Pattern of neuronal activation in rats with CHF after myocardial infarction

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Vahid-Ansari, Faranak, and Frans H. H. Leenen. Pattern of neuronal activation in rats with CHF after myocardial infarction. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H2140–H2146, 1998.—To identify neuronal populations possibly contributing to the sympathetic hyperactivity in rats with congestive heart failure (CHF) after myocardial infarction (MI), immunohistochemical detection of Fra-like immunoreactivity (Fra-LI) was used as a marker of long-term neuronal activation. In adult Wistar rats, 2 and 4 wk after left coronary artery ligation, left ventricular (LV) peak systolic pressure and LV end-diastolic pressure were measured, immediately followed by transcardial perfusion and removal of the heart and brain. The brains were processed using an antibody that recognizes Fos, FosB, Fra-1, and Fra-2 for the detection of Fra-LI and using an antibody that only recognizes Fos-like immunoreactivity (Fos-LI). At both 2 and 4 wk after large MI, LV peak systolic pressure was significantly decreased and LV end-diastolic pressure increased. At 2 wk post-MI or sham surgery, Fra-LI was observed in several areas of either group but was significantly higher in the MI versus the sham group in the magnocellular division of the paraventricular nucleus (PVN), supraoptic nucleus (SON), subfornical organ, and caudal part of the nucleus of the solitary tract. At 4 wk after large MI, Fra-LI was clearly detected in the parvocellular and magnocellular divisions of the PVN, SON, and locus ceruleus. Modest expression was noted in these nuclei in rats with small MI, whereas Fra-like positive immunoreactive neurons were barely detectable in the sham group 4 wk postsurgery. In these nuclei, the extent of expression of Fra-LI correlated significantly with the LV end-diastolic pressure. Fos-LI was only noted in the cerebral cortex. These results indicate clear activation of neurons as identified by Fra-LI in specific cardiovascular control centers in rats with CHF 2 and 4 wk post-MI.

heart failure; immediate-early genes; central nervous system; hemodynamics

IN CONGESTIVE HEART FAILURE (CHF), sympathetic activity increases in parallel with the impairment of cardiac performance (17, 22) and may contribute to the progression of heart failure (26). Little is known regarding the central pathways contributing to the sympathetic hyperactivity in CHF. Patel et al. (27) measured hexokinase activity in brain nuclei of rats with CHF 3 wk after myocardial infarction (MI). Significant increases in activity were noted in the magnocellular division of the paraventricular nucleus (PVN) and the locus ceruleus (LC) but not in nuclei such as the supraoptic nucleus (SON), subfornical organ (SFO), or median preoptic nucleus (MnPO). Basu et al. (3) measured progressive increases in norepinephrine release in the PVN of rats 2, 4, and 8 wk post-MI, relative to sham rats. Recent advances in the understanding of the regulation of gene expression associated with activation of neurons may provide better insight into the central nervous system (CNS) pathways activated in CHF. Immediate-early genes encode transcriptional factors that are considered to reflect activation of neurons responding to synaptic activation (32). Immunohistochemical detection of Fos has become a sensitive marker of short-term neuronal activation including neurons involved in cardiovascular homeostasis (2, 23). Because Fos downregulates quickly during chronic exposure to a variety of treatments, it is not useful as a marker of chronic neuronal activation (24, 30). However, with continuous stimulation, Fos expression is followed by expression of other Fos family proteins such as Fos-B, Fra-1, and Fra-2, and their increased expression persists during chronic exposure (24, 25). Immunohistochemical detection of these proteins has become a useful tool to identify chronically activated neuronal populations in a wide variety of CNS regions (9, 11, 15, 35), including regions involved in cardiovascular homeostasis (4, 5). In the present study, we used immunohistochemical detection of Fos family proteins as Fra-like immunoreactivity (Fra-LI) to map activation of CNS areas during the development of sympathetic hyperactivity and CHF in rats post-MI.

METHODS

Male Wistar rats weighing 200–250 g (Charles River Breeding Laboratories, Montreal, PQ, Canada) were given food and water ad libitum and kept on a 12-h light-dark cycle. After an acclimatization period of 4 days, they were randomized to surgery for coronary artery ligation or sham ligation. Subsequently, animals were kept two per cage. All animals were treated in accordance with the procedures outlined in the “Guide for the Care and Use of Experimental Animals” endorsed by the Medical Research Council of Canada.

Surgery for Application of Coronary Occluder

Rats were anesthetized with 4% halothane and intubated with a no. 14 catheter (Insite, Becton-Dickinson, Sandy, UT) through which 1.0% halothane in oxygen was ventilated at 10 ml/kg body weight, 60 times/min, to maintain anesthesia. After the thorax was opened at the fourth or fifth left intercostal space, a coronary occluder was passed around the left coronary artery 2–3 mm from the origin by inserting the needle into the left ventricular (LV) wall under the overhanging left atrial appendage and bringing it out high on the pulmonary conus. The guide tubing with the other end of the occluder was then exteriorized at the back of the neck (16). Buprenorphine HCl was used for pain relief for 2 days.
Coronary Artery Ligation

In conscious rats, 7–10 days after the open-chest surgery, the occluder was carefully pulled until it was no longer possible to remove the occluder in relation to the outer guide. The exposed occluder was melted down with cautery to form a bubble adjacent to the distal end of the outer guide tubing, fixing it in place. Mortality was 47 and 30% in the 2- and 4-wk experiments, respectively, and death mainly occurred during the first hours after ligation. Animals allocated to sham surgery underwent the same procedures except that the coronary artery was not ligated.

Experimental Protocols

Hemodynamics and immunohistochemical detection of Fra-LI and Fos-like immunoreactivity were evaluated in two separate experiments at 2 and 4 wk after coronary artery ligation or sham ligation.

After 2 or 4 wk, a PE-50 catheter was placed in the LV via the right carotid artery, as described previously (16). Immediately after recovery, resting LV end-diastolic pressure (LVEDP), LV peak systolic pressure (LVPSP), and heart rate were assessed in conscious, unrestrained rats. Immediately after hemodynamic measurements, animals were deeply anesthetized by injection of pentobartital sodium (100 mg/kg; Somnotol, MTC Pharmaceuticals, Cambridge, ON, Canada) through the right carotid artery catheter and perfused transcardially with 200 mL of 0.9% saline followed by 150 mL of 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde (PFA) at room temperature. Brains and hearts were ultrasonically washed in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde at room temperature. Brains and hearts were then removed, and brains were postfixed in 4% PFA for 24–48 h at 4°C. Coronal sections (30 µm thick) were cut with a vibratome from the forebrain starting at the vertical limb of the diagonal band of Broca and ending at the arcuate nucleus and from the medulla starting at the level of the obex to the parabrachial nucleus according to the atlas of Paxinos and Watson (28).

Fra-LI was detected using an affinity-purified rabbit polyclonal antibody (c-fos K-25, Santa Cruz Biotechnology, Santa Cruz, CA) recognizing amino acids 128–152 in the NH2-terminal region of Fos. According to the manufacturer, this antibody recognizes Fos, Fos-B, Fra-1, and Fra-2 proteins. To detect Fos-like immunoreactivity, an affinity-purified sheep antibody (OA-11–823, Cambridge Research Biochemical, Cambridge, UK) was used that recognizes amino acids 2–16 in the NH2-terminal region of Fos. Immunohistochemistry was performed using a standard procedure previously described (29, 35). Briefly, sections (30 µm thick) were washed in 0.01 M PBS containing 0.3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Sections were then randomized three times in PBS and incubated in PBS containing 0.3% Triton X-100, 0.02% sodium azide, and primary antibody, using in alternate sections either c-fos K-25 (1:2,000) or OA-11–823 (1:2,500) for 48 h at 4°C. The sections were then washed three times with PBS and incubated with either biotin-labeled donkey anti-rabbit or donkey anti-sheep secondary antisera (1:2,000; Jackson Laboratories, West Grove, PA) for 16 h at 4°C. The sections were then washed three times with PBS and incubated for 3 h with PBS containing 0.3% Triton X-100 and streptavidin-horseradish peroxidase at room temperature (1:100, Amersham Canada, Oakville, ON, Canada). After three washes in PBS, the sections were rinsed in 0.1 M acetic acid buffer, pH 6.0. The reaction was visualized using a glucose oxidase-diaminobenzidine-nickel method. The reaction was terminated by washing in acetic acid. Subsequently, the sections were mounted on chrom-alum-coated slides, dried, dehydrated through a graded series of alcohols and two changes of xylene, and coverslipped for microscopic observation.

Positive immunoreactive neurons were quantified in the different brain areas using an image analysis system equipped with Image 1.47 software (35). Digitization of sampled areas (660 x 800) was performed at x100 magnification using a charge-coupled device camera linked to a microscope. Thresholding was performed on the digitized image to eliminate small positive profiles such as fragments of nuclei and weakly stained cells from the final analysis. To ensure consistency between measurements, the similar threshold value was chosen on the basis of the nuclear size and background staining. In every rat, for each area two measurements were performed on two separate sections. Averages from these measurements per area per rat were used for statistical analysis.

The hearts were used for measurement of infarct size. After LV weight was measured, four or five incisions were made in the LV so that the LV tissues could be pressed flat. The circumferences of the entire flat LV and the visualized infarcted area, as judged from both epicardial and endocardial sides, were outlined on a clear plastic sheet. The ratio in weight between the two marked areas on the sheet was used to determine the infarct size (7). The brains from rats with infarct size >35% of the LV at 4 wk postligation (n = 8; sham: n = 4) and at 2 wk postligation (n = 4; sham: n = 4) were processed. At 4 wk post-MI, Fra-LI was also evaluated in four rats with infarct size <35%.

Statistical Analysis

Data are presented as means ± SE. Factorial ANOVA was performed on the cell count for each region, followed by the Newman-Keuls test to determine significant differences in the number of positive immunoreactive nuclei for one area between MI and sham groups (36).

RESULTS

Hemodynamic Changes at Two and Four Weeks Post-MI

Acute coronary artery ligation caused an average infarct size of 47% in the 2-wk experiment and 43% in the large-MI group and 23% in the small-MI group in the 4-wk experiment (Table 1). Right ventricle weight was significantly increased at both 2 wk (103 ± 9 vs. 54 ± 9 mg/100 g body wt, P < 0.01) and 4 wk (110 ± 11 vs. 53 ± 3 mg/100 g body wt, P < 0.01) but not in the small-MI group (65 ± 6 mg/100 g body wt). Final body weight was significantly decreased at 2 wk post-MI by 10.2 ± 0.3 on May 27, 2017 from http://ajpheart.physiology.org/ Downloaded by 10.1722.320.146 on May 27, 2017

Table 1. Hemodynamic changes at 2 and 4 wk post-MI

<table>
<thead>
<tr>
<th></th>
<th>Final BW, g</th>
<th>LVPSP, mmHg</th>
<th>LVEDP, mmHg</th>
<th>HR, beat/min</th>
<th>Infarct Size, %</th>
</tr>
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<tbody>
<tr>
<td>2 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham   (n = 4)</td>
<td>400 ± 10</td>
<td>118 ± 3</td>
<td>2 ± 1</td>
<td>415 ± 24</td>
<td></td>
</tr>
<tr>
<td>MI     (n = 4)</td>
<td>353 ± 13*</td>
<td>94 ± 2*</td>
<td>21 ± 2*</td>
<td>415 ± 20</td>
<td>47</td>
</tr>
<tr>
<td>4 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham   (n = 4)</td>
<td>446 ± 12</td>
<td>119 ± 2</td>
<td>1 ± 1</td>
<td>381 ± 8</td>
<td></td>
</tr>
<tr>
<td>Large MI (n = 8)</td>
<td>431 ± 14</td>
<td>93 ± 2*</td>
<td>14 ± 2*</td>
<td>369 ± 7</td>
<td>43</td>
</tr>
<tr>
<td>Small MI (n = 4)</td>
<td>427 ± 17</td>
<td>97 ± 6</td>
<td>6 ± 2*</td>
<td>379 ± 7</td>
<td>23</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. MI, myocardial infarction; BW, body weight; LVPSP, left ventricular (LV) peak systolic pressure; LVEDP, LV end-diastolic pressure; HR, heart rate. *P < 0.01, sham vs. MI; tP < 0.01, large MI vs. small MI.
compared with sham but not in the 4-wk experiment. At both 2 and 4 wk post-MI, LVPSP was significantly decreased and LVEDP increased but not in rats with small MI (Table 1). Heart rate did not show significant differences at 2 and 4 wk post-MI.

Fos-Like Immunoreactivity at Two and Four Weeks Post-MI

The whole brain was scanned for the presence of positive immunoreactive neurons after 2 and 4 wk. At the level where Fra-LI was quantified in the PVN, Fos-like immunoreactivity was homogeneously detected in the cortex. This expression was not quantified. Otherwise, Fos-like immunoreactive neurons were undetectable in all examined regions at 2 and 4 wk post-MI as well as in the sham groups.

Fra-LI at Two and Four Weeks Post-MI

In contrast to Fos-like immunoreactivity, Fra-LI-positive neurons were detected in areas regulating cardiovascular function including different parts of the hypothalamus, the parvo- and magnocellular divisions of the PVN, SON, and LC. At 4 wk postsurgery, Fra-LI was clearly present in the MI groups, but the sham group showed barely any staining. Figure 1 shows the typical distribution of Fra-LI in the PVN in a sham rat and a rat with large MI at 4 wk.

PVN. Fra-LI was quantified in two divisions of the PVN, parvocellular and magnocellular. At 2 wk postsurgery, Fra-LI was detected in both divisions in both sham and MI groups but was significantly higher in the magnocellular PVN in the MI versus the sham group (Fig. 2). At 4 wk post-MI, scattered positive immunoreactive neurons were detected in the parvocellular PVN in the large-MI group, less in the small-MI group, and barely any in the sham group. A large number of Fra-LI-positive neurons were stained in the neurosecretory cells of the magnocellular part of the PVN (Figs. 1 and 3) in the large-MI group compared with few in the small-MI group and barely any in the sham group.

SON. In the SON at 2 wk postsurgery, Fra-LI was clearly present in both groups but significantly higher in the MI group (Fig. 2). At 4 wk after large MI, a number (14 ± 2) of Fra-LI-positive neurons of the magnocellular type (Fig. 3) were densely stained in the SON. Rats with small MI also showed some expression (5 ± 1), and the sham group showed barely any (Fig. 3).

LC. At 2 wk postsurgery, both groups showed similar, modest expression (Fig. 2). At 4 wk in the large-MI group, a densely packed cluster of Fra-LI-positive cells was observed in the dorsal part of the LC, where the cells are longitudinally arranged and caudally situated at the lateral border of the central gray. Fra-LI was barely detected in both the small-MI and sham groups (Fig. 3).

Correlation analysis. As depicted in Fig. 4, at 4 wk postsurgery, significant correlations between LVEDP and number of Fra-positive neurons were found in both parvocellular and magnocellular divisions of the PVN, SON, and LC.

Other areas. At 2 wk postsurgery, Fra-LI was also detected in several other areas (Table 2). The sham and MI groups showed similar expression in the anterior hypothalamic area, dorsal MnPO, and central gray.

The MI group showed a significant increase in the SFO exclusively in neurons situated at the border of the third ventricle (with none in the sham group) and the caudal nucleus tractus solitarii (NTS). At 4 wk, none of these areas showed expression.
DISCUSSION

In the present study we utilized immunohistochemical detection of Fra-LI as a sensitive marker for long-term neuronal activation in rats developing CHF post-MI. At 2 wk after sham MI (i.e., 3 wk after major surgery) sham rats showed clear expression in several areas. By 4 wk after sham MI, this presumably surgery-related expression had disappeared and sham rats showed barely any expression. Relative to the sham rats, MI rats showed a very specific pattern of activation. The SON and the magnocellular division of the PVN exhibited clear activation at both 2 and 4 wk post-MI. By 4 wk post-MI, the parvocellular division of the PVN and the LC showed some activation as well. At 4 wk post-MI, the extent of activation of these nuclei correlated significantly with LVEDP in the individual rats. On the other hand, at 2 but not 4 wk post-MI, enhanced expression was also noted in the SFO and caudal NTS.

These studies using sensitive molecular biological markers extend previous studies by Patel et al. (27), showing enhanced hexokinase activity in both divisions of the PVN and LC at 3 wk post-MI, and by Basu et al. (3), showing progressive increases in norepinephrine release in the PVN of rats at 2, 4, and 8 wk post-MI.

Methodological Considerations

The K-25 antibody recognizes all proteins of the Fos family. As a consequence, the observed immunoreactivity may represent not only an increase in Fra proteins but also increases in Fos or Fos-B. However, Fos typically peaks at \( \sim 30 \) min to 1 h, starts decreasing at 2 h, and is essentially absent at 4 h after introduction of the stimulus (24). With continuous stimulation, the elevated Fos levels decrease and stay at baseline values (24, 30, 37). Fra appears between 1 and 3 h after the stimulus, and can persist for up to 7 days (24, 31). In the setting of the developmental phase of CHF and absence of intervening acute stimuli, one would expect little Fos-like immunoreactivity, and this was indeed the case in the present study.

Sham rats demonstrated clear Fra-LI in several brain areas at 3 wk after thoracic surgery but no longer after 5 wk. This time course likely reflects the slow disappearance of increased Fra-LI related to this surgery and the subsequent recovery phase. This “aspecific” effect may explain the higher expression in the SON of MI rats at 2 versus 4 wk, or alternatively obscure differences at 2 wk post-MI in the LC. This study evaluated Fra-LI at 2 and 4 wk post-MI, i.e., the developmental phase of sympathetic hyperactiv-
Previously, studies demonstrated that both brain "ouabain" and the brain renin-angiotensin system play a major role in the sympathetic hyperactivity and impairment of baroreflex function in rats with CHF post-MI (12, 16, 19, 41). The distribution of "ouabain"-containing neurons has been studied in the rat, macaque, and dog. Dense "ouabain" immunoreactivity was detected in magnocellular neurons in the PVN and SON. Parvocellular neurons of the PVN showed significantly less immunoreactivity. In the PVN and SON, "ouabain" neurons are distributed in areas that mediate cardiovascular regulation, including septal nuclei, the preoptic region, hypothalamic nuclei, and the midbrain, including the locus ceruleus and nuclei of the medulla oblongata (6, 10). Among these areas, the highest concentrations of ANG II nerve cell body and terminal immunoreactivity were found in the PVN and the SON (6, 10). Lind et al. (19) suggested that angiotensinergic projections may include those from the PVN to sympathoexcitatory neurons in the intermediolateral cell column of the spinal cord. Brain "ouabain" and the brain renin-angiotensin system exhibit therefore colocalization in several brain nuclei, including the PVN and the SON. How and where these two systems interact cannot be assessed from the present studies and will require both double-staining and functional studies.

Two nuclei exhibited increased Fra-LI at 2 wk post-MI but no longer at 4 wk post-MI. The SFO showed distinct expression in neurons situated at the border of the third ventricle at 2 wk post-MI (and none in the sham group). This part of the SFO also shows increased expression after peripheral administration of hypertonic saline and intracerebroventricular ANG II but not after hypovolemia, hemorrhage, or intravenous ANG II (2, 20, 33). The SFO shows a large number of "ouabain"-positive fibers (39), as well as expresses high numbers of ANG II binding sites (21). Whether this specific expression of Fra-LI in the SFO at 2 wk post-MI represents a primary event leading to activation of, e.g., PVN and SON (14, 19) or is secondary cannot be assessed from the present studies.

The caudal NTS receives input from cardiovascular afferents, and both hypotension and hypertension can increase Fos expression in the NTS (18). In post-MI rats, both the decrease in arterial blood pressure and the increase in cardiac filling pressures may therefore lead to increased expression of Fos followed by other proteins of the Fos family. When we consider that baroreceptors reset to chronically higher or lower load, it is somewhat surprising that at 2 wk post-MI increased Fra expression was still present. This may indicate that resetting occurs to a lesser extent in this situation. Alternatively, other stimuli may have influ-

**Possible Functional Significance**

Previous studies demonstrated that both brain "ouabain" and the brain renin-angiotensin system play a major role in the sympathetic hyperactivity and impairment of baroreflex function in rats with CHF post-MI (12, 16, 19, 41). The distribution of "ouabain"-positive neurons in brain areas and left ventricular end-diastolic pressure (LVEDP) at 4 wk after small and large MI. Correlation coefficient (r) and P value for each area are shown.

**Table 2. Fra-like positive neurons in brain areas at 2 wk after large MI**

<table>
<thead>
<tr>
<th>Area</th>
<th>No. of Fra-like Positive Neurons</th>
<th>Coordinate From Bregma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (n = 4)</td>
<td>MI (n = 4)</td>
<td></td>
</tr>
<tr>
<td>Anterior hypothalamic area</td>
<td>12 ± 3</td>
<td>10 ± 1; −1.8 mm</td>
</tr>
<tr>
<td>Dorsal median preoptic nucleus</td>
<td>7 ± 1</td>
<td>9 ± 2; −0.4 mm</td>
</tr>
<tr>
<td>Subfornical organ</td>
<td>0 ± 1</td>
<td>15 ± 3*; −1.4 mm</td>
</tr>
<tr>
<td>Central grey</td>
<td>15 ± 2</td>
<td>22 ± 1; −7.64 mm</td>
</tr>
<tr>
<td>Caudal nucleus of solitary tract</td>
<td>3 ± 1</td>
<td>8 ± 1*; −14.30 mm</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. *P < 0.01, sham vs. post-MI.
enced Fra expression in the NTS. As stated above for the SFO, the pathophysiological role of this temporary activation of the NTS in the processes leading to sympathetic hyperactivity post-MI cannot be determined from the present studies.

In conclusion, the present study mapped long-term neuronal activation in rats post-MI using immunohistochemical detection of Fra-like proteins. The results suggest that activation of neurons in the PVN and SON may play a major role in the processes leading to sympathetic hyperactivity in rats with CHF post-MI.

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