In vitro effects of epinephrine, norepinephrine, and dobutamine on lipopolysaccharide-stimulated production of tumor necrosis factor-α, interleukin-6, and interleukin-10 in blood from healthy dogs

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OBJECTIVE
To determine the in vitro effects of epinephrine, norepinephrine, and dobutamine on lipopolysaccharide (LPS)-stimulated production of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-10 (IL-10) in blood from healthy dogs.

SAMPLES
Blood samples from 9 healthy dogs.

PROCEDURES
Blood samples were incubated with LPS from *Escherichia coli* O127:B8 or PBSS (control) for 1 hour. Afterward, the samples were incubated with 10μM epinephrine, norepinephrine, or dobutamine or with saline (0.9% NaCl) solution (control) for 23 hours. Leukocyte viability was assessed by use of trypan-blue exclusion in blood from 2 dogs to ensure cell viability was not altered by the catecholamines. Tumor necrosis factor-α, IL-6, and IL-10 concentrations were measured in the supernatant in duplicate with a canine-specific multiplex bead-based assay. Blood samples from 2 dogs were used to create dose-response curves to evaluate whether the observed cytokine modulation was dependent on catecholamine concentration.

RESULTS
Incubation of blood with epinephrine and norepinephrine significantly increased LPS-stimulated production of IL-10, compared with the control. Epinephrine and norepinephrine significantly decreased LPS-stimulated production of TNF-α, compared with the control. Epinephrine and norepinephrine did not significantly alter LPS-stimulated production of IL-6. Dobutamine did not alter catecholamine production.

CONCLUSIONS AND CLINICAL RELEVANCE
Epinephrine and norepinephrine, but not dobutamine, had immunomodulatory effects on LPS-stimulated TNF-α and IL-10 production in blood from healthy dogs in this in vitro model of sepsis. Data suggested that dobutamine may have immune system–sparing effects in dogs with sepsis. (*Am J Vet Res* 2021;82:374–380)

Both immune and neuroendocrine systems are activated during sepsis, leading to the production of cytokines and catecholamines.[1] Additionally, exogenous catecholamines, most commonly α- and β-adrenoceptor agonists, are administered to patients with septic shock. The α-adrenoceptor agonists epinephrine and norepinephrine are administered to patients with septic shock to mitigate vasodilation and improve blood pressure.[2] Medications that primarily have β-adrenoceptor agonist effects such as dobutamine are used to improve cardiac contractility and output.[2] These catecholamines can have deleterious effects when used alone in patients with shock and, therefore, are often used together.[2] In many species including humans, catecholamines have immunomodulatory properties, most commonly inhibiting LPS-stimulated production of proinflammatory cytokines, including TNF-α, IL-6, and IL-1β, and stimulating production of the anti-inflammatory cytokine IL-10.[3] This effect of catecholamines on inflammatory cytokines is one of the pathogenic mechanisms leading to immunoparalysis in people with sepsis.

Like people, dogs with sepsis develop various forms of immunodysfunction.[4] Despite the routine use of catecholamines in dogs with sepsis, information pertaining to the immunomodulatory effects of catecholamines in dogs is sparse. Understanding the immunologic implications of catecholamine administration could help guide treatment decision-making. Therefore, the objective of the study reported here

ABBREVIATIONS

IL	Interleukin
LPS	Lipopolysaccharide
NF-κβ	Nuclear factor-κβ
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α

was to evaluate the effects of epinephrine, norepinephrine, and dobutamine on LPS-stimulated production of TNF-α, IL-6, and IL-10 in healthy dogs in a focused, in vitro model of cytokine signaling. We hypothesized that epinephrine, norepinephrine, and dobutamine would downregulate LPS-stimulated production of TNF-α and IL-6 and upregulate production of IL-10.

Materials and Methods

Samples

The study protocol was approved by the University of Missouri Animal Care and Use Committee. Ten dogs (neutered male, 5; spayed female, 5) owned by residents, faculty members, and students of the University of Missouri were included in the study, with informed consent from the owners. Seven dogs were of a mixed breed, and 1 each was a Siberian Husky, German Shepherd Dog, and Beagle; dogs ranged in age from 2 to 8 years. Dogs were housed in traditional homes and fed commercially available dog food. Dogs were determined to be healthy on the basis of history and physical examination performed by the primary investigator (KMM) on the day of enrollment. Dogs had not received any vaccine or medication within 1 month of enrollment, with the exception of monthly parasitic preventives. To be included in the study, each dog had to have a standard response to LPS, defined as at least a 1-fold increase in concentrations of TNF-α and IL-10 after LPS-stimulation, compared with PBSS.

Blood sample collection and processing

A 6-mL blood sample was collected from each dog through jugular venipuncture with a 20-gauge needle. These samples were placed in blood collection tubes containing sodium heparin. Next, each sample was diluted 1:2 with complete RPMI culture medium (RPMI 1640 with 200 U of penicillin/mL and 200 mg of streptomycin/mL),[a] and then LPS from *Escherichia coli* O127:B8 (final concentration, 100 ng/mL)[b] or PBSS (control) was added. The mixtures were incubated in the dark for 1 hour at 37°C in 5% CO_2.

Following incubation, samples were transferred to a 12-well plate, and 10μM dobutamine, 10μM epinephrine, 10μM norepinephrine, or saline (0.9% NaCl) solution (control) was added. Dobutamine,[c] epinephrine,[d] and norepinephrine bitartrate[e] were each diluted in 10 mL of sterile saline solution to create a 100μM stock solution of each; 10μM of each was added to wells as described. Samples were then incubated in the dark for 23 hours at 37°C in 5% CO_2. Selection of the concentration of catecholamines was determined by extrapolation from previous studies[5,6] performed with human blood samples. After incubation, plates were centrifuged at 400 × *g* for 7 minutes at room temperature (21°C). The supernatant was retrieved and stored at −80°C until batch analysis.

Leukocyte viability in the blood samples from 2 dogs following the final incubation period (after 23-hour incubation) was assessed with a trypan-blue exclusion test as previously described.[7] After centrifugation and retrieval of supernatant, ammonium chloride–potassium lysis buffer solution (8.26 g of NH_4Cl, 1.0 g of $KHCO_3$, and 0.037 g of Na_2EDTA in 1 L of deionized distilled H_2O; pH, 7.2) was added to lyse the RBCs. Leukocytes were twice washed with PBSS and then resuspended with 50 μL of PBSS. Cell viability was evaluated by the addition of 50 μL of trypan blue and then by use of a hemocytometer, counting of a minimum of 100 leukocytes and recording the percentage of those that were viable (dye-excluding cells) and those that were not viable (positive-stained cells). The test was performed in duplicate.

Tumor necrosis factor-α, IL-6, and IL-10 concentrations were measured in the supernatant in duplicate with a canine-specific multiplex bead-based assay.[8,f] Samples were thawed and centrifuged at 400 × *g* for 7 minutes to pellet fibrin and other debris. Then, the supernatant was retrieved and mixed with anticytokine mono- or polyclonal antibody–charged polystyrene microspheres in a 96-well plate. Samples were incubated overnight at 4°C with agitation. Next, a biotinylated polyclonal detection antibody and streptavidin-phycoerythrin were added. Each sample was analyzed in duplicate with appropriate controls and associated data analysis software[g] to determine the median fluorescence intensity and cytokine concentrations. The assay's limit of detection for each cytokine was 48 pg/mL; intra-assay coefficient of variation was 5%, and interassay coefficient of variation was 15%.

Dose-response curves were created for the catecholamines that significantly altered LPS-stimulated cytokine production in the blood samples of 2 dogs. To create each dose-response curve, 6-mL blood samples were obtained and processed as described and catecholamines were added, starting at 1,000μM followed by serial 10-fold dilutions to 0.1μM.

Statistical analysis

Statistical analysis was performed with commercially available software.[h] Dose-response relationships were reported as descriptive statistics. The Shapiro-Wilk test was used to assess the data for normality. Data among cell treatment groups were compared with a 2-way repeated-measures ANOVA, and if a difference was indicated, the multiple-comparisons Fisher least significant difference method was performed. Values of $P < 0.05$ were considered significant.

Results

Incubation of blood samples with dobutamine, epinephrine, or norepinephrine did not alter leukocyte viability, compared with the control, at 24 hours (data not shown). Nine of 10 dogs had standard responses to LPS; therefore, 9 dogs were included in the study.

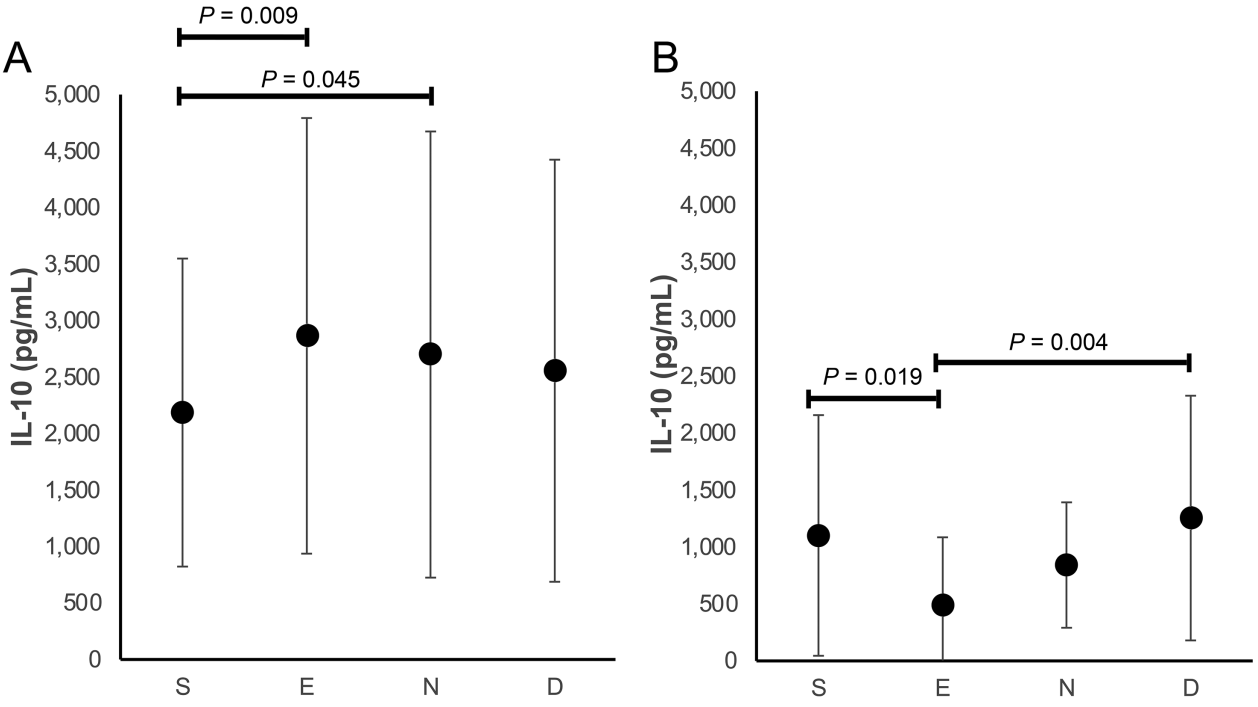

Figure 1—Mean (circle) ± SD (whiskers) LPS-stimulated IL-10 concentrations (A) and constitutive (not LPS-stimulated) IL-10 concentrations (B) for blood samples from 9 healthy dogs that were incubated with saline (0.9% NaCl) solution (S), epinephrine (E), norepinephrine (N), or dobutamine (D). Brackets denote compared treatments with significant differences and associated *P* values.

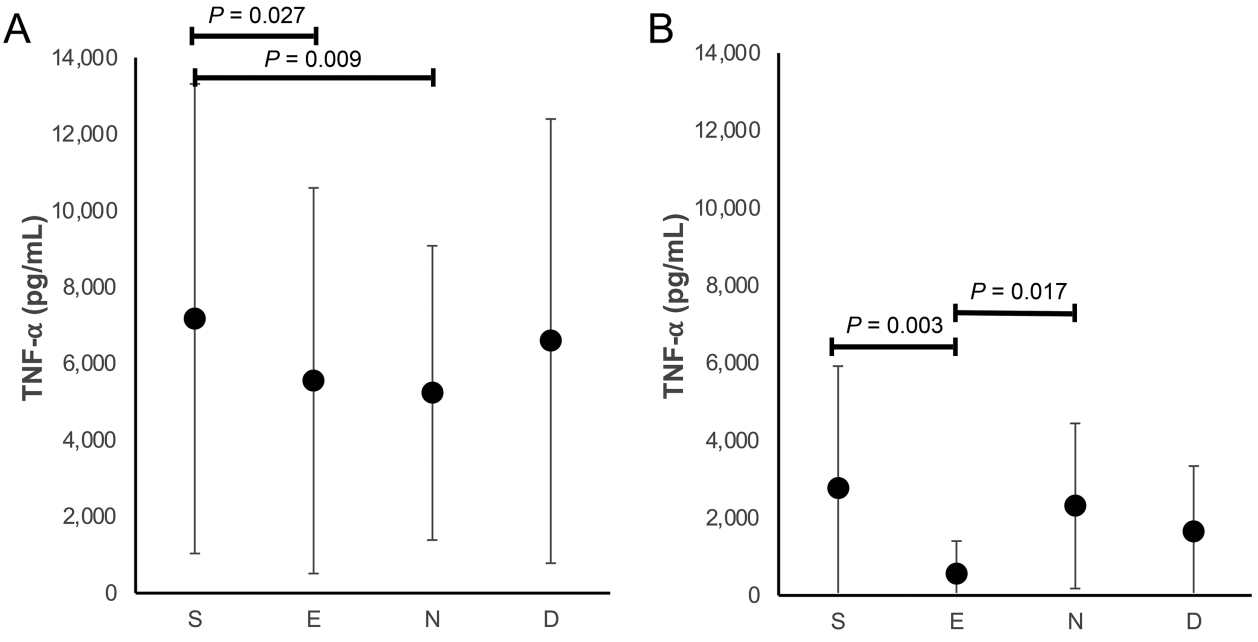

Figure 2—Mean ± SD LPS-stimulated TNF-α concentrations (A) and constitutive TNF-α concentrations (B) for the blood samples dogs of Figure 1. ***See*** Figure 1 for key.

Epinephrine and norepinephrine significantly (*P* = 0.009 and *P* = 0.045, respectively) increased LPS-stimulated production of IL-10, compared with the control **(Figure 1)**. Epinephrine also significantly decreased constitutive IL-10 production versus dobutamine (*P* = 0.004) and the control (*P* = 0.019). Dobutamine did not significantly alter LPS-stimulated or constitutive IL-10 production.

Epinephrine and norepinephrine significantly (*P* = 0.027 and *P* = 0.009, respectively) decreased LPS-stimulated production of TNF-α, compared with the control **(Figure 2)**. Epinephrine also significantly de-

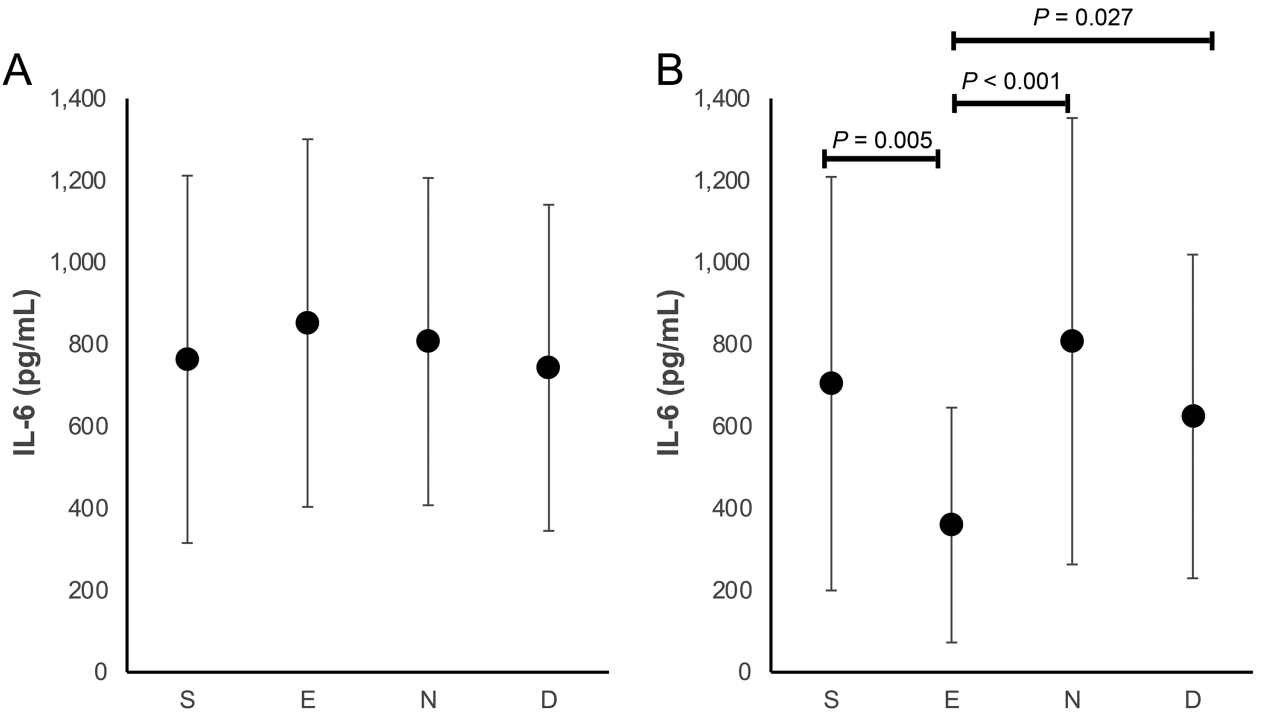

Figure 3—Mean ± SD LPS-stimulated IL-6 concentrations (A) and constitutive IL-6 concentrations (B) for the blood samples of Figure 1. ***See*** Figure 1 for key.

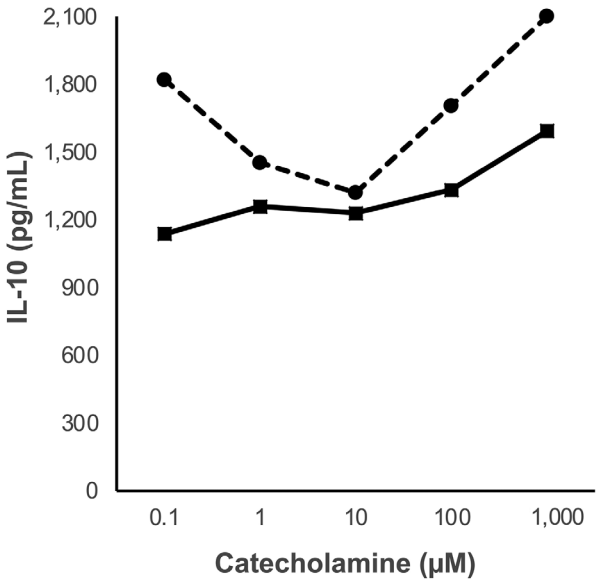

Figure 4—Dose-response curve for the production of IL-10 in the blood samples from 2 dogs of Figure 1 following incubation with various concentrations of epinephrine (solid line) and norepinephrine (dashed line).

creased constitutive production of TNF-α, compared with the control (P = 0.003) and norepinephrine (P = 0.017). Dobutamine did not significantly alter LPS-stimulated or constitutive production of TNF-α.

Epinephrine, norepinephrine, and dobutamine did not significantly alter the production of LPS-stimulated IL-6 **(Figure 3)**. Epinephrine significantly decreased constitutive production of IL-6, compared with the control (P = 0.005), norepinephrine (P < 0.001), and dobutamine (P = 0.027).

On the basis of visual inspection of the dose-response curves, epinephrine increased LPS-stimulated production of IL-10 in a concentration-dependent manner (10µM, 100µM, and 1,000µM; **Figure 4**). Lipopolysaccharide-stimulated IL-10 concentrations were lower at norepinephrine concentrations of 1µM and 10µM, compared with norepinephrine concentrations of 100µM and 1,000µM. Epinephrine and norepinephrine decreased LPS-stimulated TNF-α production at epinephrine and norepinephrine concentrations ranging from 1µM to 1,000µM, compared with 0.1µM **(Figure 5)**. However, the concentration-dependent effect of norepinephrine began to dissipate between 100µM and 1,000µM.

Discussion

The objective of the study reported here was to evaluate the effects of epinephrine, norepinephrine, and dobutamine on the LPS-stimulated production of TNF-α, IL-6, and IL-10 in blood samples from healthy dogs in an in vitro model of cytokine signaling. Incubation of the blood samples with LPS plus epinephrine or norepinephrine yielded significantly increased IL-10 concentrations and decreased TNF-α concentrations but had no effect on IL-6 concentrations. Likewise, dobutamine did not alter LPS-stimulated production of IL-6, TNF-α, or IL-10. Epinephrine, norepinephrine, and dobutamine were hypothesized to downregulate LPS-stimulated production of

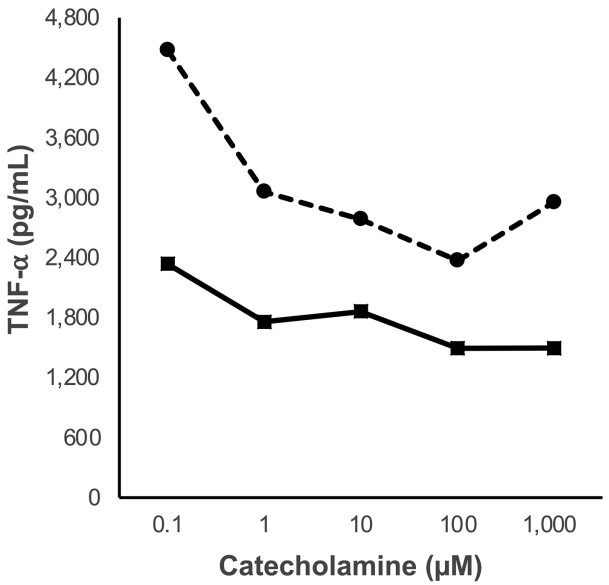

Figure 5—Dose-response curve for the production of TNF-α in the blood samples from 2 dogs of Figure 1 following incubation with various concentrations of epinephrine (solid line) and norepinephrine (dashed line).

TNF-α and IL-6 and upregulate production of IL-10. Portions of the hypothesis were confirmed, but other portions were not.

Canine leukocytes express α_1-adrenoceptors and β-adrenoceptors, including β_1- and β_2-adrenoceptors.[9,10] Signaling through these receptors affects blood cytokine production through alterations in the activation of NF-κβ.[11,12] In many species, α-adrenoceptor signaling results in upregulation of proinflammatory cytokine production, and β-adrenoceptor signaling results in downregulation of proinflammatory cytokine production.[13–16] On the basis of the results of the present study, dogs were similar to other species in that adrenoceptor stimulation altered blood cytokine production.

Interleukin-10 is a part of the host protective mechanisms during endotoxemia.[1] In the present study, epinephrine and norepinephrine increased LPS-stimulated production of IL-10, which is similar to that observed in a study[17] with human blood samples. In that study,[1,17] epinephrine and norepinephrine intensified LPS-stimulated IL-10 production, whereas propranolol, a β_1- and β_2-adrenoceptor antagonist, inhibited this intensification. Conversely, phentolamine, an α_1- and α_2-adrenoceptor antagonist, did not inhibit this intensification.[17] Furthermore, isoproterenol, a β_1- and β_2-adrenoceptor agonist, and terbutaline, a β_2-adrenoceptor agonist, promoted LPS-stimulated IL-10 production. Yet phenylephrine, an α_1-adrenoceptor agonist, mildly increased LPS-stimulated production of IL-10, and UK-14,304, an α_2-adrenoceptor agonist, did not alter production of IL-10.[17] Data from that study[17] suggest that the augmented LPS-stimulated production of IL-10 by epinephrine and norepinephrine observed in the present study may have occurred primarily through a β-adrenoceptor signaling mechanism.

In the present study, incubation of blood samples with epinephrine and norepinephrine yielded decreased concentrations of constitutive TNF-α. This is similar to what occurs in people, in which acute spikes in plasma endogenous epinephrine and norepinephrine concentrations result in decreased TNF-α production.[18] As occurred in the blood of dogs reported in the present study, epinephrine also downregulates LPS-stimulated TNF-α production in human blood.[1] In both healthy people and people with prolonged sepsis, epinephrine reduces LPS-stimulated TNF-α production in blood.[3]

The mechanism by which epinephrine and norepinephrine suppress TNF-α production is directly related to adrenoceptor stimulation. Although epinephrine and norepinephrine have α- and β-adrenoceptor effects, β-adrenoceptor–mediated effects predominate in the presence of bacteria.[19] β-Adrenoceptor activation causes a decrease in nuclear translocation of NF-κβ, which is mediated by an increase in cAMP.[20] Activation of β-adrenoceptors leads to strong immunosuppressive effects, which include decreased production of TNF-α, IL-1β, and IL-6 by cells in some species.[1,3] Additionally, epinephrine downregulates TLR4, the major TLR involved with LPS recognition.[21] Downregulation of TLR4 was not suspected to be a major mechanism of decreased TNF-α production in the present study because downregulation of TLR4 is more often a chronic effect (lasting > 24 hours) and the present study focused on the acute effects (lasting < 24 hours) of catecholamines, including epinephrine.

Epinephrine and norepinephrine had opposite effects on LPS-stimulated production of TNF-α (decreased) and IL-10 (increased). This finding was expected because β-adrenoceptor signaling in leukocytes typically increases cAMP, resulting in decreased activation and nuclear translocation of NF-κβ and decreased production of proinflammatory cytokines, including TNF-α. Conversely, alterations in IL-10 production are mediated through cAMP-induced activation of protein kinase A and not through the same alterations in downstream signaling pathways.[22] Unlike TNF-α, the 5′-regulatory region of the IL-10 gene does not have a binding site for NF-κβ.

The effects of epinephrine and norepinephrine on TNF-α production appear to vary, depending on the species. In the present study, epinephrine and norepinephrine had a dose-dependent suppressive effect on TNF-α production, although the dose-dependent effect appeared to plateau for norepinephrine at 100μM. In people, epinephrine, but not norepinephrine, acts in a similar dose-dependent manner on the production of TNF-α and TLR4 mRNA.[1,23] In macrophages obtained from the peritoneal cavity of rats, lower concentrations of epinephrine (10 ng/mL) promoted secretion of TNF-α, IL-1β, and IL-10, compared with higher concentrations (50 to 100 ng/

mL) that inhibited their secretion.[24] In mice, norepinephrine augments LPS-stimulated production of TNF-α from macrophages, possibly through the α_2-adrenoceptor.[25] Furthermore, in a human saphenous vein model of inflammation, norepinephrine inhibits LPS-stimulated TNF-α production in a dose-dependent manner.[26] Also, norepinephrine inhibits IL-10 production in a dose-dependent manner in LPS-stimulated human blood,[27] which is similar to what we observed in dog blood with higher concentrations of norepinephrine.

The finding that dobutamine did not alter constitutive or LPS-stimulated cytokine production was unexpected because dobutamine is a potent inhibitor of proinflammatory cytokine release from monocytes and is a promoter of IL-10 production, as noted in a previous study[6] performed with human blood that was stimulated with LPS ex vivo. However, dobutamine is considered a β_1-adrenoceptor agonist with mild β_2- and also α_1-adrenoceptor agonisms at therapeutic doses in vivo.[28] One hypothesis for the differences in the immunomodulatory effects of dobutamine between dogs and people is that canine cells may not have been exposed to sufficiently high concentrations of dobutamine to attain an expected response; a typical dose of dobutamine for a dog is at least 10X that for epinephrine and norepinephrine. However, a drug's concentration that effects change in a cell-culture system does not always correlate with the concentration needed to effect change in vivo. Dobutamine, epinephrine, and norepinephrine alter LPS-stimulated cytokine production at equimolar concentrations in human cell-culture models.[29] Alternatively, β_1-adrenoceptor agonism is not as involved in cytokine signaling in dogs as are β_2-adrenoceptor and α-adrenoceptor agonisms. The major cytokine signaling effects of epinephrine and norepinephrine are speculated to be mediated through β_2-adrenoceptors, which are minimally stimulated by dobutamine.

Additionally, epinephrine and norepinephrine were believed to inhibit LPS-stimulated production of IL-6 in dogs, similar to that in people.[29] However, epinephrine, norepinephrine, and dobutamine did not alter LPS-stimulated IL-6 production in the present study. This difference may be related to a species-specific immunologic response to catecholamines. Alternatively, this difference may reflect the various cells involved with IL-6 production. In human neutrophils, epinephrine induces cAMP production but without a resultant effect on LPS-stimulated production of IL-6.[29]

The present study had several limitations that could be addressed in future studies. The sample size was small, which may have resulted in an inability to detect differences in the production of cytokines secondary to dobutamine. The present study was performed in vitro and thus may not fully represent what would happen in vivo. Specific adrenoceptor-subtype antagonists were not used, and specific cell types were not identified to elucidate the specific receptors and cells involved with augmentation of cytokine production. Future studies evaluating specific receptors, signaling pathways, and cell types may improve the understanding of catecholamine-induced immunomodulation.

In conclusion, epinephrine and norepinephrine, but not dobutamine, had immunomodulatory effects on LPS-stimulated TNF-α and IL-10 production in blood from healthy dogs. Dobutamine may have limited or no immunologic effects in patients with gram-negative infections. Further study is needed to determine whether targeted selection of catecholamines to manage dogs with sepsis and hypotension could have ancillary immunomodulatory benefits.

Acknowledgments

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Footnotes

a. Gibco RPMI culture medium, Thermo Fisher Scientific, Waltham, Mass.
b. Sigma-Aldrich Co, St Louis, Mo.
c. Hospira Inc, Lake Forest, Ill.
d. BPI Labs LLC, Largo, Fla.
e. Claris Lifesciences Ltd, North Brunswick, NJ.
f. Milliplex assay for Luminex, MilliporeSigma, Billerica, Mass.
g. Milliplex analyst, version 5.1, MilliporeSigma, Billerica, Mass.
h. SigmaStat, Systat Software Inc, San Jose, Calif.

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