Increased interleukin-6 receptor expression in the paraventricular nucleus of rats with heart failure

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The paraventricular nucleus (PVN) of the hypothalamus is a forebrain nucleus with projections to brainstem and spinal cord regions regulating cardiovascular homeostasis (10). The PVN is also a key component of the hypothalamic-pituitary-adrenal (HPA) axis. Using cytokines as messengers, bidirectional communication between the nervous and immune systems occurs along the HPA axis (16, 17, 39, 41, 54, 59). Among the cytokines, IL-6 plays a significant role in HPA activation by stimulating the PVN neurons, an effect that is dependent upon transcription of the IL-6R (62). Thus, the initial steps in understanding neural-immune links between IL-6 and the PVN in the inflammatory state of HF are dependent upon determination of PVN IL-6R expression.

Changes in IL-6R expression are influenced by processing of the immature form of IL-6R, glycosylation of the protein, and generation of splice variants, resulting in expression of a soluble form of the IL-6R (9). Consistent with the majority of proteins (8, 56), analysis of IL-6R mRNA expression for IL-6R only provides limited information about changes in the presence of the functional protein. As a result, the current study employed both gene and protein analyses to test the hypothesis that PVN IL-6R protein and gene expression are upregulated in HF vs. sham-operated rats. Gene and protein levels of the gp130 subunit were not altered by HF in either tissue analyzed. Collectively, these data suggest that within the brain of HF rats, IL-6R expression is not a global change. Rather the increased IL-6 levels characteristic of HF may alter PVN-mediated physiological responses via enhanced expression of the IL-6R.

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Rats were anesthetized initially with a 5% isoflurane-95% oxygen mixture. Rats were intubated, connected to a rodent respirator (model 680, Harvard Apparatus), and maintained on 2% isoflurane-98% oxygen mixture. The heart was exteriorized, following a left-side thoracotomy, through an opening between the fifth and sixth rib and the pericardial sac opened. The left main coronary artery was ligated between the pulmonary artery and left atrium, as described previously (24). Briefly, a 6–0 silk suture was placed around the left main coronary artery and tied. Sham operations were completed using the same surgical procedures, except that the left main coronary artery was not ligated. The lungs were then hyperinflated and the ribs approximated with 3–0 gut suture. Muscles of the thorax were sewn together with 4–0 gut suture, and the skin incision was closed with a 3–0 silk suture. Ampicillin (50 mg/kg sc) and buprenorphine (0.05 mg/kg sc) were administered to each animal. Anesthesia was withdrawn, and the animals were extubated. All rats were returned to individual cages. Each rat received ampicillin and buprenorphine (0.05 mg/kg sc) were administered to each animal. The plasma portion was collected, the plasma mixture was used for the real-time PCR analysis of specific cytokine expression. The reaction was carried out for 60 min at 37.0°C, and the cDNA mixture was used for the real-time PCR analysis of specific cytokine receptor gene expression.

**Immunoblotting.** Protein expression was analyzed via immunoblotting protocols as described previously (24). Equivalent amounts of protein were incubated with Laemmli sample buffer at 95°C for 5 min and separated via SDS PAGE using a 4–15% gel (Bio-Rad, Hercules, CA) containing 10, 12, or 15 wells. Upon completion of the run, proteins were transferred to nitrocellulose membranes via electrophoretic transfer in a tank system with plate electrodes. Membranes were blocked for 1 h at room temperature in 5% nonfat dry milk and Tris-buffered saline containing 0.1% Tween-20 (TBS-Tween). Membranes were then washed and incubated, with agitation, in IL-6R (Santa Cruz Biotechnology), gp130 (Santa Cruz Biotechnology), or actin (Sigma, St. Louis, MO) primary antibody for 1 h at room temperature (IL-6R and actin) or overnight at 4°C (gp130). Membranes were washed five times with 0.1% TBS-Tween. Horseradish peroxidase-labeled secondary antibody was added, and membranes were incubated at room temperature for 1 h. Following five washes with TBS-Tween blots were visualized with chemiluminescence (Pierce Chemical, Rockford, IL) and recorded on radiographic film. After detection of either IL-6R or gp130, the membrane was probed for actin. Appropriate primary and secondary antibody concentrations, incubation times, protein loads, and chemiluminescence reagents were established before collection of experimental data. Whole brain lysates were used as a positive control in each immunoblotting experiment.

**Densitometry.** Expression level of proteins in tissues was estimated via densitometry analysis using Alpha Ease Image Software (Alpha Innotech, San Leandro, CA). Films from gels loaded with identical amounts of protein and consistent exposure parameters were assigned arbitrary bands. Band density for gp130 and IL-6R was normalized to the actin value for each sample, and the means of each group compared. Immunoblotting and densitometry analyses were run in duplicate to ensure validity.

**RNA isolation.** Total RNA was isolated from frozen PVN and non-PVN samples (<5 mg) were homogenized in liquid nitrogen and total RNA was isolated using Tri Reagent, as described in the included instructions. RNA purity and concentration were determined spectrophotometrically by calculating the ratio between the absorbance at 260 and 280 nm using a NanoDrop ND-1000 (NanoDrop, Wilmington, DE). The absorbance ratio for all samples ranged between 1.8 and 2.0.

**Real-time RT-PCR analysis.** Expression changes in IL-6R and gp130 mRNA were determined using TaqMan probe-based real-time RT-PCR analysis. 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 37.0°C, and the cDNA mixture was used for the real-time PCR analysis of specific cytokine receptor gene expression.

Gene-specific PCR primer pairs and TaqMan probes for IL-6R and gp130 were obtained from Applied Biosystems (Foster City, CA). Primers and probes for β-actin were custom synthesized using published sequences (5, 31). TaqMan probes were labeled with 6-carboxyfluorescein as the reporter dye molecule at the 5’ end and 6-carboxy-tetramethylrhodamine 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) under 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. Gene expression levels were calculated as fold change relative to the gene expression of sham-operated rats. The PCR amplification efficiencies of β-actin and the target genes were calculated using the following formula: PCR efficiency = \(10^{\frac{1}{S}} - 1\) 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 \(\Delta Ct\) (Ct of target gene – Ct of β-actin gene). The fold change was calculated as \(2^{\Delta\Delta Ct} = \text{CAvg} - \text{CAvg}\text{Sham}\), where \(\text{CAvg}\) is the average Ct of the target gene in each group, and \(\text{CAvg}\text{Sham}\) is the average Ct of the target gene in the sham group.

**Data analysis.** Values are expressed as means ± SE. Results were compared between groups using Student’s t-tests. Significance for all tests was \(P < 0.05\).

## RESULTS

**Physical characteristics and hemodynamic measurements.** Physical characteristics and hemodynamic variables are presented in Table 1. LVEDP was significantly elevated in HF rats compared with sham-operated controls. Coronary ligated rats were further divided into moderate (LVEDP < 20 mmHg) and severe (LVEDP > 20 mmHg) HF groups. Right ventricle-to-body weight ratio did not differ between sham-operated controls and moderate HF rats but was significantly elevated in severe HF rats vs. moderate HF and sham-operated controls, indicating the presence of chronic HF. The presence of congestive HF was determined by the lung weight-to-body weight ratios, which were significantly elevated in severe HF compared with both sham-operated and moderate HF rats. Thus, severe HF rats \((n = 8)\) were characterized by chronic congestive cardiac failure, while moderate HF \((n = 7)\) rats exhibited noncongestive failure.

**Plasma IL-6 levels.** Plasma IL-6 levels were not different between sham-operation rats and rats with moderate HF. Comparison of IL-6 levels between sham-operated and severe HF groups revealed that IL-6 levels were significantly higher in rats with severe HF (Fig. 1). The plasma levels of IL-6 were not statistically different between rats with moderate HF and severe HF \((P = 0.08, \text{Fig. 1})\).

**Table 1. Physical and hemodynamic indices in sham-operated, moderate HF, and severe HF rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Weight, g</th>
<th>HR, beats/min</th>
<th>MAP, mmHg</th>
<th>LVEDP, mmHg</th>
<th>LV dP/dt, mmHg</th>
<th>LV Weight/ Body Weight, mg/g</th>
<th>RV Weight/ Body Weight, mg/g</th>
<th>Lung Weight/ Body Weight, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>7</td>
<td>446 ± 13</td>
<td>333 ± 17</td>
<td>95 ± 4</td>
<td>9 ± 1</td>
<td>7657 ± 285</td>
<td>2.17 ± 0.04</td>
<td>0.55 ± 0.08</td>
<td>4.12 ± 0.32</td>
</tr>
<tr>
<td>Mod HF</td>
<td>7</td>
<td>419 ± 9</td>
<td>340 ± 3</td>
<td>96 ± 5</td>
<td>17 ± 1*</td>
<td>7157 ± 312</td>
<td>2.38 ± 0.03*</td>
<td>0.55 ± 0.02</td>
<td>4.56 ± 0.39</td>
</tr>
<tr>
<td>Sev HF</td>
<td>8</td>
<td>410 ± 6*</td>
<td>322 ± 7</td>
<td>96 ± 4</td>
<td>31 ± 2*†</td>
<td>5363 ± 267†</td>
<td>2.33 ± 0.05*</td>
<td>0.97 ± 0.08†</td>
<td>9.53 ± 1.00†</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; \(n\), number of rats; HF, heart failure; Sham, sham-operated rats; Mod, Moderate; Sev, Severe; HR, heart rate; MAP, mean arterial pressure; LVEDP, left-ventricular end-diastolic pressure; LV dP/dt, LV change in pressure over time; RV, right ventricular. *\(P < 0.05\) vs. Sham. †\(P < 0.05\) vs. Moderate HF.

**Real-time PCR (mRNA expression).** Real-time RT-PCR analysis of IL-6R, gp130, and β-actin transcript expression was performed on tissue from PVN and from cortical regions dorsal to the PVN (non-PVN). IL-6R mRNA expression normalized to β-actin did not differ between sham-operated, moderate HF, and severe HF rats for PVN (Fig. 2A) and non-PVN (Fig. 2B) tissues. Furthermore, IL-6R mRNA expression in PVN and non-PVN tissue was not significantly different in moderate vs. severe HF rats. Expression of mRNA for the β-subunit of the IL-6R complex, gp130, was also normalized to β-actin for all groups and is presented in Fig. 3. Consistent with results for IL-6R mRNA expression, gp130 mRNA expression in PVN (Fig. 3A) and non-PVN (Fig. 3B) tissue was not different in moderate and severe HF rats when compared with sham-operated controls and did not differ in moderate vs. severe HF rats.

**Immunoblot analysis (protein expression).** Representative Western blots for actin expression in PVN and non-PVN tissue are shown in the top panels of Fig. 4, A and B, respectively. Actin expression was determined for all samples and was not different between groups in either PVN (4A, bottom) or non-PVN (4B, bottom) brain punches. IL-6R and gp130 PVN protein expression are shown in Fig. 5. Representative IL-6R protein expression in PVN tissue is shown in Fig. 5A (top). As estimated by densitometry analysis, IL-6R protein expression was significantly increased \((P < 0.05)\) within the PVN of moderate and severe HF rats compared with sham-operated controls (Fig. 5A, bottom). However, expression of IL-6R was not different between moderate HF and severe HF groups. A representative blot of PVN gp130 expression is shown in Fig. 5B (top). Densitometry analysis revealed that PVN expression of the gp130 protein did not differ in sham-operated, moderate HF, and severe HF rats (Fig. 5B, bottom).

Specificity of changes in PVN protein expression was further determined via analysis of non-PVN brain punches. Cor-
tical regions, dorsal to the PVN, were punched and analyzed for IL-6R and gp130 protein expression. Representative blots of IL-6R and gp130 expression in non-PVN punches are shown in Figs. 6, A (top) and B (top), respectively. Densitometry analysis of protein expression in non-PVN tissue revealed no changes in IL-6R (Fig. 6A, bottom) or gp130 (Fig. 6B, bottom) between sham-operated, moderate HF, and severe HF rats.

DISCUSSION

The current study revealed four new findings. First, expression of the IL-6R protein is significantly upregulated in the PVN of rats with HF compared with sham-operated controls. The enhanced IL-6R protein expression was region specific as sham-operated, moderate HF, and severe HF rats all expressed consistent amounts of IL-6R protein in non-PVN tissue. Second, HF-induced changes in PVN IL-6R protein expression were independent of changes in mRNA expression. Third, expression of gp130 at both the gene and protein level was not altered in PVN or non-PVN tissue from sham-operation, moderate HF, or severe HF rats. Fourth, plasma levels of IL-6 were significantly elevated in rats with severe chronic congestive HF, but not in rats with moderate noncongestive HF or in sham-operated controls. These findings demonstrate that in rats with HF, enhanced expression of IL-6 is accompanied by increased levels of the IL-6R protein in the PVN. Together, these characteristics of HF create a scenario that may allow for modulation of PVN-mediated physiological actions via IL-6.

The PVN of the hypothalamus is a key component of the HPA axis, participating in neural-immune interactions. A novel finding in the current study was that, in rats with HF, expression of PVN IL-6R protein was significantly increased compared with sham-operated rats. Because IL-6 stimulates the release of hormones from the organs of the HPA-axis, (38, 44, 51), increased expression of the IL-6R may potentiate cytokine-mediated effects within the PVN, resulting in chronic activation of the HPA axis. Specifically, sustained increases in IL-6 expression are known to prolong neural activation of parvocellular neurons in the PVN, resulting in augmented secretion of CRF, an effect that has been shown to be dependent upon expression of the IL-6R (61). Furthermore, IL-6 stimulation of PVN magnocellular neurons results in release of arginine vasopressin, a secretagogue that is also a well-defined activator of the HPA axis (40). Both CRF and arginine vasopressin are released into the hypophyseal portal system and...
activate the HPA axis by augmenting ACTH release from the anterior pituitary. Subsequent stimulation of the adrenal gland results in the secretion of glucocorticoids.

Exogenous administration of a second class of CRF-peptide family members, termed urocortins (Ucn), have recently been demonstrated to provide positive inotropic effects in heart failure (3, 7). Endogenous Ucn has been shown to be mainly synthesized in cardiac tissue (30) and is known to exist in three subtypes, termed Ucn I, II, and III (32, 50, 63). All CRF-peptide family members improve cardiac function via binding to cardiac CRF2 receptors when administered intravenously. In addition, consistent with CRF-mediated responses, Ucn I has been shown to stimulate ACTH release in healthy rats (63) and humans (12), as well as in sheep (49) and humans (11) with HF via binding to the CRF1 receptor in the PVN. Although the influence of HF on endogenous Ucn-1 production is unknown, it is possible that Ucn-1 concomitantly with CRF may contribute to the neural-humoral activation characteristic of HF as the CRF1 and CRF2 receptor subtypes (11) for Ucn-1 and CRF are expressed in PVN tissue (26). Furthermore, Ucn-1 release is known to induce IL-6 expression in aortic smooth muscle cells (28). Thus, Ucn-1 and IL-6 may work in tandem and target the PVN, thereby contributing to the neural-humoral activation characteristic of HF.

The PVN contains magnocellular neurons, parvocellular neurons, and glial cells; however, because of the limited amount of tissue derived from the micropunch technique, the contribution of each to the increased IL-6R expression in the PVN of HF rats could not be determined. However, the current study was designed to determine global changes in IL-6R expression within the PVN and not to establish contributions from individual neuronal populations. The micropunch technique has been used in previous work from Li et al. (33) to obtain tissue from the PVN of rats with HF. In addition, Durgam and Mifflin (15) have also utilized the micropunch technique to isolate tissue from the PVN and the nucleus of the solitary tract. The unique anatomy of the PVN combined with the fact that the micropunch technique is an accepted method...

Fig. 4. Actin protein expression in PVN (A) and non-PVN (B) tissue from sham-operated, moderate HF, and severe HF rats. Mean densitometry values for actin in PVN (A, bottom) and non-PVN (B, bottom) tissue from all animals was compared between moderate HF, severe HF, and sham-operated rats. Samples in all rats were run in duplicate to ensure validity of measurements. Numbers below the Western blots serve as lane markers and represent different animals. For sham-operated control rats, n = 7; moderate HF, n = 7; and severe HF, n = 8.
of isolating tissue from specific brain nuclei (15, 33, 47) validates use of this collection technique in the current manuscript. Together, the increased expression of PVN IL-6R protein combined with the established actions of IL-6 (38, 44, 51) provides a pathway by which the cytokine may mediate HPA-axis activation in the HF state. The possibility of IL-6 activating the HPA axis by directly targeting the PVN is further supported because increased IL-6 levels following endotoxemia administration promote colocalization of the IL-6R in discrete brain nuclei, including the PVN (52, 61). Increased expression of both plasma IL-6 and receptor protein in the current study are novel findings in the severe chronic congestive rat model of HF. Work performed in healthy human skeletal muscle (29) has revealed that following an infusion of recombinant human IL-6 or an acute bout of exercise, plasma IL-6 and skeletal muscle IL-6R are upregulated. Furthermore, IL-6 levels are attenuated via participation in a chronic exercise program (18) without a change in IL-6R levels. These findings suggest that in addition to ligand and receptor protein expression, alterations in IL-6 and IL-6R binding kinetics may account for alterations in protein expression in health, exercise, and disease. In contrast to the PVN, comparison of IL-6R protein expression from cortical regions (i.e., non-PVN) between sham-operated, moderate HF, and severe HF rats revealed no changes in IL-6R protein expression 6 wk after coronary ligation surgery. Collectively, results of gene and protein work in the current study indicate that upregulation of IL-6R protein within the PVN of rats with HF is not indicative of changes in whole brain IL-6R expression. Rather, it appears IL-6R expression changes IL-6 may selectively target brain regions.

Although the primary goal of the current study was to determine whether HF is characterized by increased IL-6R expression in the PVN, plasma levels of IL-6 were also determined in all groups of rats, as it is well established that in human HF patients, plasma levels of IL-6 are increased (2, 42, 57, 58). Consistent with data from human HF patients, the current results revealed that in rats with severe chronic congestive HF, plasma levels of IL-6 were significantly elevated. On the basis of our criteria for severity of HF, previous work reporting plasma IL-6 levels in rats with HF appears to have been performed in rats with moderate noncongestive HF. For
instance, Schulze et al. (53), investigating cytokine-modulated expression of insulin-growth factor, determined that plasma IL-6 levels were not elevated in rats with HF compared with control rats. Additionally, work by Lou et al. (36) also failed to show an increase in plasma IL-6 levels in rats with adriamycin-induced cardiomyopathy leading to HF. Consistent with these findings rats with moderate noncongestive HF in the current study did not display elevations in plasma IL-6 levels. In addition to the finding that plasma IL-6 levels were increased in rats with severe chronic congestive HF it has also been shown that in response to lipopolysaccharide administration IL-6 mRNA is expressed in brain nuclei (52, 61). Furthermore, astrocytes and microglia cells can be stimulated to produce IL-6 (4, 19). Thus, in HF sources of IL-6 that may serve as a ligand for the IL-6R in the PVN include plasma, glial cells, and neurons.

Although protein expression of the IL-6R in the PVN was increased in HF rats compared with sham-operation controls, mRNA expression did not differ between groups. The cellular events leading to this discrepancy are not known, however, these results are not unusual of mRNA and protein studies. Comparison between mRNA and protein expression reveals that less than 40% of transcripts accurately reflect the corresponding protein level (8, 56). Furthermore, of those proteins with a high degree of correlation to mRNA levels, the majority were the most abundantly expressed proteins; and the correlation between mRNA and protein expression was further reduced in diseased states (8, 56). The low correlation is largely attributed to splice variants and posttranslational modifications that occur as proteins mature. Events during translation of the IL-6R result in both soluble and membrane-bound forms of the protein. Splicing of mRNA transcripts or proteolytic shedding of a mature IL-6R can give rise to a soluble form of the receptor consisting of an extracellular domain but lacking the intracellular and transmembrane domains; portions that are present in membrane-bound IL-6R (27). With regards to posttranslational modifications, following biosynthesis of soluble and membrane IL-6R, the extracellular domain undergoes glycosylation resulting in a biologically active form of the protein (9). Because both soluble and membrane forms of the IL-6R are able to mediate cellular responses through gp130, determination of the predominantly expressed IL-6R subtype
was not an aim of the current study. However, the majority of expressed IL-6R in the current study is likely the membrane form as Keller et al. (29) found that in human skeletal muscle an overwhelming majority of expressed IL-6R protein resides at the cellular membrane. Finally, additional discrepancies in IL-6R protein and gene expression may be attributed to the fact that maintenance or re-establishment of physiological homeostasis following trauma is achieved by removal of excess cytokines; (23) a process dependent upon increased expression of the mature IL-6R protein.

Upon binding of IL-6 to the alpha receptor, IL-6R, the signal transducing membrane protein gp130 associates with the complex (6, 43). Because the IL-6R is a nonsignaling subunit, recruitment of gp130 to the complex is a critical step that initiates signal transduction in the target cell. In the current study the transcribed and translated forms of gp130 were not different between sham-operation, moderate HF, and, severe HF rats in each of the tissues examined. This finding was not surprising as gp130 is required for signal propagation in all IL-6 family cytokines, resulting in ubiquitous cellular expression of the gp130 subunit. Furthermore, the only known function of the alpha subunit of the complex (e.g., IL-6R) is to render the cell responsive to IL-6 (22). Thus alterations in the IL-6R protein would affect all downstream signaling events and have significantly greater effects on IL-6 mediated actions compared with the gp130 subunit.

In summary, the current study found that in rats with chronic congestive HF, PVN expression of IL-6R protein was up-regulated, a finding that was not characteristic of non-PVN tissue from the same rats. Additionally, gene and protein expression of gp130 was not altered by HF in PVN or non-PVN tissues. Together these results suggest that in HF increased IL-6R expression provides an augmented route by which IL-6 may contribute to PVN-mediated physiological changes.

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INTERLEUKIN 6 RECEPTOR EXPRESSION IN HEART FAILURE


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