CENTRAL ACTIVATION OF SYMPATHETIC NEURAL CIRCUITS ALTERS SPLENIC CYTOKINE GENE EXPRESSION

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Anatomy and Physiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2006

ABSTRACT

Important bidirectional interactions exist between the central nervous system and the immune system. Neural-immune interactions provide a regulatory system in the body and disturbances in these interactions may lead to disease. Although the sympathetic nervous system is thought to play a key role in mediating neural-immune interactions, central neural mechanisms mediating sympathetic-immune interactions and the effect of centrally-induced alterations in sympathetic nerve discharge on immune function is not known. We tested the hypothesis that central activation of sympathetic neural circuits alters splenic cytokine gene expression. In a separate study, we tested the hypothesis that hypothermia-induced changes in visceral sympathetic nerve discharge (SND) would be attenuated in middle-aged and aged compared with young rats. Previous studies have demonstrated that skin sympathoexcitatory responses to skin cooling are attenuated in aged compared with young subjects, suggesting that advancing age influences sympathetic nerve responsiveness to cooling. The effect of age on sympathetic nerves innervating other targets organs during acute cooling remains unknown. Central activation of splenic SND was produced using three different experimental interventions: increased core body temperature produced by acute heating, intracerebroventricular injection of angiotensin II (ANG II), and decreased core body temperature produced by acute cooling. Changes in gene expression profiles were analyzed using inflammatory cytokine-specific gene-array and further validated using real-time RT-PCR analysis. The following observations were made. 1) Splenic SND increased in response to each experimental intervention except in acute cooled young rats where there was a decrease in splenic SND. 2) Splenic cytokine gene expression of pro-inflammatory cytokines (e.g., IL-1β, IL-6, IL-2) and chemokines (GRO1, CXCL2, CCCL2 and, CXCL10) was increased in response to each experimental intervention. 3) Expression of splenic cytokine genes was reduced after splenic-denervation except in acute cooled rats. 4) Progressive hypothermia reduced splenic, renal, and adrenal SND in rats and was generally attenuated in middle-aged and aged rats. These results demonstrate the functional significance of changes in sympathetic nerve activity on splenic immune cell activation and the effect of age on SND responses to core body cooling.

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ABSTRACT

Important bidirectional interactions exist between the central nervous system and the immune system. Neural-immune interactions provide a regulatory system in the body and disturbances in these interactions may lead to disease. Although the sympathetic nervous system is thought to play a key role in mediating neural-immune interactions, central neural mechanisms mediating sympathetic-immune interactions and the effect of centrally-induced alterations in sympathetic nerve discharge on immune function is not known. We tested the hypothesis that central activation of sympathetic neural circuits alters splenic cytokine gene expression. In a separate study, we tested the hypothesis that hypothermia-induced changes in visceral sympathetic nerve discharge (SND) would be attenuated in middle-aged and aged compared with young rats. Previous studies have demonstrated that skin sympathoexcitatory responses to skin cooling are attenuated in aged compared with young subjects, suggesting that advancing age influences sympathetic nerve responsiveness to cooling. The effect of age on sympathetic nerves innervating other targets organs during acute cooling remains unknown. Central activation of splenic SND was produced using three different experimental interventions: increased core body temperature produced by acute heating, intracerebroventricular injection of angiotensin II (ANG II), and decreased core body temperature produced by acute cooling. Changes in gene expression profiles were analyzed using inflammatory cytokine-specific gene-array and further validated using real-time RT-PCR analysis. The following observations were made. 1) Splenic SND increased in response to each experimental intervention except in acute cooled young rats where there was a decrease in splenic SND. 2) Splenic cytokine gene expression of pro-inflammatory cytokines (e.g., IL-1β, IL-6, IL-2) and chemokines (GRO1, CXCL2, CCCL2 and, CXCL10) was increased in response to each experimental intervention. 3) Expression of splenic cytokine genes was reduced after splenic-denervation except in acute cooled rats. 4) Progressive hypothermia reduced splenic, renal, and adrenal SND in rats and was generally attenuated in middle-aged and aged rats. These results demonstrate the functional significance of changes in sympathetic nerve activity on splenic immune cell activation and the effect of age on SND responses to core body cooling.

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DEDICATION

I would like to dedicate my thesis to my late grandfather and my beloved family.

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MANUSCRIPTS

1. **Ganta C**. **K**., F. Blecha, R. R. Ganta, B. G. Helwig, S. Parimi, N. Lu, R. J. Fels, T. I. Musch, and M. J. Kenney. Hyperthermia-enhanced splenic cytokine gene expression is mediated by the sympathetic nervous system. *Physiol Genomics.* 2004 Oct 4; 19(2):175-83.

2. **Ganta C**. **K**., N. Lu, F. Blecha, R. R. Ganta, B. G. Helwig, S. Parimi, L. Zheng, R. J. Fels, T. I. Musch, and M. J. Kenney. Central angiostensin II administration alters splenic cytokine gene expression. *Am J Physiol Heart Circ Physiol*. 2005 Oct; 289(4):H1683-91.

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6. **Ganta C**. **K**., F. Blecha, R. R. Ganta, B. G. Helwig, R. J. Fels, and M. J. Kenney. Effect of age on sympathetic nerve discharge (SND) and splenic cytokine gene expression responses to hypothermia. *Experimental Biology,* 20(4): 2006.

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4. Phi Zeta Research Day, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas. Central angiotensin II-enhanced splenic cytokine gene expression is mediated by the sympathetic nervous system. March, 2005.

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CHAPTER 1. HYPERTHERMIA-ENHANCED SPLENIC CYTOKINE GENE EXPRESSION IS MEDIATED BY THE SYMPATHETIC NERVOUS SYSTEM

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ABSTRACT

Whole body hyperthermia (WBH) has been used in experimental settings as an adjunct to radio-chemotherapy for the treatment of various malignant diseases. The therapeutic effect of WBH has been hypothesized to involve activation of the immune system, although the effect of hyperthermia-induced activation of sympathetic nerve discharge (SND) on splenic immune function is not known. We tested the hypothesis that heating-induced splenic sympathoexcitation would alter splenic cytokine gene expression as determined using gene array and real-time RT-PCR analyses. Experiments were performed in splenic-intact and splenic-denervated anesthetized Sprague-Dawley rats (n = 32). Splenic SND was increased during heating (internal temperature increased from 38^{o} to 41^{o}C) in splenic-intact rats but remained unchanged in nonheated splenic-intact rats. Splenic interleukin-1β (IL-1β), interleukin-6 (IL-6), and growth regulated oncogene 1 (GRO 1) mRNA expression was higher in heated than in nonheated splenic-intact rats. Splenic IL-1β, IL-6, and GRO 1 mRNA expression was reduced in heated splenic-denervated compared with heated splenic-intact rats, but did not differ between heated splenic-denervated and nonheated splenic-intact rats. These results support the hypothesis that hyperthermia-induced activation of splenic SND enhances splenic cytokine gene expression.

Key words: Whole body hyperthermia; Splenic SND; Splenc cytokine gene expression

INTRODUCTION

Whole body hyperthermia (WBH) profoundly influences sympathetic nerve regulation. Increased internal body temperature produced by whole body heating increases muscle sympathetic nerve discharge (SND) in conscious humans (10-12, 50), splanchnic SND in conscious rats (36), and renal, splanchnic, and splenic sympathetic nerve activity in anesthetized rats (22, 29-31, 33) and decreases SND directed to the caudal ventral artery in the rat (27). Moreover, moderate body warming decreases skin sympathetic nerve activity in human subjects (13). Hyperthermia-induced increases in visceral SND are attenuated during acute heating in cervical transected rats (33), supporting an important role for supraspinal neural circuits in mediating visceral sympathetic nerve responses to increased internal body temperature.

WBH has been used in experimental settings as an adjunct to radio-chemotherapy for the treatment of various malignant diseases (20, 58). The therapeutic effect of WBH in these conditions has been hypothesized to involve activation of the immune system (5, 51, 52). Robins et al. (52) reported elevated plasma levels of granulocyte-colony stimulating factor, interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8, interleukin-10, and tumor necrosis factor-α (TNF-α) when WBH was used in conjunction with chemotherapy in human patients. Atanackovic et al. (5) reported that human patients receiving WBH along with chemotherapy demonstrated immediate and significant increases in peripheral natural killer cells, $CD56^{+}$ cytotoxic T lymphocytes, and serum concentrations of IL-6 and TNF-α, changes that were not observed in patients receiving chemotherapy alone. These results support the hypothesis that WBH provides a stimulus to the immune system.

The sympathetic nervous system, along with cytokines and the hypothalamic-pituitary-adrenal axis, is thought to play an important role in mediating bidirectional neural-immune

interactions (1, 2, 14, 46). A role for the sympathetic nervous system in immune regulation is supported by the innervation of lymphoid organs (including the spleen) by sympathetic nerves (15-19) and the presence of adrenergic receptors on immune cells (8, 34, 39, 42, 48). A role for the immune system in sympathetic nerve regulation is supported by the increased rate of norepinephrine release and turnover in spleen and bone marrow following activation of antigen-specific T cells and B cells by a soluble protein antigen (35) and changes in the level of SND produced by administration of IL-1β antibody (43) and IL-1β (25, 56). As reviewed by Madden (45), complex functional interactions exist between the sympathetic nervous system and the immune system and it has been shown that the sympathetic nervous system can both enhance and inhibit immune responses, depending on experimental conditions, the type of stress paradigm used, activation state of the sympathetic nervous system, and types of immune cells activated. In addition, immune cell products can influence the functional state of the sympathetic nervous system. For example, Rogausch et al. (53, 54, 55) have demonstrated that locally produced IL-1β inhibits the release of norepinephrine from postganglionic splenic sympathetic nerves which in turn leads to an increase in splenic blood flow.

Although sympathetic nerves are considered an important component of the communication pathway between the brain and the immune system, the influence of hyperthermia-induced activation of splenic sympathetic nerve outflow on splenic immune function remains poorly defined. In the present study we determined the effect of WBH on splenic SND and splenic cytokine gene expression in urethane-chloralose anesthetized rats. Because the sympathetic innervation of the spleen provides a direct link between central sympathetic neural circuits and immunocompetent cells in the spleen (15, 19), we tested the hypothesis that hyperthermia-induced activation of splenic SND would alter splenic cytokine

gene expression as determined using gene array and real-time RT-PCR analyses. To demonstrate a role for the sympathetic nervous system in mediating splenic cytokine gene expression responses to WBH, experiments were completed in splenic-intact and splenic-denervated rats. The current study provides new findings supporting the idea that hyperthermia-induced activation of splenic SND enhances splenic cytokine gene expression.

METHODS

General procedures. The Institutional Animal Care and Use Committee approved the experimental procedures and protocols used in the present study and all procedures were performed in accordance with the American Physiological Society's guiding principles for research involving animals (3). Experiments were performed on Sprague-Dawley rats (381 ± 6 g, n = 32). Anesthesia was induced by isoflurane (3%) and maintained during surgical procedures using isoflurane (1.5%), α-chloralose (80 mg/kg, ip), and urethane (800 mg/kg, ip) (31, 32, 43). During the experimental protocol, maintenance doses of α-chloralose were infused intravenously (femoral vein, 35-45 mg/kg/hr). The trachea was cannulated with a polyethylene-240 catheter. Femoral arterial pressure was monitored using a pressure transducer connected to a blood pressure analyzer. Heart rate (HR) was derived from the pulsatile arterial pressure output of the blood pressure analyzer. Colonic temperature (Tc) was measured with a thermistor probe inserted approximately 5 cm into the colon. Tc was maintained between 37.8^{o}C and 38.0^{o}C during surgical procedures by a homeothermic blanket.

Neural recordings. Splenic sympathetic nerve activity was recorded biphasically with a platinum bipolar electrode after preamplification (bandpass 30-3,000 Hz). In splenic-denervated rats, renal sympathetic nerve activity was recorded using similar recording and preamplification

procedures. Using a lateral approach, splenic and renal sympathetic nerves were dissected free of surrounding connective tissue and isolated. Nerve-electrode preparations were covered with silicone gel. The filtered neurograms were routed to an oscilloscope and a nerve traffic analyzer for monitoring during the experiment and for subsequent data analysis. Sympathetic nerve potentials were full-wave rectified, integrated (time constant 10 ms) and quantified as volts x seconds (V·s) (29-31, 33). The level of activity in sympathetic nerves was corrected for background noise after administration of the ganglionic blocker trimethaphan camsylate (10-15 mg/kg iv).

Splenic denervation. A two-step denervation procedure was performed. Initially, the splenic bundle (including the splenic artery, vein and nerve) was visualized using a lateral approach and the splenic nerve was dissected free of surrounding connective tissue and sectioned at the base of the bundle. Subsequently in the same operation, the individual arteries projecting to the spleen were identified and the sympathetic nerve adjoining each vessel was sectioned. Denervation was considered complete when splenic nerve recordings taken at a site distal to the lesions demonstrated no sympathetic nerve activity.

Splenic blood flow determination. Catheters were placed in the right carotid artery and the femoral artery. The right carotid artery catheter was advanced towards the heart and secured in position just inside the aortic arch. The femoral artery catheter was advanced towards the descending aorta and secured in place. The carotid catheter was connected to a pressure transducer and femoral artery catheter was connected to a 1 ml syringe placed in a Harvard withdrawal pump. For each blood flow determination, blood withdrawal from the femoral artery catheter was initiated at a rate of 0.25 ml/min. At the same time, arterial blood pressure was recorded from the carotid artery catheter. After 30 seconds of blood withdrawal, the carotid

artery catheter was disconnected from the pressure transducer and radioactive microspheres were injected into the aortic arch. Labeled microspheres were 15±3 μm in diameter. The microspheres were suspended in normal saline containing 0.01% Tween 80 with a specific activity ranging from 7-15 mCi/g. Before each injection, the microspheres 6-7 x 10^5 were thoroughly mixed and agitated by sonication to prevent clumping. Microspheres were injected into the ascending aorta in a volume of approximately 0.10 ml and the different radioactive labels (^{46}Sc, ^{85}Sr, ^{113}Sn, and ^{141}Ce) were used in random order. At the end of each experiment, the rat was killed with an overdose of methohexital sodium (150 mg/kg iv). The placement of each catheter was verified by anatomical dissection.

Spleens and kidneys were removed, blotted, weighed and placed immediately into counting vials. The radioactivity of tissue samples was determined on a Packard Cobra II Auto-Gamma Spectrometer set to record the peak energy activity of each isotope for 5 min, and analyzed by computer, taking into account the cross-talk fraction between the different isotopes. Tissue blood flow was calculated by the reference sample method (26) and expressed as ml/min/100g of tissue. Adequate mixing of the microspheres was verified for each injection by demonstrating a <15% difference in blood flows to the right and left kidneys.

Experimental protocol. After completion of the surgical procedures, anesthetized rats were allowed to stabilize for 60 min. After the stabilization period, a 30 min control period was completed during which Tc was maintained at 38^{o}C in all rats. At the end of the control period, heating experiments were initiated in splenic-intact and splenic-denervated rats by increasing Tc at a rate of 0.1^{o}C/min from 38 to 41^{o}C (30 min) followed by a maintenance phase in which Tc was maintained at 41^{o}C for an additional 90 min. Increases in Tc were produced using a heat lamp (29-33). Nonheated experiments were completed in splenic-intact and splenic-denervated

rats by maintaining Tc at 38°C for an additional 120 min beyond the initial 30 min control period. Mean arterial pressure (MAP), HR, and SND were measured continuously during the control periods and the heating and nonheating protocols. Splenic blood flow (BF) measurements were completed at the end of the control period and at 30 and 120 min of the heating and nonheating protocols in nonheated splenic-intact (n = 3), heated splenic-intact (n = 5) and heated splenic-denervated (n = 4) rats.

Spleens were collected at the end of each experiment (with the exception of those used in experiments analyzing splenic blood flow) for splenic cytokine gene expression analysis and stored at -80°C. Gene array analysis was performed on spleens collected from four rats in each experimental group (nonheated splenic-intact, heated splenic-intact, and heated splenic-denervated). To validate the gene array results, TaqMan probe-based real-time RT-PCR analysis was performed on spleens used for gene array analysis (n = 4 for each group) and spleens from additional experiments in each group (nonheated splenic-intact, n = 2; heated splenic-intact, n = 3; heated splenic-denervated, n = 3).

RNA isolation. Frozen spleens were homogenized in liquid nitrogen and the total RNA was isolated using the TRI Reagent RNA isolation kit according to the manufacturer's protocol (Sigma Chemical Co., St. Louis, MO). RNA purity and concentration were determined spectrophotometrically by calculating the ratio between the absorbances at 260 nm and 280 nm. The absorbance ratio for all samples ranged between 1.8 and 2.0. The quality of RNA for all samples was confirmed by resolving them on a 1.5% formaldehyde agarose gel (47).

Gene array analysis. Splenic cytokine gene expression was evaluated using a mouse inflammatory cytokine cDNA array system from Superarray Biosciences (Bethesda, MD) as reported earlier (44). The cDNA array blot contained 23 inflammatory cytokine and chemokine

gene fragments spotted in duplicate wells. In addition, β-actin and GAPDH were included as positive controls and pUC18 DNA was included as a negative control. Biotin-labeled cDNA probes were synthesized from total RNA by reverse transcription using an RT-Labeling Kit (SuperArray Biosciences, Bethesda, MD). The labeled probes were hybridized to gene-specific cDNA fragments spotted on the gene array membranes. Membranes were washed to remove any unincorporated probe and incubated with alkaline phosphatase conjugated streptavidin (AP-streptavidin). Relative expression levels of specific genes were detected from signals generated by chemiluminescence from the alkaline phosphatase substrate, CDP-*Star*. The luminizing blots were used to expose X-ray films and quantified by spot densitometry with the aid of AlphaEase v5.5 software (Alpha Innotech, San Leandro, CA). The relative gene expression levels were estimated by comparing the signal intensity of the target gene to the signal intensity derived from β-actin.

Real-time RT-PCR analysis. To validate the gene-array results, TaqMan® probe-based real-time RT-PCR analysis was performed. Total RNA (2μg) was reverse-transcribed in a 20 μl volume containing 1 μM of oligo(dT) primers, 0.5 mM of each dNTP, 0.5 U/μl of RNase inhibitor and 0.2 U/μl of Omniscript Reverse Transcriptase (Qiagen, Valencia, CA) in RNase-free water. The reaction was carried out for 60 min at 37.0^{o}C and the cDNA mixture was used for the real-time PCR analysis of specific cytokine gene expression.

Gene-specific PCR primer pairs and a TaqMan® probe for growth regulated oncogene 1 (GRO 1) was obtained from Applied Biosystems (Foster City, CA). The primers and probes for β-actin, IL-6 and IL-1β genes were custom synthesized using published sequences (7, 38). TaqMan® probes were labeled with 6-carboxyfluorescin (FAM) as the reporter dye molecule at the 5' end and 6-carboxy-tetramethyl-rhodamine (TAMRA) as the quencher dye molecule at the 3' end. Real-time PCR reactions were performed with 2 μl of cDNA using Universal PCR

Master Mix (Applied Biosystems, Foster City, CA), containing 0.9 μM each of the forward and reverse primers and 0.25 μM TaqMan® probes in a 25 μl reaction. Real-time PCR analysis was performed in a Smart Cycler (Cepheid, Sunnyvale, CA) with the following PCR conditions: one cycle each of 50°C for 2 min and 95°C for 5 min, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 min.

The threshold cycle (Ct) value for each gene was defined as the PCR cycle at which the emitted fluorescence rose above a background level of fluorescence, i.e., 30 fluorescence units. Gene expression levels were calculated as fold change relative to the gene expression of nonheated splenic-intact rats. The PCR amplification efficiencies of β-actin and the target genes were calculated using the following formula: PCR efficiency = $(10^{(1/-S)} - 1)$ where S is the slope (21). The amplification efficiency was greater than 90% for all genes. The comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to quantify the results obtained by real time RT-PCR (41). Data were normalized by determining differences in Ct values between the target gene of interest and β-actin, defined as ΔCt (Ct of target gene – Ct of β-actin gene). The fold change was calculated as $2^{(SAvg\Delta Ct - CAvg\Delta Ct)}$ where SAvgΔCt – CAvgΔCt is the difference between the sample (heated-intact/heated-denervated/nonheated-denervated) ΔCt and the control (nonheated intact) ΔCt. For nonheated intact samples, ΔΔCt equaled zero and 2^0 equaled one, so that the fold change in gene expression relative to the nonheated intact samples equaled one. For the treated samples, evaluation of $2^{-\Delta\Delta Ct}$ was defined as the fold change in gene expression relative to nonheated intact samples.

Data and statistical analysis. Values are means ± SE. Control values of SND were taken as 0%. Statistical analysis of SND, MAP, HR and splenic BF responses were analyzed using analysis of variance techniques with a repeated-measures (ANOVA-R) design. When a

significant F-ratio was demonstrated by the ANOVA-R, the appropriate post-hoc tests (simple effects, Least Significant Difference, Bonferroni, Student-Newman-Keuls) were applied to describe significant MAP, HR, splenic SND, and BF versus time of heating interactions. Results from gene array and RT-PCR analyses in heated and nonheated rats were compared using Student's *t*-tests or Mann-Whitney tests. The overall level of statistical significance was $p < 0.05$.

RESULTS

SND, MAP and HR responses to WBH. Figure 1 shows SND traces from three representative experiments (A, *nonheated splenic-intact*; B, *heated splenic-intact*; C, *heated splenic-denervated*). Tc was maintained at 38°C during the control period in each rat. In the nonheated splenic-intact rat (A), Tc was held constant at 38°C for 120 min after control and splenic SND was unchanged from control at 30 and 120 min. In the heated splenic-intact rat (B), Tc was increased from 38 to 41°C during the first 30 min after control and was maintained at 41°C for an additional 90 min (120 min total heating time). Splenic SND was increased from control during heating at 30 and 120 min in the splenic-intact rat (B). In the heated splenic-denervated rat (C), no measurable splenic SND was detected during control or heating (30 and 120 min), although renal SND was increased during heating. Renal SND was recorded in the splenic-denervated rat to demonstrate specificity in the denervation procedure.

Figure 2 summarizes splenic SND (top), MAP (middle) and HR (bottom) responses in nonheated (n=4) and heated (n=4) splenic-intact rats and heated splenic-denervated (n=4) rats. In nonheated splenic-intact rats, Tc was held constant at 38°C for 150 min (30 min of control,

-30 to 0 min, and 120 min after control) and splenic SND, MAP and HR remained unchanged from control in these rats. In heated splenic-intact and -denervated rats, Tc was increased from 38 to 41°C during the first 30 min (0 to 30 min) after the control period (-30 to 0 min) and was maintained at 41°C for an additional 90 min (30 to 120 min). During heating, splenic SND was increased significantly from control in splenic-intact rats and was undetectable in splenic-denervated rats. MAP was increased significantly from control during the first 60 min of heating in splenic-intact rats and during the first 30 min of heating in splenic-denervated rats. HR was significantly increased from control during heating in splenic-intact and -denervated rats.

Splenic BF responses to WBH. Figure 3 summarizes splenic BF responses in nonheated splenic-intact (n=3), heated splenic-intact (n=5), and heated splenic-denervated (n=4) rats. Tc was maintained at 38°C during a 30 min preheating control period (Control) in all rats. In nonheated splenic-intact rats, Tc was held constant at 38°C for 120 min after the control period and splenic BF was unchanged from control values at 30 and 120 min following cessation of the control period. In heated splenic-intact and splenic-denervated rats, Tc was increased from 38-41°C during the first 30 min after control and was maintained at 41°C for an additional 90 min. Splenic BF in splenic-intact and splenic-denervated rats was significantly increased from preheating control values at 30 min of heating but decreased to control values in both groups of rats at 120 min of heating. Splenic BF responses during heating did not differ between splenic-intact and splenic-denervated rats.

Gene array analysis of splenic gene expression responses to WBH. Figure 4 shows splenic gene array results from three representative experiments (nonheated splenic-intact, *left*; heated splenic-intact, *middle*; heated splenic-denervated, *right*). β-actin and GAPDH served as internal control genes. Expression of IL-1β, IL-6, and GRO 1 genes was increased in the heated

splenic-intact rat (marked by broken lines) compared to the nonheated splenic-intact rat and heated splenic-denervated rat. Expression of TGF-β1 (Transforming growth factor-β1), interleukin-2 (IL-2), and interleukin-16 (IL-16) was observed in each experiment but did not consistently differ between groups.

Quantitative values relative to β-actin for splenic IL-1β, IL-6, GRO 1, TGF-β1, IL-2, and IL-16 mRNA expression in nonheated splenic-intact (n=4), heated splenic-intact (n=4), and heated splenic-denervated (n=4) rats are summarized in Figure 5. Expression of IL-1β, IL-6, and GRO 1 genes was significantly higher in heated splenic-intact compared with nonheated splenic-intact and heated splenic-denervated rats. Expression of TGF-β1, IL-2, and IL-16 genes did not differ between groups.

Real-time RT-PCR analysis of splenic gene expression responses to WBH. Real-time RT-PCR analysis was performed for four genes (β-actin, IL-1β, IL-6, and GRO 1) from three experimental groups of rats; nonheated splenic-intact, heated splenic-intact, and nonheated splenic-denervated. Representative amplification plots are shown in Figure 6. β-actin mRNA expression did not differ between groups. As indicated by the lower Ct values (a reflection of increased mRNA levels), expression of IL-1β, IL-6 and GRO 1 mRNA was higher in the heated splenic-intact rats compared to nonheated splenic-intact and heated splenic-denervated rats.

Ct values in heated splenic-intact rats for IL-1β, IL-6 and GRO 1 genes were significantly lower than those in nonheated splenic-intact and heated splenic-denervated rats (Table 1). Ct values did not differ between nonheated splenic-intact and heated splenic-denervated rats (Table 1). When the Ct values were translated to fold change, mRNA expression of IL-1β (2.3 to 4.0 fold), IL-6 (4.7 to 16.2), and GRO 1 (3.9 to 9.1 fold) was higher in heated splenic-intact rats (n=7) than in nonheated splenic-intact rats (n=6). The fold change for mRNA expression of IL-

1β (2.3 to 4.0 fold), IL-6 (2.7 to 9.4 fold), and GRO 1 (3.2 to 9.5 fold) was lower in heated splenic-denervated rats (n=7) compared to heated splenic-intact rats and did not differ between heated splenic-denervated and nonheated splenic-intact rats.

The role of the sympathetic nervous system in regulation of splenic gene expression under basal conditions was determined by comparing gene expression responses in nonheated splenic-intact (n=6) and nonheated splenic-denervated rats (n=3). Ct values for IL-1β (*nonheated splenic-intact*, 24.13 ± 0.45*; nonheated splenic-denervatedt,* 27.2±0.3) and GRO 1 (*nonheated splenic-intact*, 29.51 ± 0.53*, nonheated splenic-denervatedt,* 31.0±0.6) were significantly higher in nonheated-denervated compared with nonheated-intact rats. Ct values for β-actin (*nonheated splenic-denervated*, 19.29 ± 0.33*; nonheated splenic-intact,* 19.6±0.13) and IL-6 (*nonheated splenic-denervated*, 30.20 ± 0.44*; nonheated splenic-intact,* 31.1±0.4) did not differ between groups. When the Ct values were translated to fold change, mRNA expression of IL-1β (5.2 to 8 fold) and GRO 1 (1.4 to 2.8 fold) was lower in nonheated splenic-denervated compared with nonheated splenic-intact rats.

DISCUSSION

We present three new findings concerning the effect of WBH on sympathetic-immune interactions in anesthetized rats. First, splenic SND was increased during heating in splenic-intact rats but remained unchanged in nonheated splenic-intact rats. Second, splenic IL-1β, IL-6 and GRO 1 mRNA expression was higher in heated than in nonheated splenic-intact rats. Third, splenic IL-1β, IL-6, and GRO 1 mRNA expression was significantly less in heated splenic-denervated rats than in heated splenic-intact rats, but did not differ between heated splenic-denervated and nonheated splenic-intact rats. The observed differences in splenic cytokine gene

expression to WBH between splenic-intact and splenic-denervated rats were not dependent on differences in splenic blood flow during heating in these groups of rats. These results support the hypothesis that hyperthermia-induced activation of splenic SND enhances splenic cytokine gene expression.

Changing the level of efferent sympathetic nerve activity is a primary strategy used by mammals to respond to acute physical stress. WBH is an acute stressor that substantially changes the level of activity in efferent sympathetic nerves (10-13, 22, 27, 29-31, 33, 36, 50). Hyperthermia-induced changes in sympathetic outflow alter blood flow distribution profiles to enhance heat dissipation while maintaining arterial blood pressure and vital organ perfusion pressure. In addition to the thermoregulatory and cardiovascular consequences of heating-induced sympathoexcitation, the current results support a role for the sympathetic nervous system in immune regulation as demonstrated by the fact that the enhanced expression of splenic cytokine and chemokine genes in response to WBH was abrogated by splenic denervation.

Because the sympathetic nervous system plays an important role in mediating cardiovascular responses to heating (30, 32, 37), differences in splenic gene expression to heating in splenic-intact and splenic-denervated rats may have resulted from altered splenic blood flow responses to heating after splenic denervation. However, this was not the case in the present study as splenic blood flow responses to heating did not differ between splenic-intact and splenic-denervated rats. As demonstrated in a series of studies by Rogausch et al. (53, 54, 55), splenic blood flow regulation in the rat involves complex mechanisms. In this regard, the current study provides little insight concerning the role of the sympathetic nervous system in modulating splenic blood flow responses to WBH, however, the primary focus of the blood flow studies was not to discern mechanisms regulating splenic blood flow responses to heating. Rather, we

wanted to assess whether heating-induced changes in splenic gene expression could be ascribed to substantial changes in blood flow to this organ following splenic sympathetic denervation.

WBH has been used in experimental settings as an adjunct to cytotoxic therapy in the treatment of various malignant diseases and it is hypothesized that the beneficial effect of WBH in these conditions may relate to heating-induced expression of cytokines (23, 28, 52). The current finding that hyperthermia-induced increases in splenic IL-1β, IL-6 and GRO 1 mRNA expression were abrogated in splenic-denervated rats suggests that splenic sympathoexcitation to acute heating may contribute to the induction of cytokines observed during chemotherapy plus WBH protocols. Consistent with this idea, Thyagarajan et al. (60) reported reduced splenic norepinephrine concentrations, splenic IL-2 and interferon-gamma levels, and hypothalamic dopaminergic activity in saline-treated, mammary tumor-bearing rats. In contrast, administration of L-deprenyl (a monoamine oxidase inhibitor) to rats with mammary tumors was associated with tumor regression, enhanced splenic production of IL-2, interferon-gamma, and natural killer cell activity, increased splenic norepinephrine concentrations, and increased hypothalamic dopaminergic activity. These findings demonstrate interactions between the sympathetic nervous system and splenic immune function and suggest that increased central and peripheral catecholaminergic activity produced by deprenyl administration may enhance anti-tumor immunity (60).

The physiological relevance of the enhanced expression of splenic IL-1β, IL-6, and GRO 1 genes to WBH is presently unknown, although numerous functional effects of these molecules have been established. For example, IL-1β is a proinfammatory cytokine that induces lymphocyte proliferation, fever, and production of other cytokines (6, 62). IL-6 is a multifunctional cytokine that is considered a factor in hematopoietic colony stimulation (63),

cytolytic T lymphocyte differentiation (49, 59), B cell differentiation (24), and T cell activation (24). In addition, IL-6 is involved in the acute phase reaction (9, 40) and hematopoiesis (61) and has been shown to inhibit the growth and metastasis of autologous human cancers (59) and inhibit the growth of carcinoma cell lines (57). GRO 1 is a growth-related oncogene and melanoma growth stimulatory factor and expression of the *GRO* family of genes is important in IL-1 induced inflammatory responses in fibroblasts (4).

The current results are applicable to splenic tissue only and application to other lymphoid or secondary lymphoid organs remains to be established. The current study used genomic level of analyses and may not represent the influence of WBH on splenic protein expression. The present results provide insight concerning the role of splenic sympathetic nerves in splenic immune regulation in response to a specific experimental intervention (WBH) using an in vivo preparation and cannot necessarily be applied to other experimental interventions or preparations. However, within the constraints of the current experimental protocols and analyses, the present results strongly support a role for splenic sympathetic nerves in increasing the expression of selective splenic cytokine genes in response to WBH.

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Table 1. Threshold cycle (Ct) values for β-actin, IL-1β, IL-6, and GRO 1 genes from nonheated splenic-intact, heated splenic-intact, and heated splenic-denervated rats.

Genes	Nonheated Splenic-intact	Heated Splenic-intact	Heated Splenic-denervated
β-actin	19.29 ± 0.33	19.40 ± 0.31	19.66 ± 0.22
IL-1β	24.13 ± 0.45	22.34 ± 0.31*	24.45 ± 0.43
IL-6	30.20 ± 0.44	27.18 ± 1.07*	29.77 ± 0.37
GRO 1	29.51 ± 0.53	27.04 ± 0.67*	29.76 ± 0.76

Values are means ± SE. nonheated intact (n=6), heated splenic-intact (n=7), heated splenic-denervated (n=7). * Significantly different from nonheated splenic-intact and heated splenic-denervated rats.

Figure 1

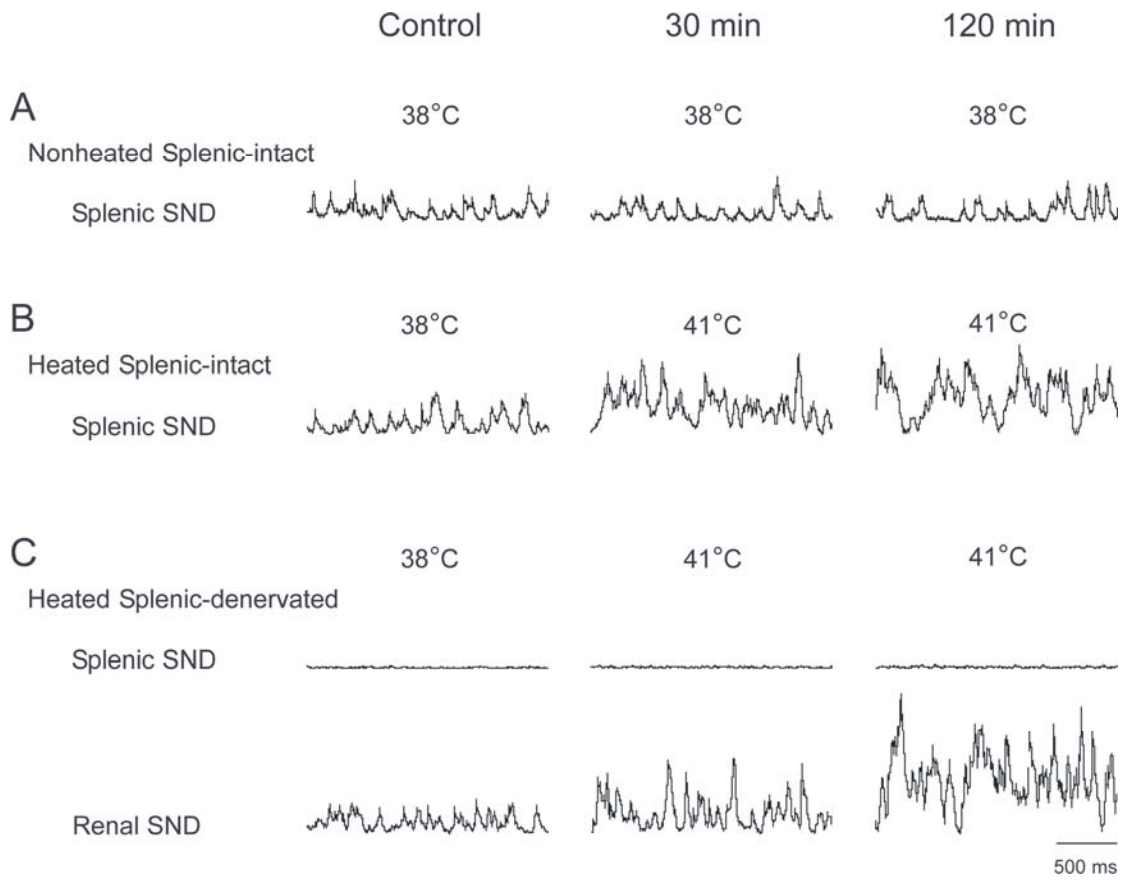

Figure 1. *(A)* Traces of splenic sympathetic nerve discharge (SND) recorded during control and at 30 min and 120 min after cessation of control in a nonheated rat with intact splenic nerves whose colonic temperature was maintained at 38°C. *(B)* Traces of splenic SND recorded before (Control) and during (30 min and 120 min) whole body heating that increased Tc from 38°C to 41°C in a rat with intact splenic nerves. *(C)* Traces of splenic and renal SND recorded before (Control) and during (30 min and 120 min) whole body heating that increased Tc from 38°C to 41°C in a splenic-denervated rat. Horizontal calibration is 500 ms.

Figure 2

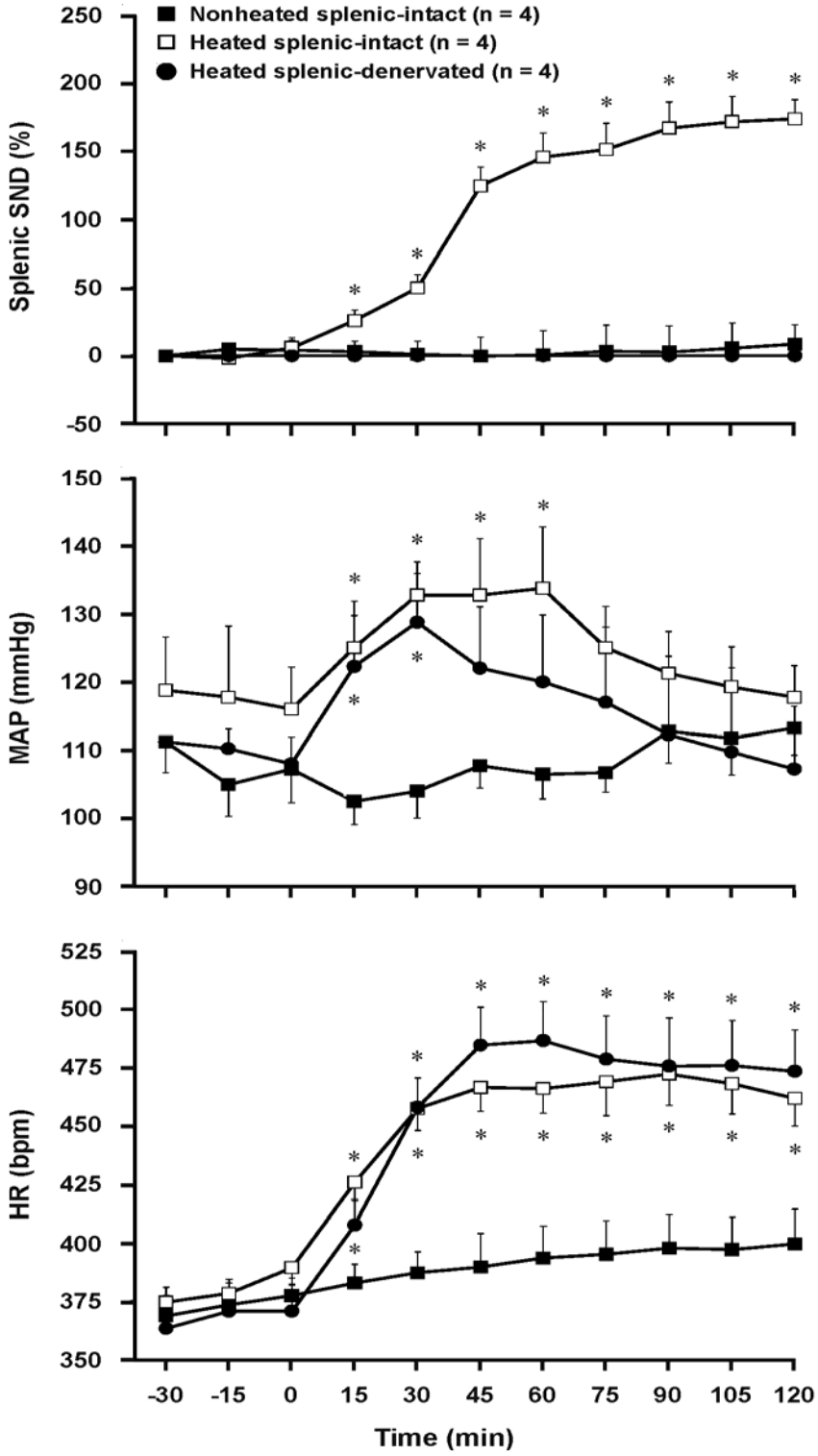

Figure 2. Splenic sympathetic nerve discharge (SND), mean arterial pressure (MAP), and heart rate (HR) measurements in nonheated splenic-intact (filled squares), heated splenic-intact (open squares), and heated splenic-denervated (filled circles) rats. Each group completed a 30 min control (-30 to 0 min) during which colonic temperature was held at 38°C. Colonic temperature

was maintained at 38°C for 120 min after cessation of control in nonheated splenic-intact rats. Colonic temperature was increased from 38°C to 41°C during the first 30 min after cessation of control and maintained at 41°C for an additional 90 min (120 min total heating time) in heated splenic-intact rats and heated splenic-denervated rats. *Significantly different from control values.

Figure 3

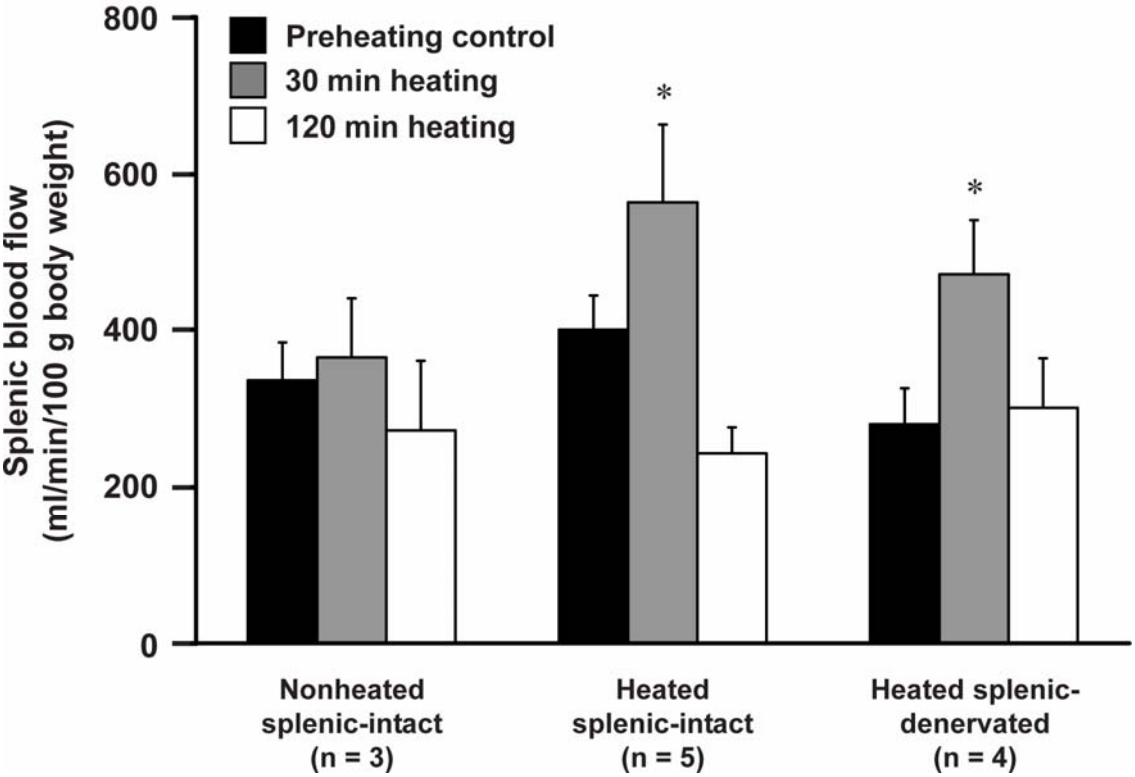

Figure 3. Splenic blood flow measurements during control and at 30 and 120 min after cessation of control in: nonheated splenic-intact rats in which colonic temperature was maintained at 38ºC during and for 120 min after the control period, heated splenic-intact rats in which colonic temperature was increased from 38ºC to 41ºC during the first 30 min after control and was maintained at 41ºC for an additional 90 min (120 min total heating time), and heated splenic-denervated rats that completed a similar heating protocol as described for heated splenic-intact rats. * Significantly different from preheating control for heated splenic-intact and heated splenic-denervated rats.

Figure 4

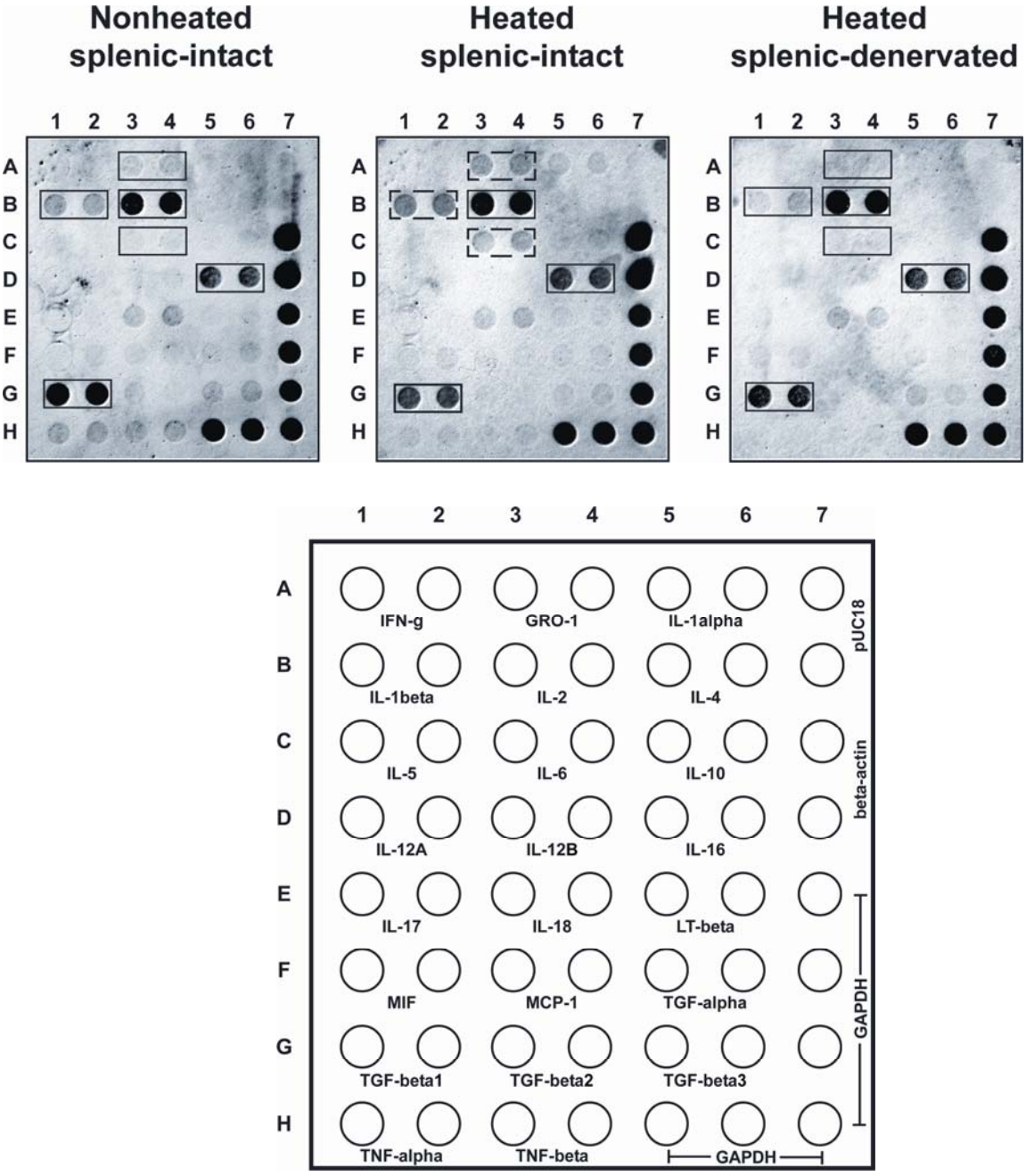

Figure 4. Gene array analysis for inflammatory cytokine gene expression. Gene array analysis was performed on splenic mRNAs using cDNA array blots containing 23 inflammatory cytokines, chemokines, and housekeeping genes. Array results for nonheated and heated splenic-intact rats and heated splenic-denervated rats are shown. β-actin and GAPDH served as controls for normalizing mRNA for each experimental group. Genes for which detectable changes in the mRNA expression levels were observed are shown as boxed wells. The broken-lined boxes in mRNA analyzed from the heated splenic-intact rat indicate the increase in the detected mRNA levels compared to the nonheated splenic-intact rat and the heated splenic-

denervated rat (shown as the solid-lined boxes). A grid identifying each of the 23 inflammatory cytokines, chemokines, and housekeeping genes is shown below the individual arrays.

Figure 5

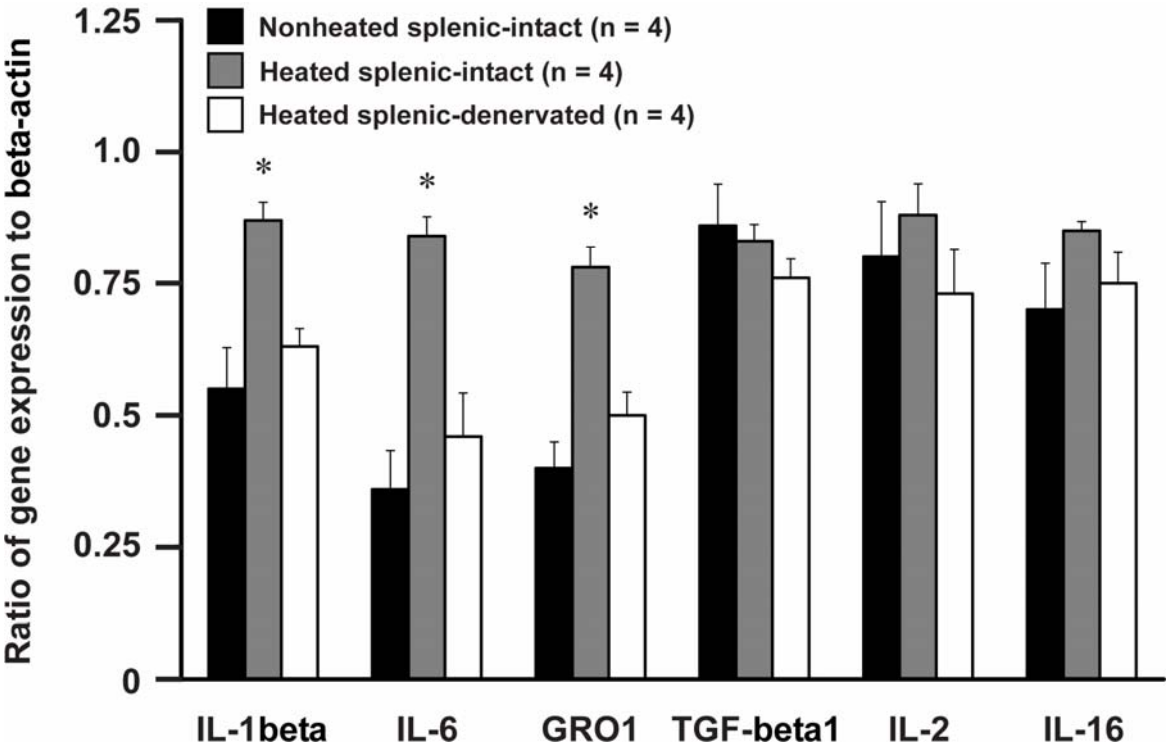

Figure 5. Quantitative values of mRNA expression levels of IL-1β, IL-6, GRO 1, TGF-β1, IL-2, and IL-16 were determined in nonheated splenic-intact (black bars), heated splenic-intact (gray bars) and heated splenic-denervated (white bars) rats. Gene expression levels are presented relative to β-actin mRNA expression. *Heated splenic-intact rats significantly different from nonheated splenic-intact and heated splenic-denervated rats.

Figure 6

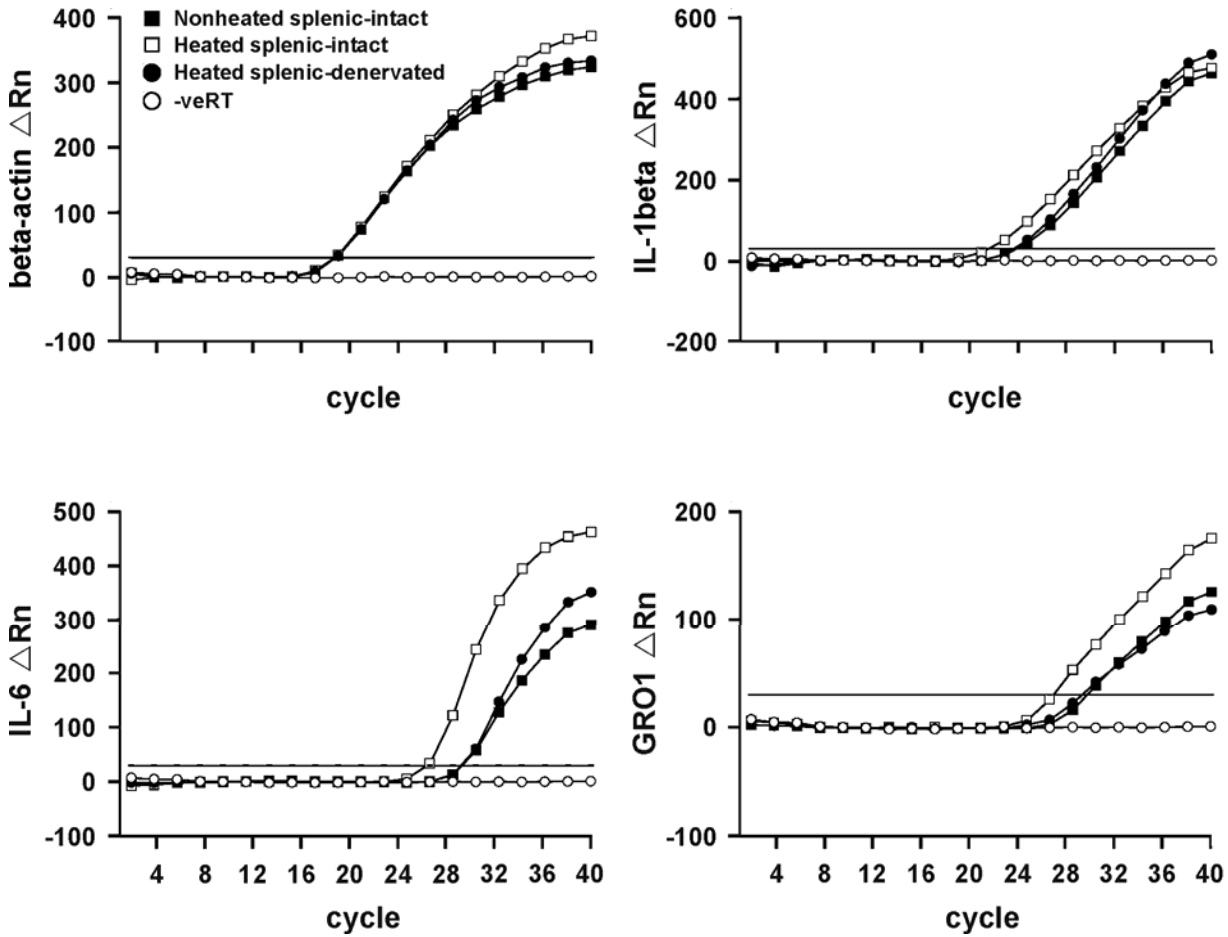

Figure 6. Real time RT-PCR analysis was performed for β-actin, IL-1β, IL-6, and GRO 1 and the amplification plots of representative experiments from three groups of rats (nonheated splenic-intact; heated splenic-intact; heated splenic-denervated) are shown. A negative RT control (-veRT) for each primer set is also presented.

CHAPTER 2. CENTRAL ANGIOTENSIN II-ENHANCED SPLENIC CYTOKINE GENE EXPRESSION IS MEDIATED BY THE SYMPATHETIC NERVOUS SYSTEM

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ABSTRACT

We tested the hypothesis that central angiotensin II (Ang II) administration would activate splenic sympathetic nerve discharge (SND) which in turn would alter splenic cytokine gene expression. Experiments were completed in sinoaortic-denervated, urethane-chloralose anesthetized, splenic nerve-intact and splenic nerve-denervated, Sprague-Dawley rats. Splenic cytokine gene expression was determined using genearray and real-time RT-PCR analyses. Splenic SND was significantly increased after intracerebroventricular (icv) administration of Ang II (150 ng/kg, 10 μl), but not artificial cerebrospinal fluid (aCSF). Splenic mRNA expression of IL-1β, IL-6, IL-2, and IL-16 genes was increased in Ang II-treated splenic-intact rats compared with aCSF-treated splenic-intact rats. Splenic IL-1β, IL-2, and IL-6 gene expression responses to Ang II were significantly reduced in splenic-denervated compared with splenic-intact rats. Splenic gene expression responses did not differ significantly in Ang II-treated splenic-denervated and aCSF-treated splenic-intact rats. Splenic blood flow responses to icv Ang II administration did not differ between splenic-intact and splenic-denervated rats. These results provide experimental support for the hypothesis that Ang II modulates the immune system through activation of splenic SND, suggesting a novel relationship between Ang II, efferent sympathetic nerve outflow, and splenic cytokine gene expression.

Key words: Angiotensin II; splenic sympathetic nerve discharge; splenic cytokine gene expression

INTRODUCTION

Bidirectional communication pathways exist between the central nervous system and the immune system (1, 2), and the sympathetic nervous system is considered an important component of the efferent arm mediating interactions between these systems (12). For example, the sympathetic innervation of the spleen provides a direct link between central sympathetic neural circuits and immunocompetent cells in the spleen (13,14), and changes in the level of efferent splenic sympathetic nerve discharge (SND) can alter immune responses in this organ (17).

Angiotensin II (Ang II) is an octapeptide that is known to be involved in central regulation of cardiovascular function and sympathetic nerve outflow (4, 8, 37, 48, 54, 57). Ang II type-1 (AT_1) receptors are found in areas of the brain associated with cardiovascular and autonomic regulation (39), intracerebroventricular (icv) administration of Ang II increases renal and splenic SND (8, 54), and microinjection of Ang II into the rostral ventral lateral medulla (4, 48), paraventricular nucleus of the hypothalamus (57), and pontine A5 region (37) increases renal SND. Central Ang II influences a diverse array of physiological responses (11, 22, 26, 27, 53), including immune responses to central administration of lipopolysaccharide (49). ICV administration of an angiotensin converting enzyme inhibitor or an AT_1 receptor antagonist attenuates brain IL-1β and febrile responses to icv administration of lipopolysaccharide (49), suggesting a link between brain Ang II and the immune system. However, the influence of Ang II-induced activation of splenic SND on splenic immune function remains poorly defined.

In the present study we determined the effect of icv (lateral ventricle) administration of Ang II on splenic SND and splenic cytokine gene expression in urethane-chloralose anesthetized rats. Because the sympathetic innervation of the spleen provides a direct link between central sympathetic neural circuits and splenic immune cells (13, 14), we hypothesized that central Ang

II administration would activate splenic SND which in turn would alter splenic cytokine gene expression as determined using genearray and real-time RT-PCR analyses. Experiments were completed in splenic nerve-intact (splenic-intact) and splenic nerve-denervated (splenic-denervated) rats.

METHODS

General procedures. The Institutional Animal Care and Use Committee approved the experimental procedures and protocols used in the present study and all procedures were performed in accordance with the American Physiological Society's guiding principles for research involving animals (3). Experiments were performed on Sprague-Dawley rats (368±5 g, n=50) anesthetized with isoflurane (during surgical procedures only; 3% induction followed by 1.5%-2.5%), α-chloralose (initial dose 80 mg/kg ip, maintenance dose of 35-45 mg/kg/hr iv), and urethane (800 mg/kg ip). The trachea was cannulated with a polyethylene-240 catheter and femoral arterial pressure was monitored using a pressure transducer connected to a blood pressure analyzer. Colonic temperature was maintained between 37.8°C and 38.0°C by a homeothermic blanket.

Arterial baroreceptors were denervated by cutting the superior laryngeal nerve near its junction with the vagus nerve, removing the superior cervical ganglion, and removing the adventitia from the carotid sinus bifurcation (31). Sinoaortic denervation was considered complete by demonstrating loss of coherence between the arterial pulse and SND (23, 29). Studies were completed in sinoaortic-denervated rats to eliminate the influence of baroreceptor afferent feedback mechanisms that may attenuate SND responses of central origin.

A lateral ventricular cannula was surgically implanted after placing the rat in a stereotaxic frame, leveling the head between lambda and bregma, and making a small hole in the skull (1.2-1.4 mm lateral to the midline and 0.8-1.0 mm posterior to bregma). A stainless steel guide cannula (22 gauge) was lowered 4 mm below the surface of the skull and an injector was introduced through the guide cannula to protrude 0.5 mm beyond the tip of the guide cannula.

Neural recordings. Splenic SND was recorded biphasically with a platinum bipolar electrode after preamplification (bandpass 30-3,000 Hz). In splenic-denervated rats, renal SND was recorded using similar recording and preamplification procedures. Splenic and renal sympathetic nerves were isolated from a lateral approach. For monitoring during the experiment and for subsequent data analysis, the filtered neurograms were routed to an oscilloscope and a nerve traffic analyzer. Sympathetic nerve potentials were full-wave rectified, integrated (time constant 10 ms) and quantified as volts x seconds (V·s) (17, 29). SND was corrected for background noise after administration of the ganglionic blocker, trimethaphan camsylate (10-15 mg/kg iv).

Splenic denervation. A two step splenic denervation procedure was performed. Initially, the splenic bundle (including splenic artery, vein and nerve) was visualized and the splenic nerve was dissected free of surrounding connective tissue and sectioned at the base of the bundle. Subsequently, the individual arteries projecting to the spleen were identified and the sympathetic nerve adjoining each vessel was sectioned. Denervation was considered complete when splenic nerve recordings completed after denervation demonstrated no sympathetic nerve activity.

Splenic and renal blood flow determination using microspheres. Catheters were placed in the right carotid artery and the femoral artery. The right carotid artery catheter was advanced

towards the heart and secured in position just inside the aortic arch. The femoral artery catheter was advanced towards the descending aorta and secured in place. The carotid catheter was connected to a pressure transducer and the femoral artery catheter was connected to a 1 ml syringe placed in a Harvard withdrawal pump. For each blood flow determination, blood withdrawal from the femoral artery catheter was initiated at a rate of 0.25 ml/min. At the same time, arterial blood pressure was recorded from the carotid artery catheter. After 30 seconds of blood withdrawal, the carotid artery catheter was disconnected from the pressure transducer and radioactive microspheres were injected into the aortic arch. Labeled microspheres were 15±3 μm in diameter. The microspheres were suspended in normal saline containing 0.01% Tween 80 with a specific activity ranging from 7-15 mCi/g. Before each injection, the microspheres, 6-7 x 10^5 were thoroughly mixed and agitated by sonication. Microspheres were injected into the ascending aorta in a volume of approximately 0.10 ml and the different radioactive labels (^{46}Sc, ^{85}Sr, and ^{141}Ce) were used in random order.

Spleens and kidneys were removed, blotted, weighed and placed immediately into counting vials. The radioactivity of tissue samples was determined on a Packard Cobra II Auto-Gamma Spectrometer set to record the peak energy activity of each isotope for 5 min, and analyzed by computer, taking into account the cross-talk fraction between the different isotopes. Tissue blood flow was calculated by the reference sample method (25) and expressed as ml/min/100g of tissue. Adequate mixing of the microspheres was verified for each injection by demonstrating no significant difference in blood flows to the right and left kidneys.

Determination of splenic artery blood velocity. A Doppler flow probe filled with ultrasonic transmission gel was placed on the splenic artery for measurement of splenic blood flow velocity (30). The flow probe wires were connected to a pulsed Doppler flowmeter. Details of the

Doppler technique, including the reliability of the method for estimation of velocity have been described previously (21). Blood velocity (in kHz Doppler shift) is directly proportional to absolute blood flow, therefore the Doppler technique provides a relative measure of changes in flow (21).

Central administration of Ang II and artificial cerebrospinal fluid (aCSF). Lateral ventricle infusions were completed using an injector that was connected via polyethylene tubing to a 100 µl microsyringe driven by a micropump (1 µl/min, 10 min). Three experimental groups were completed; rats with intact splenic nerves that received icv infusions of aCSF (aCSF-treated, splenic-intact), rats with intact splenic nerves that received icv infusions of Ang II (Ang II-treated, splenic-Intact), and splenic-denervated rats that received icv infusions of Ang II (Ang II-treated, splenic-denervated). After completion of the lateral ventricular cannulation and nerve-electrode preparations, animals were allowed to stabilize for 60 min. Measurements of SND, mean arterial pressure (MAP) and heart rate completed at the end of this stabilization period were considered as control data (-10 min in Figure 2). Following collection of control data, rats were treated with icv infusions (10 min) of aCSF (10 µl) or Ang II (150 ng/kg, 10 µl) and SND, MAP and heart rate recordings were made continuously for 60 min after cessation of icv infusions. At the end of each experiment, spleens were collected (with the exception of those used in experiments analyzing splenic blood flow) for splenic cytokine gene expression analysis and stored at -80^{o}C. Gene array analysis was performed on spleens collected from aCSF-treated splenic-intact (n=3), Ang II-treated splenic-intact (n=5), and Ang II-treated splenic-denervated (n=3) rats. TaqMan probe-based real-time RT-PCR analysis was performed on spleens used for gene array analysis and spleens from additional experiments in each group (aCSF-treated splenic-intact, n=3; Ang II-treated splenic-intact, n=3; Ang II-treated splenic-denervated, n=5).

RNA isolation. Frozen spleens were homogenized in liquid nitrogen and total RNA was isolated using the TRI Reagent RNA isolation kit according to the manufacturer's protocol (Sigma Chemical Co., St. Louis, MO). RNA purity and concentration were determined spectrophoto-metrically by calculating the ratio between the absorbance at 260 nm and 280 nm using a NanoDrop ND-1000 (NanoDrop, USA). The absorbance ratio for all samples ranged between 1.8 and 2.0. The quality of RNA for all samples was confirmed by resolving them on a 1.5% formaldehyde agarose gel (38).

GeneArray analysis. Splenic cytokine gene expression was evaluated using a mouse inflammatory cytokine cDNA array system from Superarray Biosciences (Bethesda, MD) similar to a study reported earlier (17). The cDNA array blot contained 23 inflammatory cytokine and chemokine gene fragments (interleukin -1α, -1β, -2, -4, -5, -6, -10, -12α, -12β, -16, -17, -18; transforming growth factor -α, -β1, -β2, -β3; interferon-γ; growth regulated oncogene 1; lymphocyte toxin-β; macrophage inhibitory factor -1; monocyte chemoattractant protein -1; tumor necrosis factor-α, -β) spotted in duplicate wells. In addition, β-actin and GAPDH were included as positive controls and pUC18 DNA was included as a negative control. Biotin-labeled cDNA probes were synthesized from total RNA by reverse transcription using an RT-Labeling Kit (SuperArray Biosciences, Bethesda, MD). The labeled probes were hybridized to gene-specific cDNA fragments spotted on the genearray membranes. Membranes were washed to remove any unincorporated probe and incubated with alkaline phosphatase conjugated streptavidin (AP-streptavidin). Relative expression levels of specific genes were detected from chemiluminescence signals generated after the addition of alkaline phosphatase substrate, CDP-*Star*. The luminescent blots were used to expose X-ray films and the signal intensity of each spot was quantified by spot densitometry with the aid of AlphaEase v5.5 software (Alpha Innotech,

San Leandro, CA). The relative gene expression levels were estimated by comparing the spot density of the target gene to the spot density derived from β-actin.

Real-time RT-PCR analysis. To validate the genearray results, TaqMan® probe-based real-time RT-PCR analysis was performed for a subset of genes. Total RNA (2 μg) was reverse-transcribed in a 20 μl volume containing 1 μM of oligo(dT) primers, 0.5 mM of each dNTP, 0.5 U/μl of RNase inhibitor, and 0.2 U/μl of Omniscript Reverse Transcriptase (Qiagen, Valencia, CA) in RNase-free water. The reaction was carried out for 60 min at 37°C and the cDNA mixture was used for real-time PCR analysis.

Gene-specific PCR primers and TaqMan® probe for TGF -β1 were obtained from Applied Biosystems (Foster City, CA). The primers and probes for β-actin, IL-1β, IL-6 and IL-2 genes were custom synthesized using published sequences (6, 32) and the primers and probe for IL-16 were designed using the primer quest software (IDN technologies, Coralville, IA) with sense primer 5'AAATGGACACTGCCAATGG
TGCTC3', anti-sense primer 5'AAAGGAGCTGATTCTCTGCCGGAT3', and probe 5' AAGTCAGCAGATGGCAGC ACTGTGAA3'. TaqMan® probes were labeled with 6-carboxyfluorescin (FAM) as the reporter dye molecule at the 5' end and 6-carboxytetramethyl-rhodamine (TAMRA) as the quencher dye molecule at the 3' end. Real-time PCR reactions were performed with 2 μl of cDNA using Universal PCR Master Mix (Applied Biosystems, Foster City, CA), containing 0.9 μM each of the forward and reverse primers and 0.25 μM TaqMan® probes in a 25 μl reaction. Real-time PCR analysis was performed in a Smart Cycler (Cepheid, Sunnyvale, CA) with the following PCR conditions: one cycle each of 50°C for 2 min and 95°C for 5 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. For IL-16 the real-time

PCR reaction was performed with 2 µl of cDNA using core reagent kit (Applied Biosystems, Foster City, CA) and the PCR conditions include one cycle each of 50°C for 2 min and 95°C for 5 min, followed by 45 cycles of 95°C for 30 s 58°C for 30 s and 72°C for 30 s.

The threshold cycle (C_T) value for each gene was defined as the PCR cycle at which the emitted fluorescence rose above a background level of fluorescence, i.e., 30 fluorescence units. Expression levels were calculated as fold change relative to the gene expression of aCSF-treated splenic-intact rats. The PCR amplification efficiencies of β-actin and the target genes were calculated using the following formula: PCR efficiency = $10^{(1/-S)} - 1$ where S is the slope (19). The amplification efficiency was estimated to be greater than 90% for all genes. The comparative C_T method ($2^{-\Delta\Delta C_T}$) was used to quantify the results obtained by real time RT-PCR (35). Data were normalized by determining differences in C_T values between the target gene of interest and β-actin, defined as ΔC_T (C_T of target gene – C_T of β-actin gene). The fold change was calculated as $2^{(SAvg\Delta C_T - CAvg\Delta C_T)}$ where $SAvg\Delta C_T - CAvg\Delta C_T$ is the difference between the sample ΔC_T (Ang II-treated splenic-intact or Ang II-treated splenic-denervated) and the control ΔC_T (aCSF-treated splenic-intact). For aCSF-treated splenic-intact samples, $\Delta\Delta C_T$ equaled zero and 2^0 equaled one, so that the fold change in gene expression relative to the aCSF-treated splenic-intact samples equaled one. For the treated samples, evaluation of $2^{-\Delta\Delta C_T}$ was defined as fold change in gene expression relative to aCSF-treated samples.

Brain histology. At the end of each experiment fluorescent latex microspheres (50 nm diameter) were injected into the lateral ventricle, rats received an overdose of methohexital sodium (150 mg/kg, iv), and were transcardially perfused with 0.15 M NaCl (containing 3 IU/ml heparin) followed by a fixative solution consisting of 10% buffered neutral formalin (pH 7.4). Brains were removed, blocked, post-fixed in buffered neutral formalin, and placed in 20%

sucrose for cryoprotection. Brains were frozen sectioned at 40 μm in the coronal plane, collected into phosphate buffered saline, and mounted on slides in serial sequence. The sections were rinsed in distilled water, air dried, and cleared in xylenes. Lateral ventricular injection sites were confirmed by observing fluorescent microspheres in the ventricular system using brightfield or epifluoresence.

Data and statistical analysis. Values are means ± SE. Splenic SND data are expressed as percentage change from baseline. SND, MAP and HR responses were analyzed using analysis of variance techniques with a repeated-measures design followed by Bonferroni post hoc tests. Results from genearray and RT-PCR analyses in aCSF-treated splenic-intact rats, Ang II-treated splenic-intact rats, and Ang II-treated splenic-denervated rats were compared using Student's *t*-tests or Mann-Whitney tests. The overall level of statistical significance was $p<0.05$.

RESULTS

SND, MAP and heart rate responses to icv Ang II or aCSF infusion. Figure 7 shows SND traces recorded before (Control), immediately after and 60 min after icv Ang II infusion in a rat with intact splenic nerves (A) and in a splenic-denervated (B) rat. Splenic SND was increased from pretreatment levels immediately and 60 min after icv Ang II infusion in the rat with intact splenic nerves. No measurable splenic nerve activity was detected in the splenic-denervated rat, although, renal SND was increased after icv Ang II infusion (B). Renal SND was recorded in splenic-denervated rats to demonstrate specificity in the denervation procedure.

Figure 8 summarizes splenic SND, MAP, and heart rate responses in aCSF-treated splenic-intact (n=6), Ang II-treated splenic-intact (n=8), and Ang II-treated splenic-denervated (n=8) rats. Splenic SND, MAP, and heart rate remained unchanged from pretreatment levels (-10 min)

during (0 to 10 min) and after (10 to 70 min) icv aCSF infusion in splenic-intact rats. In Ang II-treated splenic-intact rats, splenic SND, MAP and heart rate were increased significantly from pretreatment levels during and after icv Ang II infusion. In Ang II-treated splenic-denervated rats, no measurable splenic SND was recorded and MAP and heart rate were increased significantly from pretreatment levels during and after icv Ang II infusion. Splenic SND was significantly higher in splenic-intact rats after Ang II infusion compared to after aCSF infusion. MAP did not differ between Ang II-treated splenic-intact and Ang II-treated splenic-denervated rats before, during, or after Ang II infusion. MAP was significantly higher in splenic-intact and splenic-denervated rats during and after Ang II infusion compared to splenic-intact rats during and after aCSF infusion.

Genearray analysis of splenic gene expression responses to icv Ang II or aCSF infusion. Genearray analysis was completed in aCSF-treated splenic-intact (n=3), Ang II-treated splenic-intact (n=5), and Ang II-treated splenic-denervated (n=3) rats. Among the 23 cytokine genes, transcripts for IL-1β, IL-2, IL-6, IL-16, and TGF-β1 were consistently detected. Expression of IL-1β, IL-2, and IL-16 genes was significantly higher in Ang II-treated splenic-intact rats compared to aCSF-treated splenic-intact and Ang II-treated splenic-denervated rats (Figure 9). IL-6 mRNA expression levels were significantly higher in Ang II-treated splenic-intact rats compared to Ang II-treated splenic-denervated rats but did not differ between Ang II-treated splenic-intact rats and aCSF-treated splenic-intact rats (Figure 3). Expression of TGF-β1 did not differ significantly between groups (Figure 3).

Real-time RT-PCR analysis of splenic gene expression responses to icv Ang II or aCSF infusion. Real-time RT-PCR analysis was performed to verify the genearray data and representative amplification plots are shown in Figure 10. As indicated by the lower C_T values

(a reflection of increased mRNA levels), expression of IL-1β, IL-2, IL-6, and IL-16 mRNA was higher in the Ang II-treated splenic-intact rat compared to the aCSF-treated splenic-intact and the Ang II-treated splenic-denervated rat. TGF-β1 and β-actin mRNA expression did not differ between groups.

Table 2 summarizes mean absolute C_T values for β-actin and mean corrected C_T (ΔC_T) values for 5 target genes from three experimental groups. ΔC_T values for IL-1β, IL-6 and IL-2 genes were significantly lower in Ang II-treated splenic-intact rats (n=8) compared with aCSF-treated splenic-intact (n=6) and Ang II-treated splenic-denervated (n=8) rats (Table 2). The ΔC_T value for IL-16 was significantly lower in Ang II-treated splenic-intact rats compared to aCSF-treated splenic-intact rats, but did not differ between Ang II-treated splenic-intact and Ang II-treated splenic-denervated rats (Table 2). ΔC_T values for TGF-β1 did not differ between groups (Table 2). When the C_T values were translated to fold change, mRNA expression of IL-1β (2 to 11.3 fold), IL-6 (2.1 to 4.7 fold), IL-2 (1.6 to 9.3 fold), and IL-16 (1.4 to 3.2 fold) was higher in Ang II-treated splenic-intact rats compared to aCSF-treated splenic-intact rats. The fold change for mRNA expression of IL-1β (1.7 to 9 fold), IL-6 (3.2 to 7.3 fold), and IL-2 (1.4 to 8.4 fold), but not IL-16, was lower in Ang II-treated splenic-denervated rats compared to Ang II-treated splenic-intact rats, but did not differ between Ang II-treated splenic-denervated and aCSF-treated splenic-intact rats.

The role of the sympathetic nervous system in regulation of splenic gene expression under basal conditions (no icv Ang II administration) was determined by comparing gene expression responses in aCSF-treated splenic-intact rats (n=6) and aCSF-treated splenic-denervated rats (n=3) using real-time RT-PCR. Mean absolute C_T values for β-actin (aCSF splenic-intact, 15.87±0.17; aCSF splenic-denervated, 15.44±0.31) and mean corrected C_T values for IL-1β (aCSF splenic-intact, 7.76±0.87; aCSF splenic-denervated, 8.72±0.14), IL-6 (aCSF splenic-intact, 11.46±0.24; aCSF

splenic-denervated, 11.86±0.45), IL-2 (aCSF splenic-intact, 14.87±0.3; aCSF splenic-denervated, 14.13±0.04), IL-16 (aCSF splenic-intact, 6.4±0.31; aCSF splenic-denervated, 7.39±0.36) and TGF-β1 (aCSF splenic-intact, 3.31±0.28; aCSF splenic-denervated, 3.62±0.25) did not differ significantly between groups.

Splenic blood flow responses. Figure 11A summarizes splenic blood flow responses determined using microspheres in aCSF-treated (n=5) and Ang II-treated (n=7) splenic-intact rats, and Ang II-treated splenic-denervated (n=6) rats. Control levels of splenic blood flow did not differ significantly between groups. Splenic blood flow was not significantly changed from control levels immediately or 60 min after icv infusion of Ang II in splenic-intact or splenic-denervated rats; however, splenic blood flow was significantly increased from control levels 60 min after icv infusion of aCSF in splenic-intact rats. Blood flow to the right and left kidneys did not differ in splenic-intact rats (Control: left kidney 667±82, right kidney, 646±89; Immediately after Ang II infusion: left kidney 515±65, right kidney, 547±51; 60 min after Ang II infusion: left kidney 431±67, right kidney, 439±66) or splenic-denervated rats (Control: left kidney 509±55, right kidney, 516±46; Immediately after Ang II infusion: left kidney 475±85, right kidney, 482±79; 60 min after Ang II infusion: left kidney 456±79, right kidney, 435±67), demonstrating adequate mixing of microspheres during the experiments. Renal blood flow (right and left kidneys) was significantly reduced 60 min after icv Ang II infusion in splenic-intact rats (see data in previous sentence) and tended ($p>0.05$) to be reduced 60 min after Ang II infusion in splenic-denervated rats (see data in previous sentence).

In a separate group of experiments, splenic artery blood velocity was determined using Doppler flow probes to provide a continuous measure of relative blood flow before, during, and after icv Ang II infusion in splenic-intact (n=3) and splenic-denervated (n=3) rats. Splenic blood

flow did not change significantly from control levels during or for 60 min after icv Ang II infusion in splenic-intact and splenic-denervated rats and did not differ between groups during or after Ang II infusion (Figure 11B).

DISCUSSION

This study determined the effect of central administration of Ang II on splenic cytokine gene expression in anesthetized, baroreceptor-denervated rats. The current results provide experimental support for three new findings that contribute to the understanding of the role of Ang II in mediating sympathetic-immune interactions. First, splenic SND was significantly increased after icv administration of Ang II, but not aCSF. Second, splenic mRNA expression of IL-1β, IL-6, IL-2, and IL-16 was increased in Ang II-treated splenic-intact rats compared with aCSF-treated splenic-intact rats. Third, splenic IL-1β, IL-2, and IL-6 gene expression responses to Ang II were significantly reduced in splenic-denervated compared with splenic-intact rats. These results suggest that central administration of Ang II activates splenic sympathetic nerve outflow, which in turn increases the expression of selective splenic cytokine genes.

Bidirectional interactions between the nervous system and the immune system have been established (1, 2), and the sympathetic nervous system is thought to play a key role in mediating these interactions (12). The results of several studies demonstrate the central neural component of sympathetic-immune interactions (24, 28, 42, 51, 52). Icv infusion and hypothalamic microinjection of interferon-alpha reduce the cytotoxicity of splenic natural killer cells, an effect that is eliminated by splenic nerve denervation (51, 52). The cytotoxic activity of splenic natural killer cell activity is reduced after bilateral lesions of the medial part of the preoptic hypothalamus, an effect that is blocked by prior splenic denervation (28). Autonomic ganglionic

blockade, produced by intraperitoneal administration of chlorisondamine, antagonizes the immunosuppressive effect of centrally administered corticotrophin-releasing factor (24). Electrical stimulation of the ventromedial hypothalamus decreases the mitogenic response of splenic lymphocytes produced by concanavalin A administration, a response that is not observed in autonomic ganglion blocked or splenic nerve lesioned animals (42). The current study extends previous results by establishing a functional relationship between central Ang II administration, splenic nerve sympathoexcitation, and transcriptional regulation of splenic cytokine gene expression. Enhanced IL-1β, IL-2, and IL-6 splenic cytokine gene expression responses to central Ang II administration were observed in splenic-intact but not splenic-denervated rats, supporting a role for splenic sympathetic nerves in increasing the expression of selective splenic cytokine genes in response to central Ang II administration. Enhanced splenic cytokine gene expression responses were observed after central Ang II (increased splenic SND) but not central aCSF (no change in splenic SND) administration, suggesting that, under the conditions of the present experiments, activation of splenic sympathetic nerve outflow is required for upregulating the expression of selective splenic cytokine genes.

It is known that Ang II influences regulation of the sympathetic nervous system and the immune system. With regards to the sympathetic nervous system, Ang II increases sympathetic nerve outflow after central and peripheral administration (43, 54, and the current results), influences the transcriptional regulation of genes for the norepinephrine transporter, tyrosine hydroxylase, and dopamine β-hydroxylase in the brain (18, 36, 55), and enhances the release of norepinephrine from noradrenergic nerve terminals through direct activation of the ganglion and prejunctional angiotensin receptors (9, 47). Ang II modulates the immune system by augmenting the proliferation of splenic lymphocytes via a calcineurin pathway (41) and by increasing the

production of proinflammatory cytokines and transcription factors in the rat liver (5). The current results provide experimental support for Ang II modulating the immune system through activation of efferent sympathetic nerve outflow, suggesting a novel relationship between Ang II, splenic sympathetic nerve discharge, and splenic cytokine gene expression.

Because the central administration of Ang II increases splenic SND (54 and the current results), differences in splenic cytokine gene expression to icv Ang II infusion in splenic-intact and splenic-denervated rats may have resulted from altered splenic blood flow responses to Ang II after splenic denervation. This is likely not the case in the present study because splenic blood flow responses during and after Ang II infusion did not differ between splenic-intact and splenic-denervated rats. In contrast to other visceral organs, little is known about the role of the sympathetic nervous system in regulation of splenic blood flow in the rat (44-46). Therefore, we can provide little insight concerning the role of splenic sympathetic activation in modulating splenic blood flow responses to central Ang II administration. However, the primary focus of the blood flow studies was not to discern mechanisms regulating splenic blood flow responses to Ang II, rather, we wanted to assess whether Ang II-induced changes in splenic cytokine gene expression could be ascribed to substantial changes in blood flow to this organ following splenic denervation.

What is the functional significance of central Ang II-splenic SND-splenic cytokine gene expression interactions? Although the current results do not address this question, the pathophysiology of heart failure suggests an interesting possibility. Activation of the sympathetic nervous system is considered a hallmark of heart failure. Radiotracer studies of norepinephrine kinetics indicate increased norepinephrine spillover from the heart and kidneys (20, 40), microneurographic studies have demonstrated increased muscle SND in heart failure patients

(16, 33), and renal SND is significantly higher in animal models of cardiac failure than in noninfarcted controls (11, 15). The renin-angiotensin system is altered in heart failure. Icv injection of losartan decreases levels of resting renal SND in rats with chronic heart failure (11), and chronic central AT_1 receptor blockade normalizes the enhanced sympathoexcitation, reduced sympathoinhibition, and desensitized baroreflex responses observed in congestive heart failure rats after myocardial infarction (56). Congestive heart failure patients exhibit clinical features that are observed in chronic inflammatory conditions (10, 34, 50). For example, plasma levels of proinflammatory cytokines, notably IL-6 and TNF-alpha, are elevated in congestive heart failure patients and serum levels of IL-10, a potent anti-inflammatory cytokine, are reduced (10, 34, 50). It is tempting to speculate that alterations in the central Ang II-splenic SND-splenic cytokine gene expression axis may play a role in the pathophysiology of congestive heart failure, providing rationale for understanding mechanisms mediating interactions between these different physiological systems.

The current results are applicable to splenic tissue only, and their relevance to other primary and secondary lymphoid organs remains to be established. The genearray used in this study consists of a limited number of cytokine and chemokine genes, and the possibility exists that icv Ang II infusion regulates other cytokines, chemokines and their receptor genes. The present study utilized genomic level of analyses; therefore, the influence of icv Ang II infusion on splenic protein expression remains to be established. Because protein appearance can be altered by numerous transcription- and translational-related events and, because little is known about the time required for protein synthesis to occur secondary to activation of splenic nerve efferents, we believe that determining the effect of central Ang II infusion on splenic protein synthesis is reasonably beyond the scope of the current study. It must be considered that at least

part of the effect of central Ang II administration to influence splenic cytokine gene expression may have resulted from Ang II leaking from the cerebrospinal fluid into the peripheral circulation and causing norepineprine release from adrenergic nerve ending sites. Although the current data do not exclude this possibility, it seems unlikely based on the results of Bruner et al. (7) who reported that plasma Ang II levels were not increased during the fifth day of a chronic icv infusion of Ang II administered in the microgram dose range. In contrast, the present study used a single 10 min central infusion of Ang II administered in the nanogram dose range. Because Ang II can directly influence splenic immune cell function (5, 41), we did not complete peripheral control experiments in which Ang II was administered intravenously. The current study was completed in sinoaortic-denervated rats to eliminate the influence of baroreceptor afferent feedback mechanisms that may attenuate SND responses of central origin; therefore, the influence of the arterial baroreflex on the functional relationship between Ang II, splenic sympathetic nerve discharge, and splenic cytokine gene expression remains to be established.

In summary, the current data provide insight concerning the role of splenic sympathetic nerves in splenic immune regulation in vivo to a specific experimental intervention (icv Ang II infusion) and, within the constraints of the current experimental protocol and analyses, the present results strongly support a role for splenic sympathetic nerves in increasing the expression of splenic cytokine genes in response to icv Ang II infusion.

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Table 2. Corrected (ΔC_T) threshold cycle (C_T) values for IL-1β, IL-6, IL-2, IL-16, and TGF-β1 genes from aCSF-treated splenic-intact, Ang II-treated splenic-intact, and Ang II-treated splenic-denervated rats. Absolute threshold cycle (C_T) values for β-actin served as internal controls.

	β-actin C_T	IL-1β ΔC_T	IL-6 ΔC_T	IL-2 ΔC_T	IL-16 ΔC_T	TGF-β1 ΔC_T
aCSF Splenic-intact	15.87±0.17	7.76±0.87	11.46±0.24	14.87±0.30	6.40±0.31	3.31±0.28
Ang II Splenic-intact	15.84±0.1	5.51±0.4*	9.81±0.2*	12.93±0.5*	5.36±0.2†	3.03±0.3
Ang II Splenic-denervated	15.47±0.19	7.49±0.95	12.12±0.12	14.72±0.21	5.93±0.29	2.67±0.35

Values are means ± SE. ΔC_T = Avg C_T of target gene - Avg β-actin C_T. aCSF-treated splenic-intact (n=6), Ang II-treated splenic-intact (n=8), Ang II-treated splenic-denervated (n=8). * Significantly different from aCSF-treated splenic-intact and Ang II-treated splenic-denervated rats. † Significantly different from aCSF-treated splenic-intact rats.

Figure 7

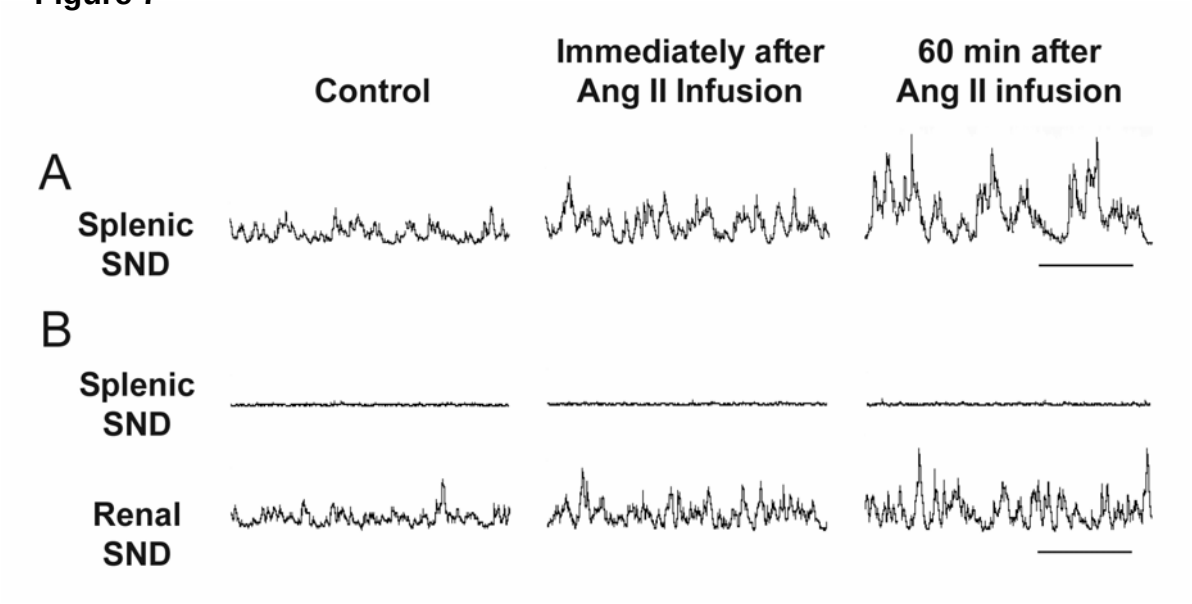

Figure 7. *(A)* Traces of splenic sympathetic nerve discharge (SND) recorded before (Control), immediately after, and 60 min after cessation of icv Ang II infusion in a rat with intact splenic nerves. *(B)* Traces of splenic and renal SND recorded before (Control), immediately after, and 60 min after cessation of icv Ang II infusion in a splenic-denervated rat. Horizontal calibration is 500 ms.

Figure 8

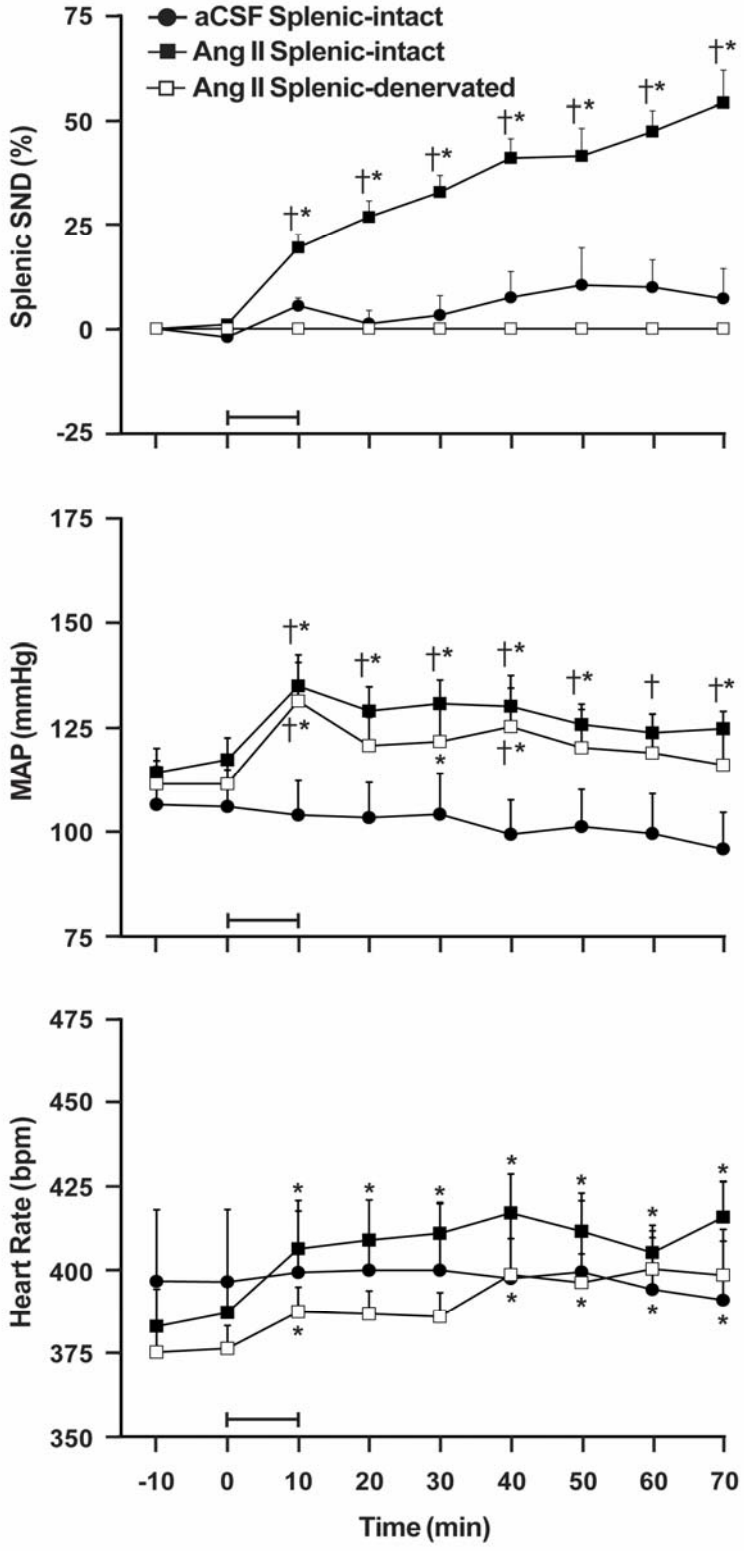

Figure 8. .Splenic sympathetic nerve discharge (SND), mean arterial pressure (MAP), and heart rate measurements in aCSF-treated splenic-intact (filled circles), Ang II-treated splenic-intact

(filled squares), and Ang II-treated splenic-denervated (open square) rats. Measurements were obtained before (-10 min), during (0-10 min, indicated by horizontal bar), and for 60 min after (10-70 min) icv infusion of aCSF or Ang II. ***** Significantly different from control values (-10 min). † Significantly different from aCSF splenic-intact rats.

Figure 9

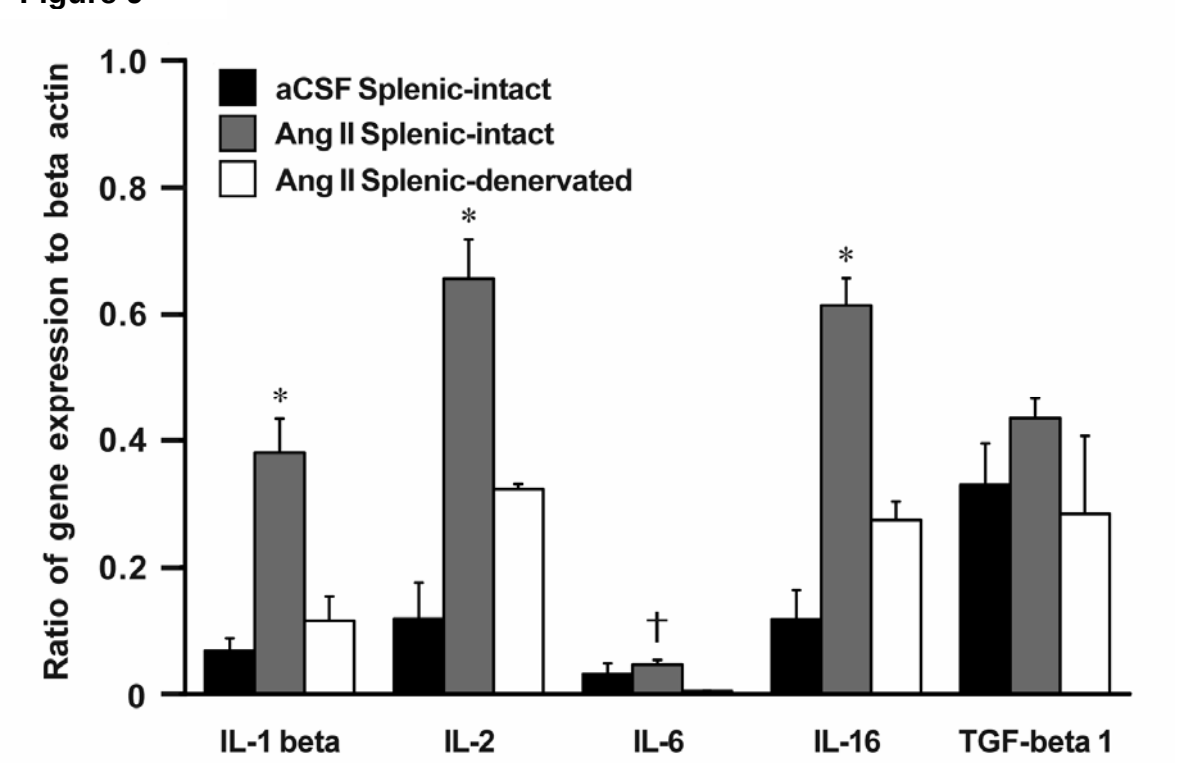

Figure 9. Levels of mRNA expression for IL-1β, IL-2, IL-6, IL-16, and TGF-β1 genes determined in aCSF-treated splenic-intact (black bars), Ang II-treated splenic-intact (gray bars), and Ang II-treated splenic-denervated (white bars) rats. Gene expression levels are presented relative to β-actin mRNA expression. * Ang II-treated splenic-intact rats significantly different from aCSF-treated splenic-intact and Ang II-treated splenic-denervated rats. † Ang II-treated splenic-intact rats significantly different from Ang II-treated splenic-denervated rats.

Figure 10

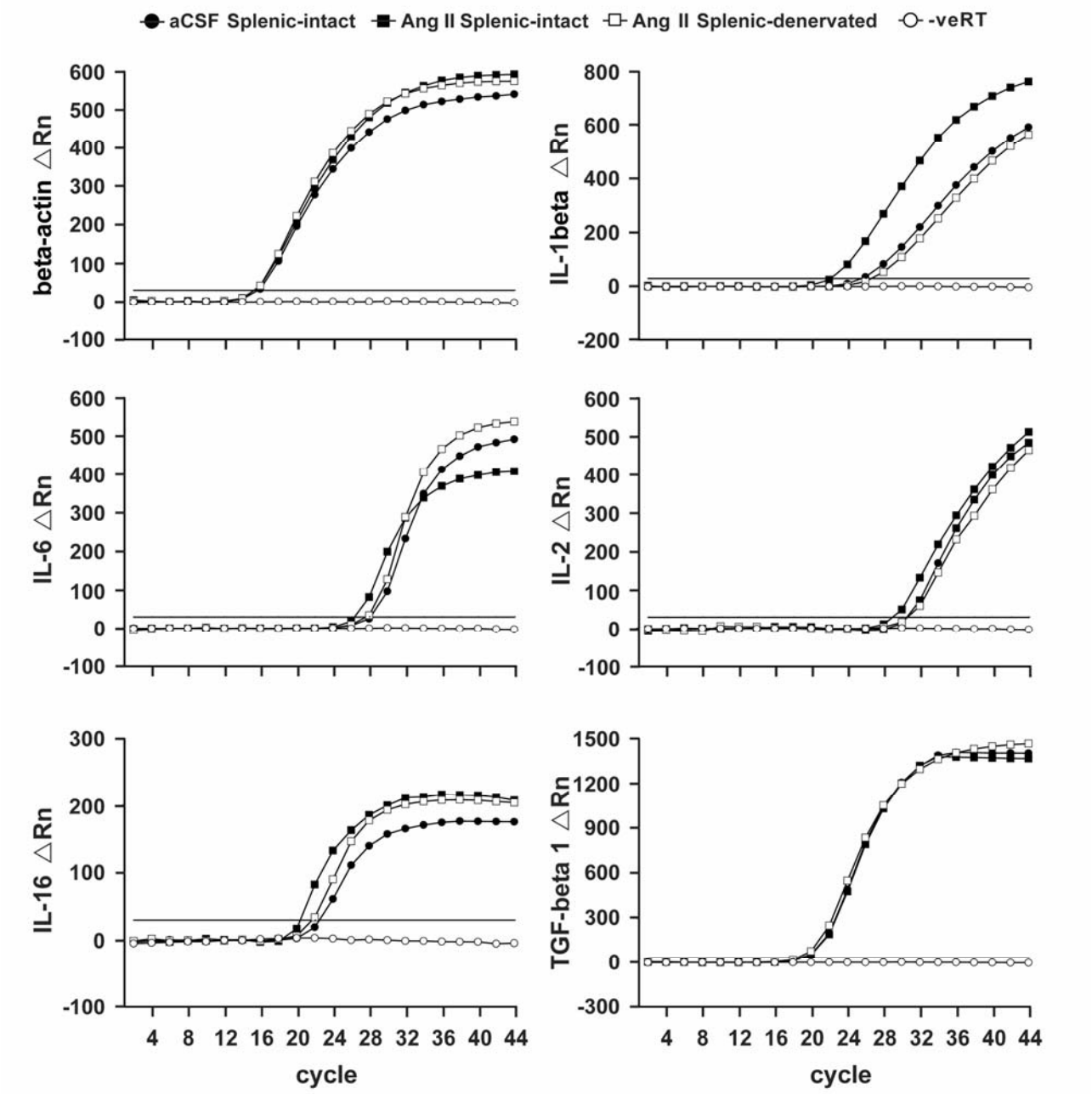

Figure 10. Real time RT-PCR analysis was performed for β-actin, IL-1β, IL-6, IL-2, IL-16, and TGF- β1 and the amplification plots of representative experiments from three groups of rats (aCSF splenic-intact; Ang II splenic-intact; Ang II splenic-denervated) are shown. A negative RT control (-veRT) for each primer set is also presented.

Figure 11

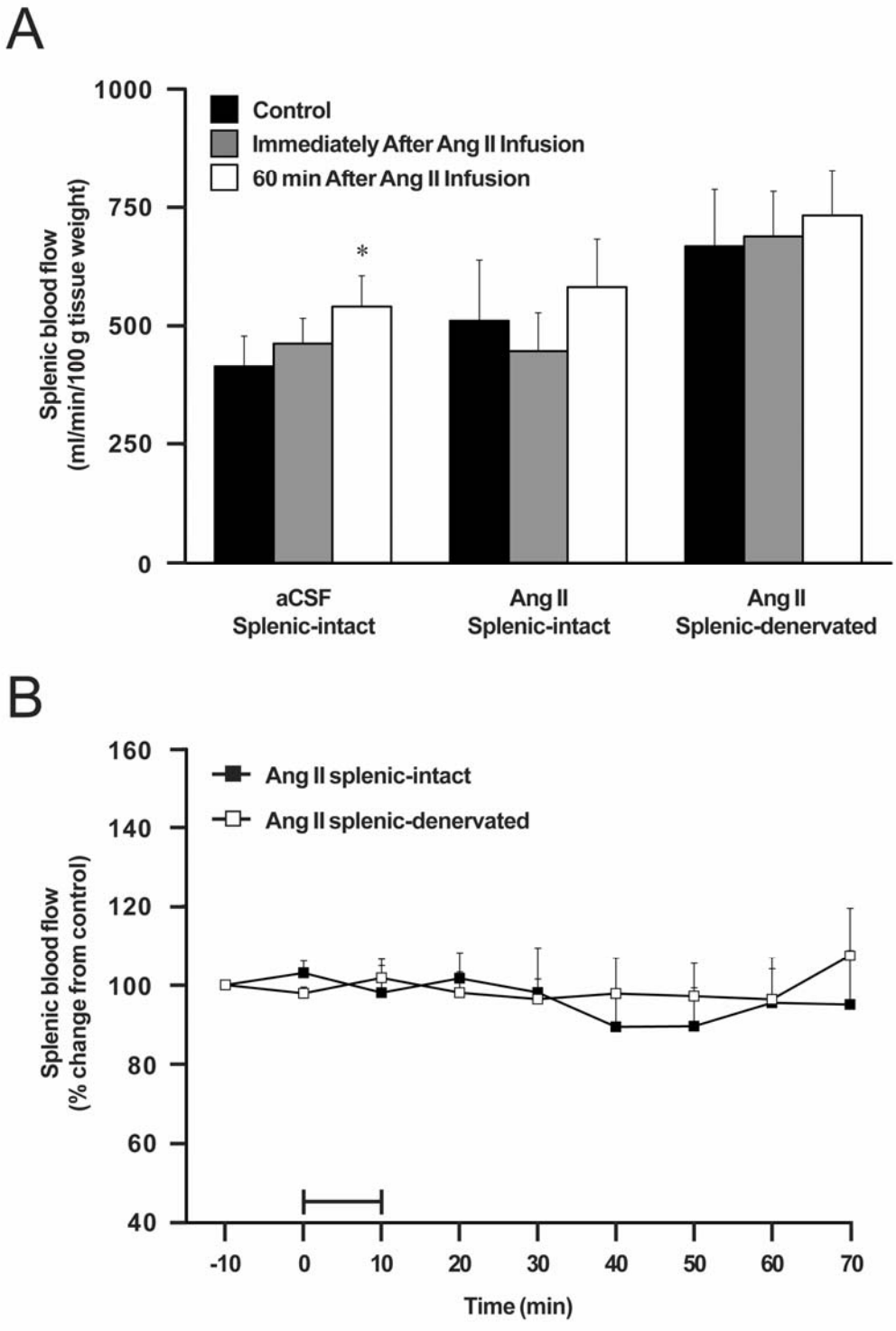

Figure 11. (A) Splenic blood flow measurements during control (black bars), immediately after a 10 min infusion of aCSF or Ang II (gray bars), and 60 min after cessation of aCSF or Ang II Infusion (white bars) in splenic-intact and splenic-denervated rats. * Significantly different from control values. (B) Doppler splenic blood flow measurements in Ang II splenic-intact and Ang II splenic-denervated rats expressed as percent change from control levels (-10 min). Continuous

measurements were obtained before (-10-0 min), during (0-10 min), and for 60 min after (10-70 min) icv infusion of Ang II.

CHAPTER 3. HYPOTHERMIA-ENHANCED SPLENIC CYTOKINE GENE EXPRESSION IS INDEPENDENT OF THE SYMPATHETIC NERVOUS SYSTEM

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ABSTRACT

Splenic nerve denervation abrogates enhanced splenic cytokine gene expression responses to acute heating, demonstrating that hyperthermia-induced activation of splenic sympathetic nerve discharge (SND) increases splenic cytokine gene expression. Hypothermia alters SND responses; however, the role of the sympathetic nervous system in mediating splenic cytokine gene expression responses to hypothermia is not known. The purpose of the present study was to determine the effect of hypothermia on the relationship between the sympathetic nervous system and splenic cytokine gene expression in anesthetized F344 rats. Gene expression analysis was performed using a microarray containing 112 genes representing inflammatory cytokines, chemokines, cytokine/chemokine receptors and housekeeping genes. A subset of differentially expressed genes was verified by real-time RT-PCR analysis. Splenic SND was decreased significantly during cooling (core temperature decreased from 38 to 30ºC) in splenic-intact rats but remained unchanged in sham-cooled splenic-intact rats (core temperature maintained at 38ºC). Hypothermia upregulated the transcripts of several genes including, chemokine ligands CCL2, CXCL2, CXCL10 and CCL20, and interleukins IL-1α, IL-1β and IL-6. Gene expression responses to hypothermia were similar for the majority of cytokine genes in splenic-intact and splenic-denervated rats. These results suggest that hypothermia-enhanced splenic cytokine gene expression is independent of splenic SND.

Key words: Hypothermia, splenic cytokine gene expression, splenic sympathetic nerve discharge.

INTRODUCTION

Evidence from the disciplines of neuroscience and immunology demonstrate bidirectional communication pathways between the sympathetic nervous system and the immune system (2, 3, 13, 38). Sympathetic innervation to the spleen provides a connection between central sympathetic neural circuits and immunocompetent cells in the spleen (1, 6, 9, 16, 17). For example, chemical sympathectomy alters splenic T and B cell proliferation and natural killer cell activity (36-40, 45) and diminishes splenic production of immunoglobulin M (31). Results of our recent studies demonstrate that in rats with intact splenic nerves, whole body hyperthermia (19) and central angiotensin II infusion (20) increase splenic sympathetic nerve discharge (SND) and the expression of selective splenic cytokine genes. Splenic cytokine gene expression responses to whole body hyperthermia and central angiotensin II infusion are significantly reduced in splenic nerve-denervated compared with splenic nerve-intact rats (19, 20), suggesting that activation of splenic SND can enhance splenic cytokine gene expression.

Hypothermia, a common side effect of extreme cold environments, anesthesia, and serious traumatic injuries, alters the sympathetic nervous system and immune system regulation (8, 13-15, 21, 27-29, 47-50). Acute cold stress increases plasma concentrations of norepinephrine and epinephrine (18, 26, 46, 52), changes the pattern of synchronized SND bursts (28), influences the frequency-domain relationships between discharge bursts in regionally selective sympathetic nerves (28), and produces nonuniform changes in the level of sympathetic nerve activity to selected target organs (28). With regards to the latter, hypothermia increases lumbar and decreases renal SND without significantly changing the level of activity in the splanchnic and adrenal nerves in anesthetized rats (28) and activates preganglionic cervical SND in anesthetized rabbits (27). Considering immune system regulation, hypothermia alters the activity of splenic natural killer cells (11, 25, 36), augments the production of inflammatory

cytokines in a cell line of peripheral blood monocytes (15), and increases cytokine gene expression in cultures of peripheral blood mononuclear cells (49). In addition, plasma levels of proinflammatory cytokines are increased in patients suffering accidental hypothermia (4).

Despite the substantial literature demonstrating that hypothermia alters regulation of the sympathetic nervous and immune systems, the influence of hypothermia on sympathetic-immune interactions is not well established for the following reasons. First, the effect of hypothermia on splenic SND is not known. Second, the effect of hypothermia on splenic cytokine gene expression is poorly understood. Third, if hypothermia alters splenic cytokine gene expression, it is not known if this effect is dependent on the sympathetic innervation to the spleen. In the present study we used splenic SND recordings, splenic nerve denervations, and splenic gene expression analyses (microarray and real-time RT-PCR) to determine the effect of hypothermia on the relationship between the sympathetic nervous system and splenic cytokine gene expression in urethane-chloralose anesthetized Fischer (F344) rats. We tested the hypothesis that hypothermia would alter splenic SND thereby modifying the expression of selective splenic cytokine and chemokine genes.

METHODS

General procedures. The Institutional Animal Care and Use Committee approved the experimental procedures and protocols used in the present study and all procedures were performed in accordance with the American Physiological Society's guiding principles for research involving animals (5). Experiments were performed on F344 rats (n=30) anesthetized with isoflurane (during surgical procedures only; 3% induction followed by 1.5%-2.5%), α-chloralose (initial dose 80 mg/kg ip, maintenance dose of 35-45 mg/kg/hr iv), and urethane (800

mg/kg ip). The trachea was cannulated with a polyethylene-240 catheter and femoral arterial pressure was monitored using a pressure transducer connected to a blood pressure analyzer. The pulsatile arterial pressure output of the blood pressure analyzer was used to derive heart rate. Colonic temperature (Tc) was maintained during surgical procedures between 37.8ºC and 38.0ºC by a homeothermic blanket.

Neural recordings. Following completion of the cannulation procedures, splenic and renal SND was recorded biphasically (bandpass 30-3000 Hz) with a platinum bipolar electrode after preamplification. In splenic-denervated rats, renal SND was recorded using similar recording and preamplification procedures. Splenic and renal sympathetic nerves were isolated from a lateral approach. For monitoring during the experiment and for subsequent data analysis, the filtered neurograms were routed to an oscilloscope and a nerve traffic analyzer. Sympathetic nerve potentials were full-wave rectified, integrated (time constant 10 ms) and quantified as volts x seconds (V·s) (19, 20, 28, 29). SND was corrected for background noise after administration of the ganglionic blocker, trimethaphan camsylate (10-15 mg/kg iv).

Splenic denervation. A two-step splenic denervation procedure was performed. Initially, the splenic bundle (including splenic artery, vein and nerve) was visualized and the splenic nerve was dissected free of surrounding connective tissue and sectioned at the base of the bundle. Subsequently, the individual arteries projecting to the spleen were identified and the sympathetic nerve adjoining each vessel was sectioned. Denervation was considered complete when splenic nerve recordings completed after denervation demonstrated no sympathetic nerve activity.

Experimental protocol. After completion of the surgical procedures, chloralose-anesthetized, gallamine-paralyzed, baroreceptor-innervated rats were allowed to stabilize for 60 min. After the stabilization period, a 30 min control period was completed during which Tc was

maintained at 38°C in all rats. At the end of the control period, use of the homeothermic heating blanket was discontinued and ice packs were placed in close proximity to the dorsal and ventral surfaces of splenic-intact and splenic-denervated rats. The rate of cooling was controlled by altering the position of the ice packs to maintain a reduction in Tc of 0.1°C/min from 38ºC to 30ºC. End-tidal CO_2 was kept between 4.8 and 5.2% by adjusting the frequency of respiration during hypothermia. Sham-cooled experiments were completed in splenic-intact rats by maintaining Tc at 38ºC for an additional 80 min beyond the initial 30 min control period. Mean arterial pressure (MAP), HR, and SND were measured continuously during the control periods and during the cooling and sham cooling protocols. Spleens were collected at the end of each experiment and stored at -80ºC. Gene array analysis was performed on spleens collected from four rats in each experimental group (Sham-cooled splenic-intact, cooled splenic-intact, and cooled splenic-denervated). To validate the gene array results, TaqMan probe-based real-time RT-PCR analysis was performed on spleens used for gene array analysis (n = 4 for each group) and spleens from additional experiments in each group (sham cooled splenic-intact, n = 5; cooled splenic-intact, n = 8; cooled splenic-denervated, n = 5).

RNA isolation. Frozen spleens were homogenized in liquid nitrogen and total RNA was isolated using the TRI Reagent RNA isolation kit according to the manufacturer's protocol (Sigma Chemical Co., St. Louis, MO). RNA purity and concentration were determined spectrophoto-metrically by calculating the ratio between the absorbance at 260 nm and 280 nm using a NanoDrop ND-1000 (NanoDrop, USA). The absorbance ratio for all samples ranged between 1.8 and 2.0. The quality of RNA for all samples was confirmed by resolving them on a 1.5% formaldehyde agarose gel.

Microarray analysis. Splenic cytokine gene expression was evaluated using a rat inflammatory cytokines and receptors microarray (Superarray Biosciences, Bethesda, MD). The oligo/microarray blot contained 96 inflammatory cytokine and chemokine gene fragments with each gene spotted in four wells. In addition, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein L32, lactate dehydrogenase A, aldolase A and biotinylated artificial sequence 2 complementary sequence were included as internal positive controls and PUC18 plasmid DNA was included as an internal negative control. Biotin-labeled cRNA probes were synthesized from total RNA TrueLabeling-AMP^{TM} Linear RNA amplification kit (SuperArray Biosciences, Bethesda, MD). The labeled cRNA probes were hybridized to oligonucleotide fragments spotted on the gene array membranes. Membranes were washed to remove any unincorporated probe and incubated with alkaline phosphatase conjugated streptavidin (AP-streptavidin). Relative expression levels of specific genes were detected from signals generated by chemiluminescence from the alkaline phosphatase substrate, CDP-*Star*. The luminizing blots were used to expose X-ray films and quantified by spot densitometry with the aid of GEArray expression analysis suite (Superarray Biosciences, Bethesda, MD). The relative gene expression levels were estimated by comparing the signal intensity of the target gene to the signal intensity derived from GAPDH.

Real-time RT-PCR analysis. To validate the gene-array results, TaqMan® probe-based real-time RT-PCR analysis was performed. Total RNA (2μg) was reverse-transcribed in a 20 μl volume containing 1 μM of oligo(dT) primers, 0.5 mM of each dNTP, 0.5 U/μl of RNase inhibitor and 0.2 U/μl of Omniscript Reverse Transcriptase (Qiagen, Valencia, CA) in RNase-free water. The reaction was carried out for 60 min at 37.0^{o}C and the cDNA mixture was used for the real-time PCR analysis of specific cytokine gene expression.

Gene-specific PCR primer pairs and TaqMan® probes for chemokine (C-X-C motif) ligand 10 (CXCL10), chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-X-C motif) ligand 2 (CXCL2), chemokine (C-C motif) ligand 20 (CCL20) and, transforming growth factor beta 1 induced transcript 4 (TGFβ1i4) were obtained from Applied Biosystems (Foster City, CA). Primers and probes for β-actin, lL-6 and IL-1β genes were custom synthesized using published sequences (7, 33). TaqMan® probes were labeled with 6-carboxyfluorescin (FAM) as the reporter dye molecule at the 5' end and 6-carboxy-tetramethyl-rhodamine (TAMRA) as the quencher dye molecule at the 3' end. Real-time PCR reactions were performed with 2 µl of cDNA using Universal PCR Master Mix (Applied Biosystems, Foster City, CA), containing 0.9 µM each of the forward and reverse primers and 0.25 µM TaqMan® probe in a 25 µl reaction. Real-time PCR analysis was performed in a Smart Cycler (Cepheid, Sunnyvale, CA) with the following PCR conditions: one cycle each of 50^{o}C for 2 min and 95^{o}C for 5 min, followed by 45 cycles of 95^{o}C for 15 seconds and 60^{o}C for 1 min.

The threshold cycle (Ct) value for each gene was defined as the PCR cycle at which the emitted fluorescence rose above a background level of fluorescence, i.e., 30 fluorescence units. Gene expression levels were calculated as fold change relative to the gene expression of sham-cooled splenic-intact rats. The PCR amplification efficiencies of β-actin and the target genes were calculated using the following formula: PCR efficiency = $(10^{(1/-S)} - 1)$ where S is the slope (21). The amplification efficiency was greater than 90% for all genes. The comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to quantify the results obtained by real time RT-PCR (35). Data were normalized by determining differences in Ct values between the target gene of interest and β-actin, defined as ΔCt (Ct of target gene – Ct of β-actin gene). The fold change was calculated as $2^{(SAvg\Delta Ct - CAvg\Delta Ct)}$ where SAvgΔCt – CAvgΔCt is the difference between the sample (cooled-

intact/cooled-denervated) ΔCt and the control (sham-cooled intact) ΔCt. For sham-cooled intact samples, ΔΔCt equaled zero and 2^0 equaled one, so that the fold change in gene expression relative to the sham-cooled intact samples equaled one. For the treated samples, evaluation of $2^{-\Delta\Delta Ct}$ was defined as the fold change in gene expression relative to sham-cooled splenic-intact samples.

Determination of splenic artery blood velocity. A Doppler flow probe filled with ultrasonic transmission gel was placed on the splenic artery for measurement of splenic blood flow velocity (32). The flow probe wires were connected to a pulsed Doppler flowmeter. Details of the Doppler technique, including the reliability of the method for estimation of velocity have been described previously (23). Blood velocity (in kHz Doppler shift) is directly proportional to absolute blood flow, therefore the Doppler technique provides a relative measure of changes in flow (23).

Data and statistical analysis. Values are means ± SE. Control values of SND were taken as 0%. Statistical analysis of SND, MAP and HR were analyzed using analysis of variance techniques with a repeated-measures (ANOVA-R) design. When a significant F-ratio was demonstrated by the ANOVA-R, post-hoc tests (Bonferroni) were applied to describe significant MAP, HR, splenic SND, and BF versus cooling interactions. Results from gene array and RT-PCR analyses in cooled and sham-cooled rats were compared using Student's t-tests. The overall level of statistical significance was $p < 0.05$.

RESULTS

SND, MAP and HR responses to hypothermia. Figure 12 shows SND traces from three representative experiments (A, *sham-cooled splenic-intact*; B, *cooled splenic-intact*; C, *cooled*

splenic-denervated). Tc was maintained at 38ºC during the control period in each rat. In the sham-cooled splenic-intact rat (A), Tc was held constant at 38ºC after the control period and splenic SND remained unchanged from the control. In the cooled splenic-intact rat (B), Tc was decreased from 38 to 30ºC and splenic SND was reduced from control during cooling. In the cooled splenic-denervated rat (C), no measurable splenic SND was detected during the control period or cooling, although renal SND was decreased during cooling. Renal SND was recorded in the splenic-denervated rat to demonstrate specificity in the denervation procedure.

Figure 13 summarizes splenic SND, MAP, and HR responses to hypothermia in splenic-intact (n=7) and splenic-denervated (n=7) rats. Tc was reduced from 38 to 30ºC at a rate of 0.1ºC/1 min in both groups. During cooling, splenic SND was decreased significantly from control (38ºC) in splenic-intact rats and was undetectable in splenic-denervated rats. MAP and HR were progressively and significantly decreased from control (38ºC) during cooling in splenic-intact and splenic-denervated rats (asterisks denote statistically significantly reductions for MAP and HR in both groups). Splenic SND was modestly but significantly increased whereas MAP and HR remained unchanged in sham-cooled (Tc held constant at 38ºC for 80 min) splenic-intact rats (Table 3).

Microarray analysis of splenic gene expression responses to hypothermia. Figure 14 shows microarray results performed with RNA isolated from spleens of three representative experiments (sham-cooled splenic-intact, *left*; cooled splenic-intact, *middle*; cooled splenic-denervated, *right*). Expression of RPL32 and GAPDH genes (internal controls) was similar in each experiment. Expression of IL-1β, IL-6, CXCL2, CCL2 and CCL20 genes was increased in the cooled splenic-intact and splenic-denervated rats compared to the sham-cooled splenic-intact

rat. Expression of CXCL10 was increased in the cooled splenic-intact rat compared with the sham-cooled splenic intact rat and the cooled splenic-denervated rat. Expression of TGFβ1i4 was observed in each experiment but did not differ between experiments.

Values relative to GAPDH for splenic IL-1β, IL-6, CXCL10, CXCL2, CCL2, CCL20, TGFβ1i4 and RPL32 mRNA expression in sham-cooled splenic-intact (n=4), cooled splenic-intact (n=4), and cooled splenic-denervated (n=4) rats are summarized in Figure 15. Expression of IL-1β, IL-6, CXCL2, CCL2 and CCL20 genes was significantly increased in cooled splenic-intact and cooled splenic-denervated rats compared to sham-cooled splenic-intact rats. CXCL10 gene expression was increased in cooled splenic-intact rats compared with cooled splenic-denervated and sham-cooled splenic-intact rats. Expression of TGFβ1i4 and RPL32 genes did not differ between groups.

Real-time RT-PCR analysis of splenic cytokine gene expression responses to hypothermia. Real-time RT-PCR analysis was performed for all candidate genes identified using microarray analysis (IL-1β, IL-6, CXCL10, CXCL2, CCL2, CCL20 and TGFβ1i4) from three experimental groups of rats; sham-cooled splenic-intact, cooled splenic-intact, and cooled splenic-denervated. β-actin was used as the internal control gene. Amplification plots for eight representative genes are shown in Figure 16. β-actin mRNA expression did not differ between groups. As indicated by the lower Ct values (a reflection of increased mRNA levels), expression of IL-1β, IL-6, CXCL2, CCL2, and CCL20 mRNA was higher in the cooled splenic-intact and splenic-denervated rats compared to sham-cooled splenic-intact rat. The expression of TGFβ1i4 did not differ between groups and the expression of CXCL10 was higher in the cooled splenic-intact rat compared to the sham-cooled splenic-intact and cooled splenic-denervated rats.

Summarized real-time RT-PCR data are shown in Table 4. The ΔCt values were significantly lower (a reflection of increased mRNA levels) in cooled splenic-intact and cooled splenic-denervated rats for IL-1β, IL-6, CXCL2, CCL2 and CCL20 genes compared to sham-cooled splenic-intact rats. The ΔCt value for TGFβ1i4 did not differ significantly between the groups and the ΔCt for CXCL10 was significantly lower in cooled splenic-intact compared to sham-cooled splenic-intact rats, but not with cooled splenic-denervated rats. When the Ct values were expressed as fold change, mRNA expression of IL-1β, IL-6, CXCL2, CCL2 and, CCL20 was increased in cooled splenic-intact rats (n=11) and cooled splenic-denervated rats (n=8) compared with sham-cooled splenic-intact rats (n=10). The fold change for mRNA expression of CXCL10 was increased only in cooled splenic-intact rats compared with sham-cooled splenic-intact rats.

Splenic blood flow responses to hypothermia. Splenic blood flow responses to hypothermia were determined in splenic-intact (n=7) and splenic-denervated (n=5) rats. Splenic artery conductance was significantly increased from control (38.0ºC) in cooled splenic-intact (34ºC, +48±12%; 30ºC, +38±12%) and cooled splenic-denervated rats (34ºC, +32±20%; 30ºC, +30±18%). Splenic blood flow conductance responses to hypothermia did not differ (p=0.13) in cooled splenic-intact and cooled splenic-denervated rats.

DISCUSSION

The current study provides experimental support for three new findings concerning the influence of hypothermia on sympathetic-immune interactions in anesthetized F344 rats. First, hypothermia produced progressive and significant reductions in the level of splenic sympathetic nerve activity. Second, expression of selective splenic cytokine and chemokine genes was

higher in cooled compared with sham-cooled splenic-intact rats. Third, similar hypothermia-induced increases in splenic cytokine gene expression were observed in splenic-intact and splenic-denervated rats. These results demonstrate that hypothermia upregulates splenic cytokine gene expression, an effect that is not dependent on the sympathetic innervation to the spleen.

Changing the level of activity in peripheral sympathetic nerves in response to environmental challenges, including hypothermia, is a primary strategy used by mammals to maintain physiological homeostasis. Sabharwal (50) reported that whole body hypothermia reduces renal SND in anesthetized, cold-acclimated rats. Broman et al (8) reported that, in the absence of muscle shivering, whole body hypothermia reduces renal SND in nonacclimated, anesthetized rats. In contrast to hypothermia-induced renal sympathoinhibitory responses, Kaul et al. (27) observed that acute cold stress activates preganglionic cervical SND in anesthetized rabbits, suggesting that decreased internal body temperature may elicit nonuniform changes in the level of efferent sympathetic nerve activity. Consistent with this idea, whole body hypothermia in nonacclimated, anesthetized, paralyzed rats increases lumbar and decreases renal SND without significantly changing the level of activity in splanchnic and adrenal nerves (28). These findings demonstrate nonuniform responses to acute cold stress in sympathetic nerves innervating visceral and peripheral targets (renal SND decreased and lumbar SND increased) and in sympathetic nerves innervating visceral targets (renal SND decreased and splanchnic SND unchanged). Because the overall aim of the current study was to determine the effect of hypothermia on the relationship between splenic SND and splenic cytokine gene expression and because whole body hypothermia produces nonuniform visceral SND responses (28), the first objective was to determine the effect of acute cold stress on the level of splenic SND, which, to

the best of our knowledge, remained unknown. The present findings demonstrate that hypothermia in anesthetized rats produces progressive and significant reductions in the level of splenic sympathetic nerve activity, supporting the idea that hypothermia substantially alters efferent sympathetic nerve outflow.

In recent studies we found that whole-body hyperthermia and central angiotensin II infusion increased both the level of splenic sympathetic nerve activity and the expression of selective splenic cytokine and chemokine genes (19, 20). The enhanced splenic gene expression to these interventions was abrogated by denervation of splenic sympathetic nerves, suggesting a role for splenic sympathoexcitation in upregulation of selective splenic cytokine genes. Based on these findings, we reasoned that splenic sympathoinhibitory responses to hypothermia would reduce the expression of selective splenic cytokine and chemokine genes. However, the present findings show enhanced splenic cytokine and chemokine gene expression responses to hypothermia, despite concomitant splenic sympathoinhibition. In addition, splenic gene expression responses to hypothermia, with the exception of CXCL10, were similar in splenic nerve-intact and splenic nerve-denervated rats, indicating that changes in splenic cytokine gene expression to acute cold stress in anesthetized, nonacclimated rats are independent of sympathetic innervation to the spleen.

The results of previous studies (11, 14, 15, 25, 30, 36, 47, 49) indicate that hypothermia alters immune system regulation. Moderate cooling (*in vivo* studies) increases natural killer cell activity (11, 34), white blood cell counts, (24) and monocytic expression of IL-6 and TNF-α (11) in human subjects. *In vitro* studies using human cell lines incubated at 32ºC and 33ºC showed increased IL-10 and decreased interferon gamma cytokine gene expression in peripheral blood cultures (49), decreased production of IL-10 in peripheral mononuclear blood cells (41), and

prolonged activation of nuclear factor kappa B and increased proinflammatory cytokine gene expression in lipopolysaccharide-treated human monocytic cell lines (15). In addition, cold exposure has been shown to suppress T-lymphocyte proliferation and splenic natural killer cell activity in rodents (25, 30). The current study used a broad scale experimental approach, involving microarray and real-time PCR analyses, to determine the effect of whole-body cooling on splenic cytokine, chemokine, and receptor genes in anesthetized rats. Hypothermia significantly enhanced the splenic expression of IL-1β, IL-6, CXCL2, CXCL10, CCL2 and CCL20 mRNA. IL-1β mediates proinflammatory immune responses (12), IL-6 is a multifunctional cytokine that regulates the acute-phase response (10), CXCL2 is a macrophage inflammatory protein-2 (42, 43) and CCL20 is a macrophage inflammatory protein-3 (48), CXCL10 is a γ-interferon-inducible protein (44), and CCL2 is a monocyte chemoattractant protein (22). Collectively, the current findings indicate that acute hypothermia upregulates the expression of a functionally diverse array of splenic cytokines and chemokines.

One possible mechanism mediating hypothermia-induced increases in splenic cytokine and chemokine gene expression may be a direct effect of acute cooling on splenic immune cells. Consistent with this idea, the results of several studies demonstrate enhanced proinflammatory cytokine expression from immune cells cooled *in vitro* (15, 41, 49). The present results demonstrate significant increases in splenic artery conductance during cooling in splenic nerve-innervated and -denervated rats, suggesting that circulating factors may contribute to the enhanced gene expression observed in splenic tissue in response to hypothermia. For example, cytokines released from activated peripheral monocytes or activated peripheral monocytes themselves could circulate to the spleen and enhance the expression of proinflammatory cytokines in this tissue. Similarly, indirect activation of splenic immune cells via circulating

catecholamines may be involved because cold-stress increases plasma levels of catecholamines (18, 26, 46, 52), which in turn can activate α2-adrenergic receptors and enhance proinflammatory cytokine levels (51). Because of this possibility, the present findings suggest that the upregulation of splenic cytokine gene expression to hypothermia under the conditions of the current experiments is not dependent on the sympathetic innervation to the spleen; however, an effect of the sympathetic nervous system via circulating catecholamines cannot be discounted.

The current study is applicable to splenic tissue only, and the application to other lymphoid organs remains to be established. Furthermore, because a pathway specific microarray with a limited number of cytokines and chemokines was used in the current study, the role of hypothermia on other immune parameters needs further examination. The use of gene array and real-time RT-PCR analyses gives an estimate of the genomic levels of expression and may not represent protein expression in the spleen. However, within the constraints of the current experimental protocols and analyses, the present results show that during core-body cooling splenic SND is decreased and splenic cytokine gene expression is increased and this enhanced splenic cytokine gene expression is independent of splenic sympathetic innervation.

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Table 3. Splenic SND, MAP, and HR recorded in sham-cooled splenic-intact rats maintained at 38ºC for 80 min.

Time (min)	0	10	20	30	40	50	60	70	80
SND (%)	0	4±2	2±4	8±4	7±2	9±3	14±3*	15±3*	19±2*
MAP (mmHg)	113±4	111±5	111±5	111±4	111±5	110±5	112±5	111±5	109±5
HR (bpm)	420±6	418±8	422±7	423±7	425±8	424±8	425±8	426±9	424±9

Values are mean ± SE; SND, sympathetic nerve discharge (n=5); MAP, mean arterial pressure (n=5); HR, heart rate (n=5); bpm, beats/min. *Significantly different from control values ($p<0.05$).

Table 4. Absolute Ct values and fold change from sham-cooled splenic intact rats for IL-1β, IL-6, CXCL10, CXCL2, CCL2, CCL20 and, TGFβ1i4 genes

	Sham-cooled Splenic-intact	Cooled Splenic-intact		Cooled Splenic-denervated	
			Fold change		Fold change
	ΔCt	ΔCt	($2^{-\Delta\Delta Ct}$)	Δ Ct	($2^{-\Delta\Delta Ct}$)
IL-1B	3.3±0.3	2.0 ± 0.2*	2.5 (2.2 – 2.8)	2.2 ± 0.3*	2.1 (1.7 – 2.7)
IL-6	7.7±0.6	3.9 ± 0.4*	14.4 (11 – 19)	4.0 ± 0.4*	13.0 (10 – 17)
CXCL10	2.7±0.5	0.9 ± 0.3†	3.6 (1.5 – 2.2)	1.9 ± 0.3	1.8 (1.4 – 2.1)
CXCL2	6.9±0.8	3.1 ± 0.4*	13.9 (11 – 18)	2.9 ± 0.4*	16.1 (13 – 22)
CCL2	3.9±0.6	1.4 ± 0.1*	5.5 (5.4 – 5.5)	1.3 ± 0.1*	6.1 (5.3 – 6.5)
CCL20	8.1±0.6	4.8 ± 0.3*	10.0 (8.3 – 12)	5.2 ± 0.3*	7.5 (5.7 – 9.9)
TGFβ1i4	6.4±0.1	6.3 ± 0.04	1.1 (1 – 1.1)	6.4 ± 0.2	1.0 (0.9 – 1.1)

Values are means ± SE; ΔCt = Avg Ct of target gene – Avg Ct of β-actin, $2^{-\Delta\Delta Ct}$ = Fold change in gene expression relative to sham-cooled splenic-intact rats.
*Cooled splenic-intact and cooled splenic-denervated rats significantly differ from sham-cooled splenic-intact rats ($P<0.05$).
†Cooled-splenic-intact rats significantly differ from sham-cooled splenic-intact rats ($P<0.05$).

Figure 12

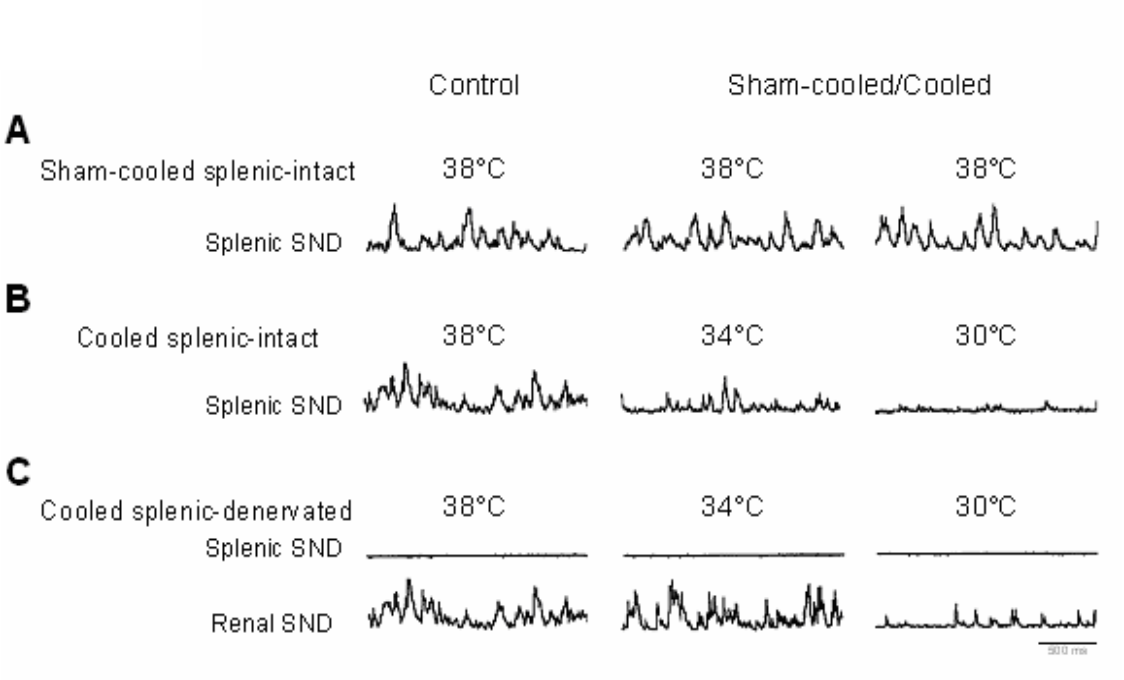

Figure 12. *(A)* Traces of splenic sympathetic nerve discharge (SND) recorded during control (38ºC) and during sham cooling in a splenic-intact rat in which internal body temperature (Tc) was maintained at 38ºC. *(B)* Traces of splenic SND recorded during control (38ºC) and during hypothermia (34ºC and 30ºC) in a splenic-intact rat. *(C)* Traces of splenic and renal SND recorded during control and during hypothermia (34ºC and 30ºC) in a splenic-denervated rat. Horizontal calibration is 500 ms.

Figure 13

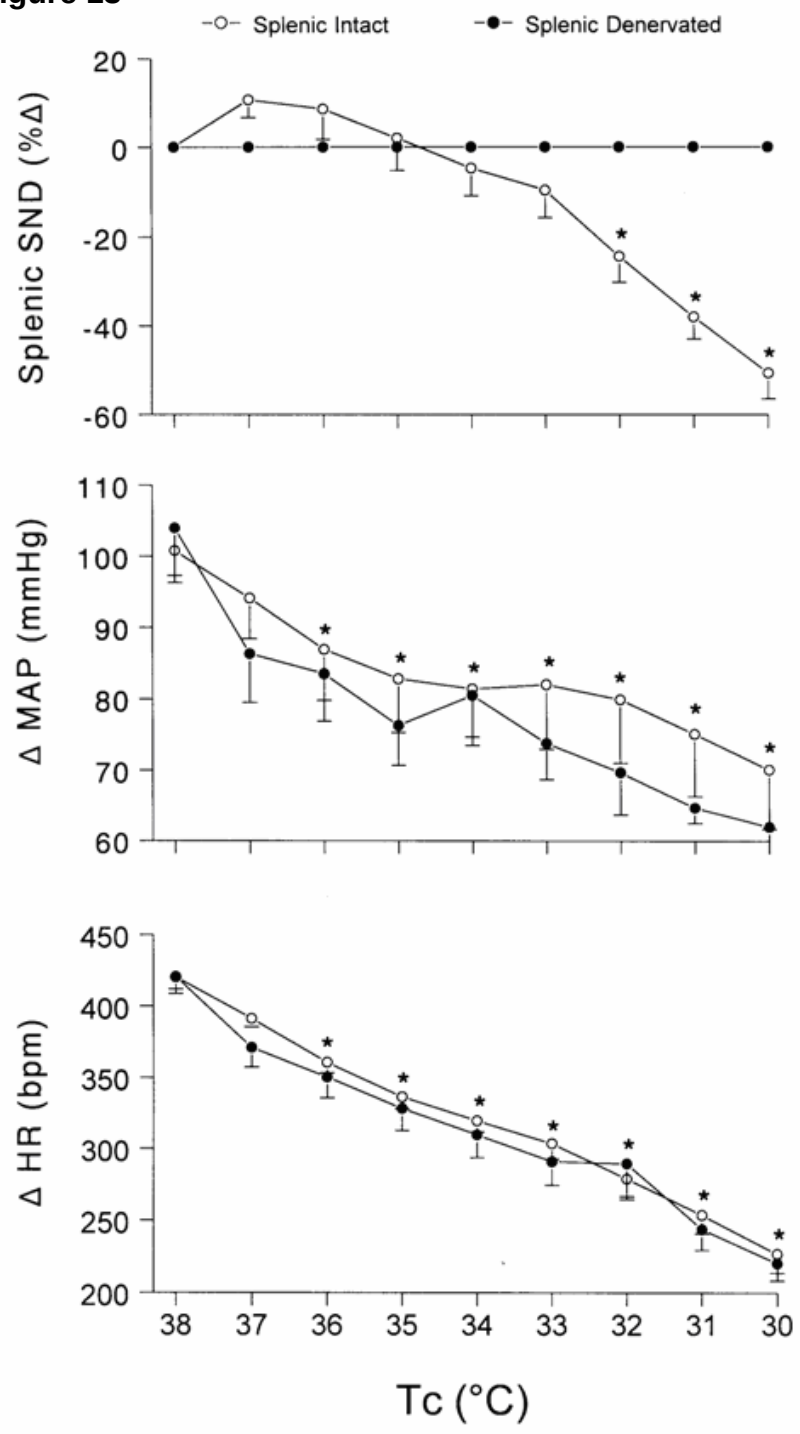

Figure 13. Splenic sympathetic nerve discharge (SND), mean arterial pressure (MAP), and heart rate (HR) during control (38ºC) and hypothermia (Tc decreased from 38ºC to 30ºC) in splenic-intact (open circles) and splenic-denervated (filled circles) rats. *Cooled splenic-intact and cooled splenic-denervated rats significantly different ($P<0.05$) from control values.

Figure 14

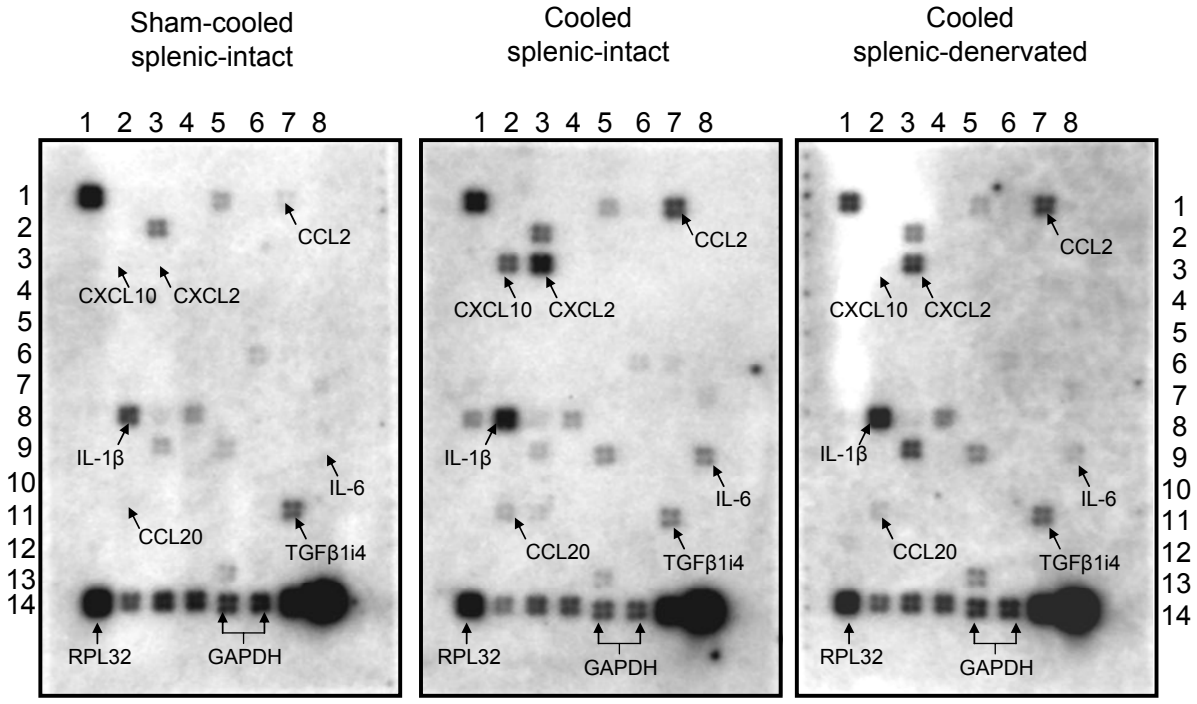

Figure 14. Microarray analysis of inflammatory cytokine gene expression. Microarray analysis was performed on splenic mRNAs using cDNA array blots containing 112 inflammatory cytokines, chemokines, cytokine/chemokine receptors, and housekeeping genes. Representative arrays from sham-cooled splenic-intact, cooled splenic-intact, and cooled splenic-denervated rats are shown. GAPDH and RPL32 served as internal controls. Genes for which detectable changes in mRNA expression were observed in cooled splenic-intact and cooled splenic-denervated rats compared with sham-cooled splenic-intact rats are indicated by the arrows, except for CXCL10 whose gene expression was decreased in the cooled splenic-denervated rat marked by arrow.

Figure 15

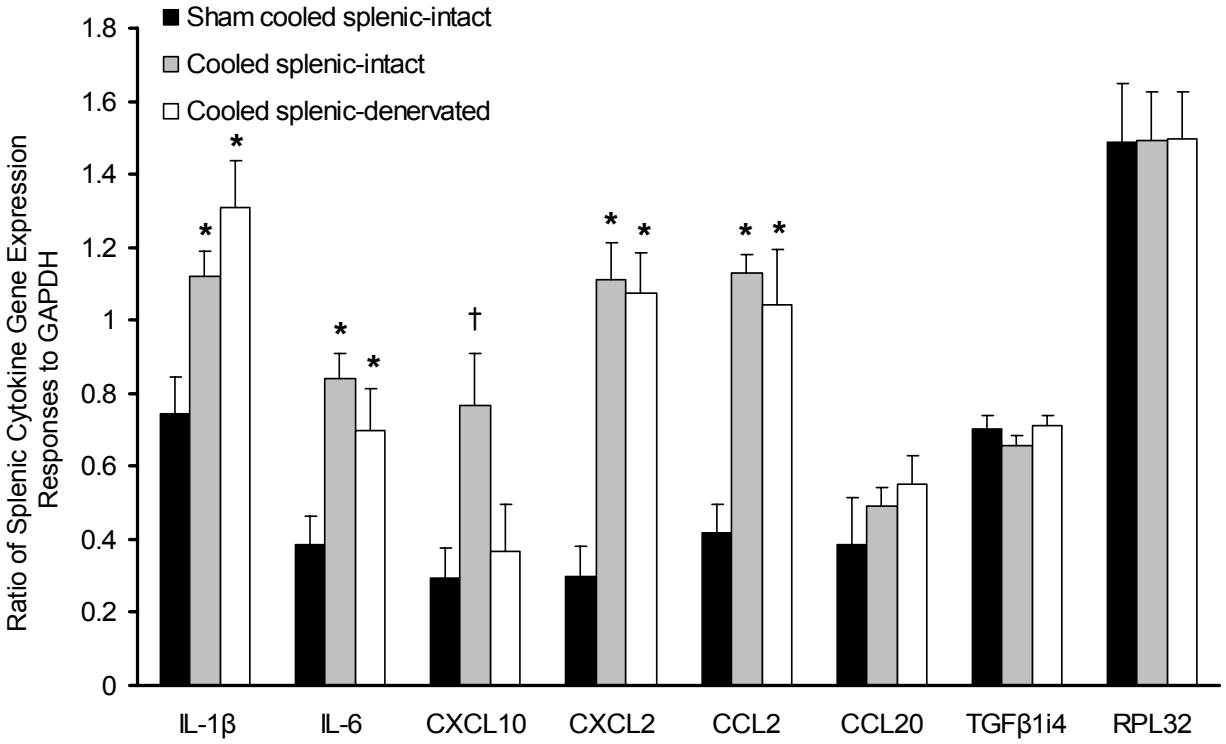

Figure 15. Values of mRNA expression levels of IL-1β, IL-6, CXCL10, CXCL2, CCL2, CCL20, TGFβ1i4, and RPL32 in sham-cooled splenic-intact (black bars), cooled splenic-intact (gray bars), and cooled splenic-denervated (white bars) rats. Gene expression levels are presented relative to GAPDH mRNA expression. *Cooled splenic-intact and cooled splenic-denervated rats significantly different ($P<0.05$) from sham-cooled splenic-intact rats, †Cooled splenic-intact rats significantly different ($P<0.05$) from sham-cooled splenic-intact and cooled splenic-denervated rats.

Figure 16

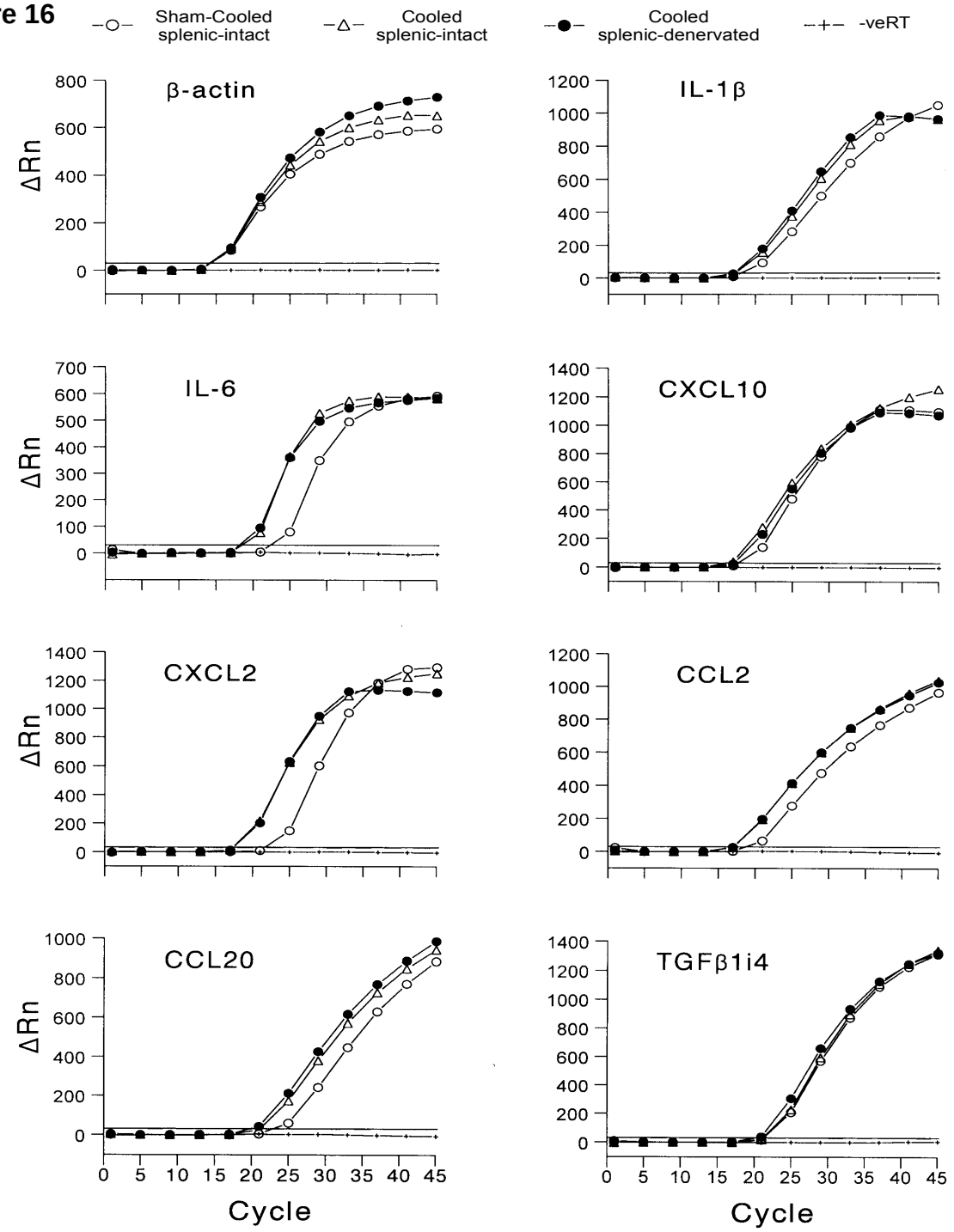

Figure 16. Real-time RT-PCR analysis was performed for β-actin, IL-1β, IL-6, CXCL10, CXCL2, CCL2, CCL20 and, TGFβ1i4 and the amplification plots of representative experiments from three groups of rats (Sham-cooled splenic-intact; Cooled splenic-intact; Cooled splenic-

denervated) are shown. A negative RT control (-veRT) for each primer set is also presented. ΔRn; change in fluroscence.

SUMMARY

Important bi-directional interactions exist between the central nervous system and the immune system. The sympathetic nervous system is considered an important componenet of the bi-directional neuro-immune interactions however, there is no direct evidence to demonstrate the modulatory role of the sympathetic nervous system in neuro-immune interactions. In the current studies we measured splenic nerve recordings and completed surgical splenic nerve denervations to demonstrate the role of splenic SND in splenic cytokine gene expression. In the first study (chapter 1) we demonstrated that hyperthermia-enhanced splenic cytokine gene expression is dependent on heating induced splenic-sympathoexcitation, indicating a role for the sympathetic nervous system in modulating splenic cytokine gene expression. In the second study (chapter 2) we demonstrated the role of central Ang II-induced increases in splenic SND in increasing splenic cytokine gene expression. These findings support the results from the first study demonstrating that enhanced splenic SND increases splenic cytokine gene expression. In addition, splenic blood flow studies demonstrated that enhanced splenic cytokine gene expression to central activation is not dependent on changes in splenic blood flow.

In the third study (chapter 3) the effect of acute cooling on splenic SND and splenic cytokine gene expression was determined. The results from this study showed that progressive hypothermia significantly decreased splenic SND and increased splenic cytokine gene expression in both splenic-intact and splenic-denervated rats, suggesting that hypothermia-induced increases in splenic cytokine gene expression is not dependent on splenic SND. In this study hypothermia significantly decreased splenic SND, according to the results from the studies 1 & 2 we expected to see a decrease in the splenic cytokine gene expression, in contrast, we found a significant increase in the splenic cytokine gene expression in both splenic-inatct and splenic-denervated

rats. These findings demonstrate that the interactions between the sympathetic nervous system and the immune system are very complex and the modulatory role of SND on the immune system system is dependent on the type of stimulus.