CALL FOR PAPERS | Physiology and Pharmacology of Temperature Regulation

Hypothermia-enhanced splenic cytokine gene expression is independent of the sympathetic nervous system

Chanran K. Ganta,1 Bryan G. Helwig,1 Frank Blecha,1 Roman R. Ganta,2 Richard Cober,1 Sujatha Parimi,1 Timothy I. Musch,1 Richard J. Fels,1 and Michael J. Kenney1

1Departments of Anatomy and Physiology and 2Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, Kansas

Submitted 5 December 2005; accepted in final form 31 January 2006

Ganta, Chanran K., Bryan G. Helwig, Frank Blecha, Roman R. Ganta, Richard Cober, Sujatha Parimi, Timothy I. Musch, Richard J. Fels, and Michael J. Kenney. Hypothermia-enhanced splenic cytokine gene expression is independent of the sympathetic nervous system. Am J Physiol Regul Integr Comp Physiol 291: R558–R565, 2006. First published February 9, 2006; doi:10.1152/ajpregu.00846.2005.—Splenic nerve denervation abrogates enhanced splenic cytokine gene expression responses to acute heating, demonstrating that hypothermia-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 30°C) in splenic-intact rats but remained unchanged in sham-cooled splenic-intact rats (core temperature maintained at 38°C). 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.

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 ANG 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 ANG 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 regard 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⁻¹·h⁻¹ 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 37.8°C and 38.0°C by a homeothermic blanket.

**Neural recordings.** After completion of the cannulation procedures, splenic and renal SND was recorded biphasically (bandpass 30–3,000 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 × 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-anaesthetized, gallamine-paralysed, 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 38°C to 30°C. End-tidal CO₂ 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 38°C for an additional 80 min beyond the initial 30 min control period. Mean arterial pressure (MAP), heart rate (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 −80°C. 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, St. Louis, MO). RNA purity and concentration were determined spectrophotometrically by calculating the ratio between the absorbance at 260 nm and 280 nm using a NanoDrop ND-1000 (NanoDrop, Columbus, OH). 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, GAPDH, ribosomal protein L32 (RPL32), 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 by using a TrueLabeling-AMP 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 GEAarray 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 (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 the real-time PCR analysis (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 β1-induced transcript 4 (TGFB1i4) were obtained from Applied Biosystems (Foster City, CA). Primers and probes for β-actin, IL-6 and IL-1β genes were custom synthesized using published sequences (7, 33). TaqMan probes were labeled with 6-carboxyfluorescein as the reporter dye molecule at the 5’ end and 6-carboxy-tetramethyl-rhodamine as the quencher dye molecule at the 3’ end. TaqMan probe-based real-time RT-PCR analysis was performed with 2 μl of cDNA using Universal PCR Master Mix (Applied Biosystems), 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°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.

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/ΔΔCt) − 1], where S is the slope (21). The amplification efficiency was greater than 90% for all genes. The comparative Ct method (2−ΔΔCt) 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 $2^{-\Delta\Delta Ct} = 2^{(\Delta C_{\text{sham-cooled intact}} - \Delta C_{\text{cooled intact}})}$, where $\Delta C_{\text{sham-cooled intact}} = C_{\text{sham-cooled intact}} - C_{\text{control}}$ and $\Delta C_{\text{cooled intact}} = C_{\text{cooled intact}} - C_{\text{control}}$, where $\Delta C_{\text{sham-cooled intact}}$ is the difference between the sample (cooled-intact/cooled-denervated) ΔCt and the control (sham-cooled intact) ΔCt. For sham-cooled intact samples, ΔCt equaled zero and 2$^{-\Delta C}$ 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 $2^{-\Delta C}$ 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 expressed as means ± SE. Control values of SND were taken as 0%. Statistical analysis of SND, MAP, and HR were analyzed using ANOVA 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 blood flow vs. 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 1 shows SND traces from three representative experiments (A, sham-cooled splenic-intact; B, cooled splenic-intact; C, cooled splenic-denervated). Tc was maintained at 38°C during the control period in each rat. In the sham-cooled splenic-intact rat (A), Tc was held constant at 38°C 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 30°C 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 2 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 30°C at a rate of 0.1°C/1 min in both groups. During cooling, splenic SND was decreased significantly from control (38°C) in splenic-intact rats and was undetectable in splenic-denervated rats. MAP and HR were progressively and significantly decreased from control (38°C) during cooling in splenic-intact and splenic-denervated rats (asterisks denote statistically significant 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 38°C for 80 min) splenic-intact rats (Table 1).

**Microarray analysis of splenic gene expression responses to hypothermia.** Figure 3 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 with 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 TGFβ1/4 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, TGFβ1/4, 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 Fig. 4. Expression of IL-1β, IL-6, CXCL2, and CCL2 genes was significantly increased in cooled splenic-intact and cooled splenic-denervated rats compared with 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 TGFβ1/4 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 TGFβ1/4) from three experimental groups of rats; sham-
cooled splenic-intact, cooled splenic-intact, and cooled splenic-denervated rats significantly different (P < 0.05) from control values.

Fig. 2. Splenic SND, mean arterial pressure (MAP), and heart rate (HR) during control (38°C) and hypothermia (Tc decreased from 38°C to 30°C) in splenic-intact (open circles) and splenic-denervated (solid circles) rats. *Cooled splenic-intact and cooled splenic-denervated rats significantly differ from control values.

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 et al. (50) reported that whole body hypothermia reduces renal SND in anesthe-

<table>
<thead>
<tr>
<th>Time, min</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>SND, %</td>
<td>0</td>
<td>4±2</td>
<td>2±4</td>
<td>8±4</td>
<td>7±2</td>
<td>9±3</td>
<td>14±3*</td>
<td>15±3*</td>
<td>19±2*</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>113±4</td>
<td>111±5</td>
<td>111±5</td>
<td>111±5</td>
<td>110±5</td>
<td>112±5</td>
<td>111±5</td>
<td>109±5</td>
<td></td>
</tr>
<tr>
<td>HR, bpm</td>
<td>420±6</td>
<td>418±8</td>
<td>422±7</td>
<td>423±7</td>
<td>425±8</td>
<td>424±8</td>
<td>425±8</td>
<td>426±9</td>
<td>424±9</td>
</tr>
</tbody>
</table>

Values are means ± 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).

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 et al. (50) reported that whole body hypothermia reduces renal SND in anesthe-
tized, 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 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 ANG 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. On the basis of 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 32°C and 33°C 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 κB and increased proinflammatory cytokine gene expression in LPS-
Fig. 5. Real-time RT-PCR analysis was performed for \( \beta \)-actin, IL-1\( \beta \), IL-6, CXCL10, CXCL2, CCL2, CCL20, and TGF\( \beta \)I4, 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. \( \Delta R_n \), change in fluorescence.

Table 2. Ct values and fold change from sham-cooled splenic intact rats for IL-1\( \beta \), IL-6, CXCL10, CXCL2, CCL2, CCL20, and TGF\( \beta \)I4 genes

<table>
<thead>
<tr>
<th></th>
<th>Sham-Cooled Splenic-Intact ( \Delta C_t )</th>
<th>Cooled Splenic-Intact ( \Delta C_t )</th>
<th>Fold Change/Range, ( 2^{-\Delta C_t} )</th>
<th>Cooled Splenic-Denervated ( \Delta C_t )</th>
<th>Fold Change/Range, ( 2^{-\Delta C_t} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1( \beta )</td>
<td>3.3( \pm )0.3</td>
<td>2.0( \pm )0.2*</td>
<td>2.5 (2.2–2.8)</td>
<td>2.2( \pm )0.3*</td>
<td>2.1 (1.7–2.7)</td>
</tr>
<tr>
<td>IL-6</td>
<td>7.7( \pm )0.6</td>
<td>3.9( \pm )0.4*</td>
<td>14.4 (11–19)</td>
<td>4.0( \pm )0.4*</td>
<td>13.0 (10–17)</td>
</tr>
<tr>
<td>CXCL10</td>
<td>2.7( \pm )0.5</td>
<td>0.9( \pm )0.3*</td>
<td>3.6 (1.5–2.2)</td>
<td>1.9( \pm )0.3*</td>
<td>1.8 (1.4–2.1)</td>
</tr>
<tr>
<td>CXCL2</td>
<td>6.9( \pm )0.8</td>
<td>3.1( \pm )0.4*</td>
<td>13.9 (11–18)</td>
<td>2.9( \pm )0.4*</td>
<td>16.1 (13–22)</td>
</tr>
<tr>
<td>CCL2</td>
<td>3.9( \pm )0.6</td>
<td>1.4( \pm )0.1*</td>
<td>5.5 (5.4–5.5)</td>
<td>1.3( \pm )0.1*</td>
<td>6.1 (5.3–6.5)</td>
</tr>
<tr>
<td>CCL20</td>
<td>8.1( \pm )0.6</td>
<td>4.8( \pm )0.3*</td>
<td>10.0 (8.3–12)</td>
<td>5.2( \pm )0.3*</td>
<td>7.5 (5.7–9.9)</td>
</tr>
<tr>
<td>TGF( \beta )I4</td>
<td>6.4( \pm )0.1</td>
<td>6.3( \pm )0.4</td>
<td>1.1 (1–1.1)</td>
<td>6.4( \pm )0.2</td>
<td>1.0 (0.9–1.1)</td>
</tr>
</tbody>
</table>

Values are means \( \pm \) SE; \( \Delta C_t \) = Avg threshold cycle (Ct) of target gene-Avg Ct of \( \beta \)-actin. \( 2^{-\Delta C_t} \) = Gene expression fold change for cooled splenic-intact and cooled splenic-denervated rats relative to sham-cooled splenic-intact rats. CXCL, chemokine (C-X-C motif) ligand. CCL, chemokine (C-C motif) ligand.

*\(^{\text{Cooled splenic-intact and cooled splenic-denervated rats significantly different from sham-cooled splenic-intact rats (} P < 0.05\). \text{\textsuperscript{t}Cooled-splenic-intact rats significantly different from sham-cooled splenic-intact rats (} P < 0.05\). \text{\textsuperscript{t}}\)
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); 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 while splenic cytokine gene expression is increased, and this enhanced splenic cytokine gene expression is independent of splenic sympathetic innervation. As stated previously, the results of our recent study indicate that whole body hyperthermia increases the expression of selective splenic cytokine genes (19), an effect that is abrogated by splenic denervation. Although the role of the sympathetic innervation to the spleen in mediating enhanced splenic gene expression differs between hypothermia and hyperthermia, it appears that both increases and decreases in internal body temperature provide a potent stimulus to splenic cytokine gene expression.

**GRANTS**

This study was supported by National Heart, Lung, and Blood Institute Grants HL-65346 and HL-69755 to M.J. Kenney.

**REFERENCES**

19. Ganta CK, Blecha F, Ganta RR, Helwig BG, Parimi S, Lu N, Fels RJ, Musch TI, and Kenney MJ. Hypothermia-enhanced splenic cytokine...


