of 10^{-8} M and higher. At a concentration of 10^{-6} M, isoprenaline reduced TNF levels to $17.9\% \pm 4.1\%$ and IL-6 levels to $61.8\% \pm 8.1\%$ of those observed after incubation with LPS alone.

Flow cytometry experiments. One mechanism by which β -adrenergic stimulation may lead to a reduction in LPS-

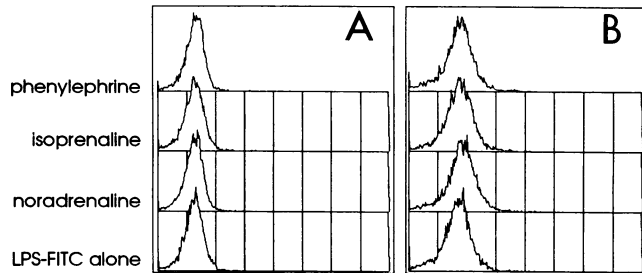


FIG. 5. Effect of adrenergic agonists on LPS-FITC binding to and internalization into mononuclear cells. Whole blood was incubated for 1 h at 4°C (A) or 37°C (B) with LPS-FITC (1 or 10 $\mu\text{g/ml}$) in the presence and absence of noradrenaline (10^{-6} M), isoprenaline (10^{-6} M), or phenylephrine (10^{-6} M). Thereafter, erythrocytes were lysed, mononuclear cells were gated, and LPS-FITC binding and internalization were measured by FACS analysis. Results of incubations with LPS-FITC at a concentration of 1 $\mu\text{g/ml}$ are shown. Similar results were obtained with LPS-FITC at 10 $\mu\text{g/ml}$ (data not shown). x axis, fluorescence intensity; y axis, number of cells.

induced TNF and IL-6 production is inhibition of LPS binding to or internalization into mononuclear cells. Therefore, we next investigated these issues by FACS analysis. Neither noradrenaline, isoprenaline, nor phenylephrine (each at a concentration of 10^{-6} M) affected LPS-FITC binding to and internalization into mononuclear cells (Fig. 5). Furthermore, noradrenaline did not alter the expression of CD14, the main receptor for LPS on mononuclear cells (20) (Fig. 6).

DISCUSSION

It has been widely recognized that LPS and cytokines can elicit the release of catecholamines *in vivo* (18, 22, 26). More recent studies indicate that catecholamines may have a bimodal effect on LPS-induced TNF production, the eventual result depending on whether the α - or β -adrenergic receptor is preferentially stimulated (20, 21). Knowledge of the effect of

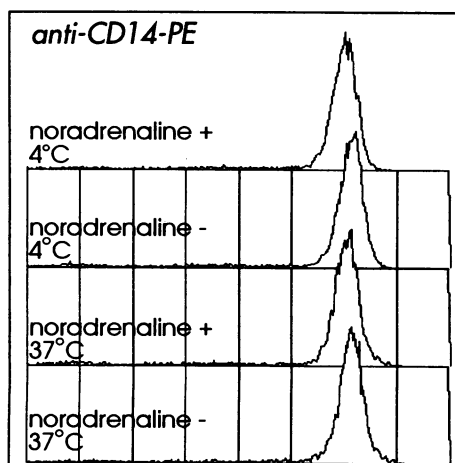


FIG. 6. Effect of noradrenaline on the expression of CD14 on mononuclear cells. Whole blood was incubated for 1 h at 4 or 37°C in the presence (+) and absence (-) of noradrenaline (10^{-6} M). Thereafter, erythrocytes were lysed, leukocytes were washed, and anti-CD14-PE (10 $\mu\text{g/ml}$) was added. Mononuclear cells were gated, and expression of CD14 was measured by FACS analysis. x axis, fluorescence intensity; y axis, number of cells.

catecholamines on cytokine production in sepsis is important not only for the unraveling of feedback loops between these two mediators systems but also because of the fact that catecholamines are frequently administered to septic shock patients as vasoactive agents. This study aimed to assess the effect of noradrenaline on LPS-induced TNF and IL-6 production in human whole blood, an *ex vivo* system that has been shown to be a suitable model to study cytokine synthesis in sepsis (3, 4). The use of whole blood eliminates artifacts that may be associated with the isolation of monocytes, such as adherence-induced expression of TNF (12). Additionally, by using whole blood the effect of a hormone on the release of cytokines evoked by LPS can be investigated under conditions with a physiological endocrine background, which is likely to be of more relevance for the *in vivo* situation. Our results demonstrate that noradrenaline causes a dose-dependent inhibition of LPS-induced release of TNF and IL-6 and that this effect is mediated by β_1 -adrenergic receptors.

Mononuclear cells, which represent major producers of TNF and IL-6, express both α - and β -adrenergic receptors on their surfaces (14, 15, 20, 21, 29). Stimulation of α -adrenergic receptors may result in an enhancement of immune cell responsiveness and augmentation of LPS-induced TNF production, whereas β -receptor stimulation may decrease immune cell activity and reduce LPS-induced TNF synthesis (15, 20, 21). Noradrenaline binds to α receptors and the β_1 receptor (28). Recently, noradrenaline, at concentrations between 10^{-9} and 10^{-8} M, has been reported to increase production of TNF by murine macrophages incubated with LPS for 4 h (i.e., similar to the incubation period used in our study), an effect that likely was solely mediated by α_2 receptors (21). At a concentration of 10^{-8} M, the potentiating effect of noradrenaline was less pronounced; the effect of higher concentrations, which may have revealed inhibition of LPS-induced TNF production by noradrenaline, was not reported (21). In the present study, downregulation of LPS-induced TNF production was not observed until a noradrenaline concentration between 10^{-8} and 10^{-7} M, an effect that could be completely prevented by the addition of a specific β_1 antagonist and mimicked by a specific β agonist. However, in contrast to the findings of the study cited immediately above (21), the stimulatory effect of noradrenaline was not observed at any concentration (lowest concentration tested, 10^{-10} M; data not shown). The reason for this apparent discrepancy remains to be established but may be related to interspecies differences and/or differences in experimental systems (i.e., isolated cells versus whole blood). Phenylephrine, the α -adrenergic agonist used in the present study, has a higher affinity for α_1 than α_2 receptors (28). Theoretically, α_1 -adrenergic stimulation may enhance TNF production by activation of protein kinase C (1, 17). Our results demonstrate that stimulation of α_1 -adrenergic receptors, in either the presence or absence of LPS, does not affect cytokine production in human whole blood. Furthermore, it is unlikely that α_2 -adrenergic stimulation enhances LPS-induced TNF production in human whole blood, considering the fact that a blockade of β_1 receptors, allowing noradrenaline to bind only to α_1 and α_2 receptors, was not associated with an increase in TNF release elicited by LPS.

One mechanism by which β -adrenergic agonists may inhibit TNF production is the well-documented capability of these agents to stimulate adenylate cyclase activity and to increase cyclic AMP (cAMP) synthesis (14, 20, 29), since elevated levels of cAMP have been shown to suppress LPS-induced transcriptional activation of the TNF gene (23). To our knowledge, the current study is the first to show that β_1 -adrenergic stimulation attenuates IL-6 production, albeit that this effect was of less

magnitude than the inhibition of TNF release. It is unlikely that increasing cAMP levels contribute to the observed inhibition of IL-6 production, since cAMP-increasing agents have been reported to induce IL-6 gene expression and production in various cell types (19, 30). In addition, it is unlikely that the reduction in IL-6 release was secondary to the inhibition of TNF production, since complete neutralization of TNF activity by the addition of an anti-TNF antibody did not reduce LPS-stimulated IL-6 release in human whole blood in a previous study (3). Therefore, we addressed another mechanism by which noradrenaline may inhibit IL-6 (and TNF) production, i.e., the binding of LPS to mononuclear cells. It appeared that neither noradrenaline, isoprenaline, nor phenylephrine affected LPS-FITC binding to and internalization into mononuclear cells. In accordance, noradrenaline did not influence the expression of the main LPS receptor CD14 on mononuclear cells, although this does not preclude an effect of noradrenaline on the expression of other LPS receptors (24). Thus, other mechanisms must be involved.

In acutely septic patients, TNF and IL-6 are among the major cytokines found in the circulation. Our study demonstrates that noradrenaline attenuates LPS-induced release of TNF and IL-6 in human whole blood via stimulation of β_1 -adrenergic receptors. These results exemplify the tight interaction between the cytokine network and the endocrine system and suggest that, in acute sepsis, noradrenaline (released at least partly by the action of TNF and IL-6) inhibits the ongoing production of these mediators as part of a negative feedback mechanism.

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