Increased Cyclooxygenase-2 Expression in Hypothalamic Paraventricular Nucleus in Rats With Heart Failure
Role of Nuclear Factor κB

Yang Yu, Yu-Ming Kang, Zhi-Hua Zhang, Shun-Guang Wei, Yi Chu, Robert M. Weiss, Robert B. Felder

Abstract—We investigated the role of nuclear factor κB (NF-κB) in the cytokine-mediated induction of cyclooxygenase-2 activity in the paraventricular nucleus of hypothalamus (PVN), a critical cardiovascular and autonomic center, in rats with heart failure (HF). Sprague–Dawley rats underwent coronary artery ligation to induce HF or sham surgery. HF rats were treated orally for 6 weeks with vehicle (tap water), the NF-κB inhibitor pyrrolidine dithiocarbamate (150 mg/kg per day), or the mineralocorticoid receptor antagonist eplerenone (30 mg/kg per day), which has been shown to reduce circulating proinflammatory cytokines in this model. Compared with sham surgery, HF rats had higher (P<0.05) levels of aldosterone, interleukin-1β and norepinephrine in plasma and prostaglandin E2 in cerebrospinal fluid. In the PVN, NF-κB p50 precursor p105 mRNA increased, and mRNA for its inhibitor, IκB, decreased (P<0.05). Cyclooxygenase-2 mRNA and protein expression was increased in perivascular cells of the PVN. Both pyrrolidine dithiocarbamate and eplerenone reduced (P<0.05) p105 mRNA and increased IκB mRNA in PVN. Both also reduced (P<0.05) cyclooxygenase-2 mRNA and protein expression in PVN, cerebrospinal fluid prostaglandin E2, and plasma norepinephrine. Eplerenone, but not pyrrolidine dithiocarbamate, reduced plasma interleukin-1β. Pyrrolidine dithiocarbamate and eplerenone had no effect on plasma aldosterone. The results suggest that activation of NF-κB is an intermediary step in cytokine-mediated induction of cyclooxygenase-2 in the PVN of HF rats. By enhancing access of prostaglandin E2 to hypothalamic neurons, this mechanism may contribute to augmented sympathetic nerve activity in HF. (Hypertension. 2007;49:511-518.)

Key Words: cytokines ■ mineralocorticoid receptor ■ cyclooxygenase-2 ■ nuclear factor-kappa B ■ heart failure

Nuclear factor κB (NF-κB) is a ubiquitously present transcription factor that regulates the expression of multiple inflammatory and immune response genes, including cyclooxygenase-2 (COX-2).1,4 NF-κB normally resides in the cytoplasm in an inactive form, as a heterodimer composed of p50 and p65 subunits, bound to inhibitory proteins (IκB). Multiple stimuli, including proinflammatory cytokines and reactive oxygen species, can rapidly phosphorylate IκB, resulting in its ubiquitination and proteolytic degradation.5,6 This permits translocation of the NF-κB complex to the nucleus, where it activates gene transcription.5,6 Mineralocorticoid (MR) antagonists can inhibit NF-κB activity and reduce NF-κB–mediated gene expression in experimental heart failure (HF), hypertension, and diabetes mellitus.1,7,8 The precise mechanism for this effect is unknown. Recent work in our laboratory has produced several relevant observations: (1) systemic administration of the MR agonist deoxycorticosterone acetate elicits a centrally mediated increase in circulating proinflammatory cytokines in normal rats;9 (2) central administration of the MR antagonist spironolactone substantially reduces circulating proinflammatory cytokines in rats with ischemia-induced HF;10 and (3) systemic administration of the MR antagonist eplerenone to HF rats lowers circulating cytokines and COX-2 expression in vessels supplying the paraventricular nucleus (PVN) of the hypothalamus,11 a key cardiovascular regulatory region of the brain.12,13 These observations are linked by the well-known effect of circulating cytokines to induce vascular COX-2 expression.14

COX-2 promotes the synthesis of prostaglandin E2 (PGE2),15 which acts within the brain to augment sympathetic drive16,17 and, thus, may contribute to the pathophysiology of HF. The present study explores the role of NF-κB as a putative mediator of COX-2 expression in the microvasculature of the PVN in rats with ischemia-induced HF and the potential involvement of perivascular cells in this process.

Methods

Animals

Adult male Sprague–Dawley rats weighing 350 to 375 g were obtained from Harlan Sprague Dawley Inc. They were housed in...
temperature- and light-controlled animal quarters and were provided with rat chow ad libitum. These studies were performed in accordance with the American Physiological Society Guiding Principles for Research Involving Animals and Human Beings.\textsuperscript{18} The experimental procedures were approved by the University of Iowa Institutional Animal Care and Use Committee.

Rats were anesthetized (ketamine 90 mg/kg + xylazine 10 mg/kg, IP) and underwent coronary ligation to induce HF (n = 63) or sham coronary ligation ([SHAM] n = 11), as described before.\textsuperscript{10,19} Twenty-five rats died within 24 hours of coronary ligation. All of the surviving rats underwent echocardiography under sedation (ketamine 25 mg/kg, IP) to assess left ventricular (LV) function and the extent of ischemic injury. Measurements of ischemic zone as a percentage of LV circumference, LV ejection fraction, and LV end-diastolic volume were made as described previously.\textsuperscript{10,19} Rats with a large myocardial infarction, defined by ischemic zone \( \geq 39\% \), were started immediately on treatment with vehicle ([VEH] tap water; n = 12), the MR receptor antagonist eplerenone ([EPL] 30 mg/kg per day; dose range: 27 to 36 mg/kg per day; n = 12), or the NF-\( \kappa \)-B inhibitor pyrrolidine dithiocarbamate ([PDTC] \( \approx 150 \) mg/kg per day; dose range: 135 to 181 mg/kg per day; n = 9) in tap water. Five rats with small myocardial infarctions were excluded from the study. SHAM rats received VEH treatment (n = 11) only. The dose for EPL was chosen from previous studies in which this dose was effective in vivo blockade of MR in rats.\textsuperscript{20,21} The PDTC dose for EPL was chosen from previous studies in which this dose was effective in vivo blockade of MR in rats.\textsuperscript{20,21} The dose range: 135 to 181 mg/kg per day; n = 9)

### Molecular Studies

Brain tissue was cut into 450-\( \mu \)m coronal sections. A punch biopsy was obtained from right and left PVN and from right and left cortex (in the same section) using a 15-gauge needle stub (ID: 1.5 mm) according to a previously described method.\textsuperscript{23} Tissues were homogenized in TRI Reagent (Molecular Research Center, Inc). The total RNA and protein were extracted according to the manufacturer’s instruction.

### Multiplex PCR

Total RNA was reverse transcribed into cDNA. The mRNA expression of NF-\( \kappa \)-B p50 precursor p105 and IkB was measured by multiplex PCR according to a method described previously.\textsuperscript{24} The intensity of each band was analyzed by National Institutes of Health image analysis software and normalized by GAPDH.

### Western Blot

COX-2 protein levels were measured with a Western blotting technique as described by others.\textsuperscript{26} The density of the bands was quantified using National Institutes of Health image analysis software.

### Immunofluorescent Studies

Four rats in each group were perfused transcardially with heparinized saline (30 U/mL; 100 mL) followed by 4% paraformaldehyde.

#### Analysis of Blood and CSF

Plasma aldosterone (ALDO) level was measured using a radioimmunoassay kit (Diagnostic Products Corporation). Plasma levels of interleukin-1\( \beta \) (IL-1\( \beta \)) and norepinephrine (NE) and CSF levels of PGE\(_2\) were measured using high-sensitivity ELISA kits (BioSource International, Inc; Rocky Mountain Diagnostics, Inc; and R&D Systems Inc, respectively).

#### Implantation Of Tissue and Administration Of Drugs

PGE\(_2\) and NF-\( \kappa \)-B inhibitor pyrrolidine dithiocarbamate ([PDTC] \( \approx 150 \) mg/kg per day; dose range: 135 to 181 mg/kg per day; n = 9) in tap water.

### Anatomic Assessment of HF

Wet lung weight and right ventricular weight, with respect to body weight, were measured as indices of pulmonary congestion and right ventricular remodeling, two indices of the severity of HF.

#### Echocardiographic, Hemodynamic, and Anatomical Measurements

<table>
<thead>
<tr>
<th>Variables at Baseline</th>
<th>SHAM (n = 11)</th>
<th>HF + V (n = 12)</th>
<th>HF + E (n = 12)</th>
<th>HF + P (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDV, mL</td>
<td>0.34 \pm 0.04</td>
<td>0.96 \pm 0.12*</td>
<td>0.94 \pm 0.15*</td>
<td>0.95 \pm 0.11*</td>
</tr>
<tr>
<td>LVEF</td>
<td>0.84 \pm 0.03</td>
<td>0.32 \pm 0.05*</td>
<td>0.29 \pm 0.04*</td>
<td>0.31 \pm 0.05*</td>
</tr>
<tr>
<td>%IZ</td>
<td>—</td>
<td>51.12 \pm 1.40</td>
<td>52.58 \pm 3.26</td>
<td>50.47 \pm 2.64</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables at 6 weeks</th>
<th>SHAM (n = 7)</th>
<th>HF + V (n = 8)</th>
<th>HF + E (n = 8)</th>
<th>HF + P (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>413 \pm 8</td>
<td>409 \pm 4</td>
<td>411 \pm 13</td>
<td>410 \pm 16</td>
</tr>
<tr>
<td>RV/BW, mg/g</td>
<td>0.62 \pm 0.10</td>
<td>1.21 \pm 0.09*</td>
<td>0.88 \pm 0.05*</td>
<td>1.05 \pm 0.14*</td>
</tr>
<tr>
<td>Lung/BW, mg/g</td>
<td>3.61 \pm 0.13</td>
<td>10.03 \pm 0.57*</td>
<td>8.13 \pm 0.65*</td>
<td>8.48 \pm 0.22*</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>341 \pm 15</td>
<td>327 \pm 12</td>
<td>343 \pm 15</td>
<td>325 \pm 10</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>106 \pm 4</td>
<td>102 \pm 7</td>
<td>99 \pm 3</td>
<td>103 \pm 3</td>
</tr>
<tr>
<td>PP, mm Hg</td>
<td>37 \pm 3</td>
<td>25 \pm 2</td>
<td>26 \pm 1</td>
<td>25 \pm 3</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>4.18 \pm 0.69</td>
<td>23.75 \pm 1.25*</td>
<td>14.38 \pm 3.22*</td>
<td>18.63 \pm 0.56*</td>
</tr>
</tbody>
</table>

HF + V indicates vehicle-treated HF; HF + E, eplerenone-treated HF; HF + P, pyrrolidine dithiocarbamate-treated HF; LVEDV, LV end-diastolic volume; LVEF, LV ejection fraction; %IZ, percent ischemic zone; BW, body weight; RV, right ventricular; MAP, mean arterial pressure; PP, pulse pressure; LVEDP, LV end-diastolic pressure. Values are expressed as mean \( \pm \) SEM.

\( *P < 0.05 \) vs SHAM; \( †P < 0.05 \) vs HF + V.
Brains were removed and fixed overnight in 4% paraformaldehyde at 4°C and immersed in 30% sucrose for ≥2 days. The forebrain containing PVN was sliced into 14-μm coronal sections. Immunofluorescent staining was used to localize COX-2 expression as described by others.27 The sections were incubated with anti-COX-2 polyclonal antibody (1:1000, Cayman Chemical Co) and anti-ED2 polyclonal antibody (1:1000, Serotec Inc), to identify perivascular cells,27 followed by Alex Fluor 488 goat antirabbit IgG and Alex Fluor 547 mouse anti-rabbit IgG (1:200 for both, Molecular Probes). The sections were further stained with To-Pro-3 (1:2000, Molecular Probes) to visualize cell nuclei. Fluorescent intensity was quantified with National Institutes of Health image analysis software. In each rat, ≥7 representative tissue samples from PVN and brain cortex were examined with a confocal laser-scanning microscope (Zeiss LSM 510, Carl Zeiss, Inc), and an average value was reported.

Statistical Analysis

All of the data are expressed as mean±SEM. The significance of differences in mean values was analyzed by 2-way repeated-measure ANOVA followed by posthoc Fisher’s least significant difference test. Echocardiographic parameters were analyzed using 1-way ANOVA followed by Fisher’s least significant difference test.

Results

Echocardiography

The HF animals assigned to treatment with EPL or PDTC versus VEH were well matched with regard to echocardiographically defined LV function. Compared with SHAM rats, rats with HF had reduced LV ejection fraction and increased LV end-diastolic volume. The estimated size of the ischemic zone was not different in HF rats assigned to VEH, EPL, or PDTC treatment (Table).

Indices of HF

At the conclusion of the treatment protocol, LV end-diastolic pressure was increased in all 3 HF groups; treatment with EPL or PDTC ameliorated but did not normalize the rise in LV end-diastolic pressure (Table). Body weight was similar among the experimental groups. However, the right ventricular weight/body weight and wet lung weight/body weight ratios were substantially higher in VEH-treated HF rats compared with SHAM rats. Both were significantly reduced in EPL-treated but not in PDTC-treated HF rats. There were no significant differences in mean arterial pressure or HR among the 4 groups, but all 3 HF groups exhibited a reduced pulse pressure.

NF-κB and IκB mRNA Expression

Multiplex PCR revealed an increase in the PVN mRNA expression of p105, the NF-κB p50 subunit precursor, and a reduction in IκB expression (Figure 1) in VEH-treated HF compared with SHAM rats. Treatment with either EPL or PDTC significantly reduced mRNA expression of p105 and increased the expression of IκB mRNA in the PVN of HF rats.

COX-2 mRNA Expression

As shown in Figure 2A, VEH-treated HF rats had a substantial increase in COX-2 mRNA expression in PVN compared with SHAM. Both EPL-treated and PDTC-treated HF rats had reduced (P<0.05) COX-2 mRNA expression in PVN. COX-2 was more abundantly expressed in brain cortex than in PVN. However, there were no statistically significant differences among the 4 treatment groups in COX-2 mRNA expression in brain cortex.

COX-2 Protein Expression

Western blot analysis confirmed that COX-2 protein expression paralleled mRNA induction (Figure 2B). VEH-treated HF rats had a substantial increase in COX-2 protein expression in PVN compared with SHAM rats. This increase was suppressed (P<0.05) similarly by treatment with either EPL or PDTC. There were no significant differences across treatment groups in COX-2 protein expression in brain cortex.

COX-2 Immunostaining

Laser confocal microscopy revealed a distinctly different distribution of COX-2 immunoreactivity in PVN and brain cortex. COX-2 immunoreactivity was located primarily in the cytoplasm of perivascular cells in the PVN but in neurons in brain cortex (Figures 3 and 4). Thus, to quantify changes in immunoreactivity related to treatment paradigm, we used the
area immediately surrounding cross-sections of blood vessels in the PVN and the cytoplasm of individual neurons in the cortex. By this method, COX-2 immunoreactivity increased in the PVN but not the cortex of VEH-treated HF rats. Treatment with EPL or PDTC prevented the increase in PVN (Figures 3 and 5). There were no significant differences across treatment groups in COX-2 immunoreactivity in brain cortex.

Plasma ALDO, IL-1β, and NE Levels
Plasma levels of ALDO, IL-1β, and NE were higher ($P<0.05$) in VEH-treated HF rats as compared with SHAM rats (Figure 6). Neither EPL nor PDTC treatment changed the plasma ALDO level (Figure 6A), but both significantly reduced the plasma NE level (Figure 6B). Plasma IL-1β level was significantly reduced by treatment with EPL but not PDTC (Figure 6C).

CSF PGE$_2$ Level
The PGE$_2$ level in CSF was increased in VEH-treated HF rats, consistent with the COX-2 mRNA and protein data. Both EPL and PDTC significantly reduced the CSF PGE$_2$ level (Figure 6D).

Discussion
We reported recently that rats with ischemia-induced HF have a marked increase in COX-2 expression in the small vessels penetrating the PVN of the hypothalamus. The present study suggests that activation of the transcription factor NF-κB is a critical step mediating that response.

Treatment of HF rats with the NF-κB inhibitor PDTC prevented the expected increase in COX-2 expression in the PVN and, more specifically, in the microvasculature supply-ling the PVN. HF rats treated with PDTC also had lower CSF levels of PGE$_2$ and lower plasma NE levels, suggesting less PGE$_2$-induced sympathoexcitation.

HF is associated with significant increases in circulating proinflammatory cytokines in humans, as well as in rodent models. NF-κB is a well-recognized mediator of the effects of proinflammatory cytokines. In the present study, we used two different approaches to examine the effects of NF-κB on COX-2 expression in the PVN: reducing the stimulus to activation of NF-κB by lowering circulating cytokines with the MR antagonist EPL, and preventing activation of NF-κB with PDTC, which may act by stabilizing IκB. The responses to these two interventions were qualitatively similar, suggesting that COX-2 expression in the microvasculature of the PVN in HF is initiated by circulating cytokines. This conclusion is consistent with previous work on the effects of acute cytokine stress on the cerebral vasculature. Like rats injected with lipopolysaccharide or IL-1β, rats with HF and high circulating cytokines have increased COX-2 activity in perivascular cells but not in the brain parenchyma itself. Neuronal COX-2 expression, which is prominent in discrete regions of the brain, appears to be unaffected by circulating cytokines. In our study, the failure to observe an increase in COX-2 in cortical tissues from HF rats likely reflects the presence of intense neuronal COX-2 expression under normal

Figure 2. Quantitative comparison of the mRNA (A) and protein (B) expression for COX-2 in the PVN of hypothalamus and brain cortex from HF rats treated with eplerenone (HF+E), pyrrolidine dithiocarbamate (HF+P), or vehicle (HF+V), and untreated SHAM rats. COX-2 mRNA and protein in the PVN increased in the HF+V group compared with SHAM. These responses were minimized but not normalized in HF+E and HF+P rats. Cortical expression of COX-2 mRNA and protein was high relative to PVN but did not change with HF or treatment. B, Representative Western blots are aligned with the matching grouped data. Values are expressed as mean±SEM (n=5 to 8 for each group) and relative to SHAM in PVN. *$P<0.05$ vs SHAM in relevant region, †$P<0.05$ vs HF+V in relevant region.
conditions rather than a lack of a perivascular COX-2 response to the circulating cytokines in HF. Cytokine-induced COX-2 activity in the dense microvascular network of the PVN, where neuronal COX-2 expression is sparse under normal conditions,27,34 may well contribute to the neuroendocrine and autonomic activation in HF. The NF-κB–dependent induction of COX-2 activity in perivascular cells of the PVN demonstrated in the present study is consistent with generally accepted notions regarding the interaction between circulating cytokines and the brain.

Two important differences between the responses to EPL and PDTC deserve comment. First, EPL and PDTC had different effects on clinical indicators of HF. Both reduced LV end-diastolic pressure, but EPL also reduced the wet lung weight/body weight ratio, an index of pulmonary congestion, and the right ventricular weight/body weight ratio, an index of pulmonary hypertension. The more prominent effect of EPL may be explained by inhibition of the effects of ALDO on volume regulation. Previous work from our laboratory11 has shown that the EPL treatment regimen used here does not alter LV systolic function. That observation, the lack of significant differences in mean arterial pressure, pulse pressure, or HR across the HF groups, and the similarity of measured responses to EPL and PDTC, all argue against improved cardiac output as a factor contributing to our results. Second, EPL reduced plasma IL-1β but PDTC did not. Thus, it appears that EPL reduced the stimulus to activation of NF-κB, whereas PDTC directly inhibited the response to the stimulus.

Alternative explanations for our findings must be considered. Systemically administered PDTC and EPL may reduce COX-2 expression in the PVN of HF rats by blocking the actions of circulating ALDO on the penetrating vessels. ALDO acts via MR to stimulate vascular remodeling35 and may also directly induce nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and the production of reactive oxygen species.36 Either mechanism might activate NF-κB with subsequent induction of COX-2. Our methods, using an MR antagonist and an NF-κB antagonist that is also an antioxidant, do not exclude either possibility. In addition,
it is conceivable that systemically administered PDTC blocks unrecognized NF-κB–dependent mechanisms that secondarily influence COX-2 expression in perivascular cells of the brain. With respect to that possibility, the lack of an effect of PDTC on circulating IL-1β (a potential gene product, as well as a stimulus to NF-κB) is notable. In any case, the induction of COX-2 only in perivascular cells of the PVN in HF rats is consistent with the known effects of blood-borne proinflammatory cytokines on the microvasculature of the brain, and proinflammatory cytokines are potent stimuli to NF-κB activity. Thus, the observation that both EPL and PDTC prevent perivascular COX-2 expression in the PVN of HF rats argues strongly in favor of a local cytokine-triggered, NF-κB–mediated process.

Induction of COX-2 enzyme activity and synthesis of PGE2 in the hypothalamic region has important implications for cardiovascular and autonomic regulation in HF. Functional studies have shown that PGE2 has a general excitatory influence on sympathetic nerve activity. Intracerebroventricular injection of PGE2 increases PVN neuronal activity, induces a sympathetically mediated pressor and tachycardic response, and increases circulating NE. Microinjection of PGE2 directly into the PVN increases the activity of neurons in another presynaptic nucleus, the rostral ventrolateral medulla, and increases renal sympathetic nerve activity, arterial pressure, and HR. A recent study demonstrated that PGE2 acts within the hypothalamus by hyperpolarizing GABAergic neurons that inhibit PVN preautonomic and neuroendocrine neurons, thereby promoting excitation of neurons in both limbs of the hypothalamic–pituitary–adrenal axis. The lower levels of NE observed in HF rats treated with EPL or PDTC may reflect, at least in part, a withdrawal of PGE2-mediated sympatho-excitation. Of course, inhibition of direct ALDO effects on central neural systems driving sympathetic activity may also have contributed to this peripheral outcome measure.
Two technical limitations of the study should be considered interpreting the data. First, the plasma NE values were obtained in an anesthetized preparation. Conscious sympathetic nerve activity, a better index of sympathetic drive, which is known to be increased in this model, was not measured. Nonetheless, plasma NE values were lower in EPL- or PDTC-treated HF rats, consistent with a treatment effect. Second, we did not examine the effects of the EPL and PDTC treatments on the SHAM rats. Because EPL in the same dose used in this study had no effects on hypothalamic COX-2 protein, CSF PGE2, or plasma NE in sham rats in a previous study, and little effect would be expected of NF-κB inhibition in the absence of an active inflammatory process, these studies were not deemed necessary.

Perspectives
Stress-induced cytokine activation of the hypothalamic–pituitary–adrenal axis via induction of COX-2 expression in cerebral vessels is a well-described phenomenon. It has usually been described in response to acute stress, and in that context can be shown to be mediated by activation of PGE2-sensitive regions of the brain stem with ascending projections to hypothalamic PVN. The present study confirms our previous observation that the chronic cytokine stress of ischemia-induced HF induces COX-2 expression in perivascular cells of the PVN. More importantly, it demonstrates that activation of NF-κB is an essential intermediary step in this inflammatory, sympatho-excitatory process. Manipulations designed to reduce NF-κB activity may be effective adjuncts to the current treatment of neurohumoral excitation in HF.

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Disclosures
None.

References


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