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Neuropeptide Y Modulation of Sympathetic Activity in Myocardial Infarction

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Objectives. **We examined the possible effect of neuropeptide Y in modulating central sympathetic activity after myocardial infarction in rats.**

Background. **Previous studies have shown the coexistence of neuropeptide Y and norepinephrine in the brain and a possible functional interaction between the two. Neuropeptide Y inhibits the release of norepinephrine at the presynaptic level and can be considered to act as a neuromodulator.**

Methods. **Two groups of rats were examined in this study—an experimental group, defined as those rats undergoing left coronary artery ligation, and a sham group without coronary artery ligation, serving as the control group. The animals in both groups underwent microdialysis in the paraventricular nucleus at 2, 4 and 8 weeks after operation. Microdialysis samples were collected with and without injecting neuropeptide Y in the paraventricular nucleus. The concentration of norepinephrine was determined by injecting purified microdialysate samples during high performance liquid chromatography. To explore the receptor's possible role, autoradiographic localization of neuropeptide Y receptors in the paraventricular nucleus was also carried out in the experimental and sham groups.**

Results. **The concentration of norepinephrine measured in the samples was decreased by 50% with neuropeptide Y in 2- and 4-week old rats after infarction, but by only 20% ($p < 0.05$) in 8-week old rats after infarction. The diminished inhibitory effects of neuropeptide Y on norepinephrine release was associated with increased sympathetic activity, as reflected by plasma norepinephrine; 8-week old rats after infarction had almost a 100% ($p < 0.05$) increase in their plasma norepinephrine level compared with the sham group. Autoradiography revealed a significant decrease in density of neuropeptide Y receptors in the paraventricular nucleus in 8-week old rats after infarction ($p < 0.05$).**

Conclusions. **The data presented in this report suggest that the reduction of the inhibitory activation of neuropeptide Y on sympathetic release may contribute to elevated norepinephrine levels after myocardial infarction.**

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Neuropeptide Y is colocalized with norepinephrine in the brain and has been identified in abundance in the central as well as the peripheral nervous system of many species (1–7). Neuropeptide Y inhibits the release of norepinephrine at the presynaptic level (8,9) and can be considered to act as a neuromodulator (10–13). In the brain, the highest densities of neuropeptide Y fibers and perikarya are found in the hypothalamic paraventricular nucleus (14,15). Stimulation of the paraventricular nucleus increases sympathetic outflow (16), which precipitates an increase in peripheral sympathetic nerve activity, as gauged by an elevation of plasma catecholamines in the systemic circulation (17). We sought to investigate how neuropeptide Y modulates sympathetic outflow in different pathophysiologic conditions.

Myocardial infarction in both patients and experimental animal models has been shown to be associated with an increased sympathetic drive (18). However, the reason for heightened sympathetic activity and concurrent pathophysiologic findings after myocardial infarction has yet to be satisfactorily explained. The main objective of this study was to examine the hypothesis that neuropeptide Y is involved in ischemic heart disease through altered sympathetic activity in the paraventricular nucleus of the hypothalamus, which is one of the prominent catecholamine-rich cardiovascular centers in the brain. Focusing on the importance of the paraventricular nucleus in the control of sympathetic activity and neuropeptide Y as a neuromodulator, we tried to determine the relation between neuropeptide Y as a neuromodulator and myocardial infarction.

Methods

Coronary ligation. Myocardial infarction was produced in male Sprague-Dawley rats weighing 175 to 225 g by occlusion of the left coronary artery (19–21). After the animals were

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anesthetized with ether, the skin was incised along the left sternal border, the fourth rib was cut proximal to the sternum and retractors were inserted. The pericardial sac was perforated and the heart was exteriorized through the intercostal space. The left coronary artery was ligated ~2 mm from its origin with 6-0 silk suture, and the heart was repositioned in the chest. Throughout the operation, the rats were maintained on a positive-pressure ventilation system delivering a mixture of 95% oxygen and 5% carbon dioxide, together with ether. Closure of the wound was accomplished by a pursestring suture. Sham-operated animals were treated similarly, except that the suture around the coronary artery was not tied.

Animals were housed on a dark–light cycle of 12/12 h and allowed to recover in a temperature-controlled environment with free access to food and water. The animals were maintained for 2, 4 and 8 weeks before the experimental assessments. The mortality rate of all animals operated on was 35% within 72 h after surgery. Each group had six animals 2, 4 and 8 weeks after infarction.

To examine the general characteristics of the animals and the weight of the scar tissue, the right and left ventricles of the heart was taken. The weight of the left ventricle did not include the scar tissue. First the lungs were dissected and their wet weight measured. Before dry weight was measured, the rats' lungs were kept inside an oven for 48 h at 60°C.

Microdialysis in vitro. Microdialysis probes (CMA/500-μm outer diameter, 1 mm exposed dialysis membrane, Carnegie Medicine/Bio Analytical Systems) were perfused with microdialysis perfusate (in mmol/liter: 147 sodium chloride, 4 potassium chloride, 1.2 calcium chloride, 1.1 magnesium chloride, pH 6) at a flow rate of 2.2 μl/min and mounted in Eppendorf vials containing a known concentration of norepinephrine (100 ng/μl) dissolved in microdialysis perfusate. Microdialysis probes were calibrated before the experiments (22,23). Relative recovery was defined as concentration of catecholamines in perfusate as a percentage of the concentration of the outer medium. After calibration, the probes were set to zero on the Kopf stereotaxic frame (David Kopf Instruments).

Microdialysis in vivo. Microdialysis was carried out 2, 4 and 8 weeks after coronary artery ligation (6 animals in both the experimental and control groups 2, 4 and 8 weeks after infarction). The animals were anesthetized with 4.0 mg/100 g ketamine:0.4 mg/100 g xylazine intraperitoneally and placed in a David Kopf stereotactic apparatus with the incisor bar elevated to 5 mm above the interaural line. Body temperature was maintained at 37°C by a Harvard homeothermic control pad (Harvard Apparatus, Inc.). Craniotomy was performed and the probe was stereotactically inserted into the paraventricular nucleus. Coordinates used were +1.0/+0.5/−8.0 for the 2- and 4-week old animals and +1.1/+0.5/−8.3 for the 8-week old animals, from the zero bar in millimeters, of the brain's anteroposterior/mediolateral/dorsoventral regions, respectively.

A stabilization period of 2 h was allowed before collecting the samples. The perfusion medium was microdialyzed in the brain at a flow rate of 2.2 μl/min. After the stabilization period, 100 μl of the microdialysate samples was collected and immediately taken for the purification procedure before catecholamine analysis in high performance liquid chromatography.

The animals were then intracerebrally exposed to a freshly prepared solution of neuropeptide Y (concentration of 10^{-8} mol/liter) dissolved in the microdialysis perfusate. After another stabilization period of 1 h, 100 μl of microdialysate sample was again collected for catecholamine analysis to examine the effect of neuropeptide Y.

Purification and norepinephrine analysis. The purification procedure was carried out by means of an alumina extraction protocol (Waters-Millipore, Mississauga, Ontario, Canada). Basically, the samples were treated with 2 mol/liter Tris and the pH was adjusted to 8.7. Acid-washed alumina (20 mg) was added to the vial and allowed to mix for 20 min. The supernatant was discarded and the alumina was washed three times with double-distilled water. The alumina was dried on filter paper and catecholamines were eluted from the alumina with 0.1 N acetic acid. Norepinephrine analysis was performed by injecting the purified samples during reverse-phase high performance liquid chromatography with an electrochemical detector. High performance liquid chromatography consisted of Waters Resolve 5-μm C18 dimethyloctadecylsilyl-bonded silica particle size in a 3.9-mm × 150-mm column, a 510 pump, a U6K liquid injector and a 460 electrochemical detector. The glassy carbon working electrode was set at +0.60 V versus a silver/silver chloride reference electrode. The mobile phase consisted of Waters catecholamine eluent reagent, which was regularly degassed and circulated at a flow rate of 0.9 ml/min. Concentrations were obtained after chromatographic comparison with a standard containing a known concentration of norepinephrine (100 ng/μl). The chromatographic data were collected on a microcomputer using Baseline 810 chromatographic workstation software.

Plasma norepinephrine analysis. Four milliliters of blood was collected intracardially. Extracted plasma was purified through an alumina extraction protocol (Waters-Millipore) similar to the microdialysate samples. Norepinephrine analysis was performed during high performance liquid chromatography with an electrochemical detector. Concentrations were obtained as in the microdialysate samples after chromatographic comparison to a standard known concentration of norepinephrine.

Perfusion and histologic analysis. After the microdialysis sampling, each animal was removed from the Kopf stereotactic frame and intracardially perfused with 0.9% saline at room temperature, followed by 4% paraformaldehyde in 0.1 mol/liter phosphate buffer (pH 7.4). The rats' heads were separated below the second cervical vertebra, and after craniotomy their brains were blocked on the stereotactic apparatus with the incisor bar 5 mm above the interaural line. The brain segments were cryoprotected in 25% sucrose:10% glycerin in 0.1 mol/liter phosphate buffer for 3 days. They were then sectioned in cryostat at a constant temperature of −17°C, with a thickness

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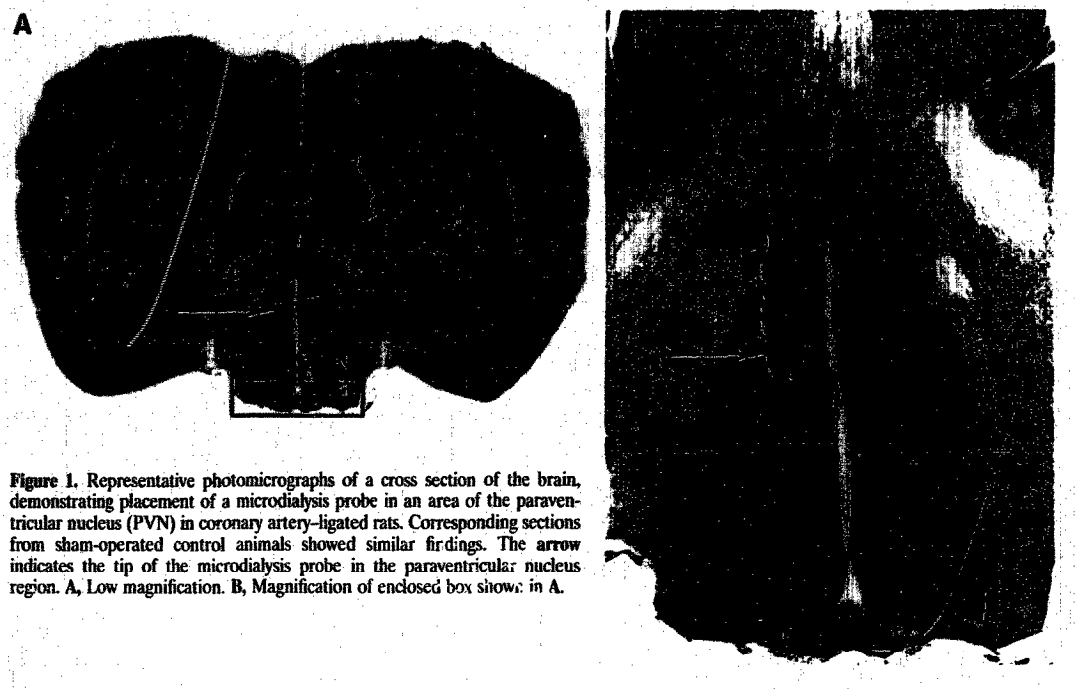


Figure 1. Representative photomicrographs of a cross section of the brain, demonstrating placement of a microdialysis probe in an area of the paraventricular nucleus (PVN) in coronary artery–ligated rats. Corresponding sections from sham-operated control animals showed similar findings. The arrow indicates the tip of the microdialysis probe in the paraventricular nucleus region. A, Low magnification. B, Magnification of enclosed box shown in A.

of 40 μm. Sections were mounted and stained with thionin Nissl stain, and probe insertion site was histologically confirmed (Fig. 1A, B).

To confirm the extent of collagen deposition, histologic sections of the hearts of 2-, 4- and 8-week old animals were taken and stained with Martius scarlet blue trichrome stain, which turns the collagen fibers a blue color.

Autoradiography for neuropeptide Y receptors. The autoradiographic technique performed in this study has been modified from the work of Mantel et al. (24). There were six animals in each of the experimental and sham groups 8 weeks after infarction. The animals in both groups were quickly decapitated and stereotactically blocked in a David Kopf apparatus. The blocked brains were immediately frozen in dry ice and cut to 20-μm thickness on a cryostat at a constant temperature of −13°C. The sections were thaw mounted on treated gelatin-coated slides and stored at −80°C for future use.

During autoradiography the sections were thawed at room temperature and then preincubated in 50 mmol/liter Tris hydrochloride, pH 7.4, at room temperature for 45 min. During incubation the brain sections were exposed to 0.2 nmol iodine-125 Bolton Hunter neuropeptide Y (NEN-Dupont) (7.4×10^7 MBq/mmol) for 120 min. It has been well established that this period is sufficient for binding iodine-125 neuropeptide Y to the brain tissue. The specificity of the receptors was determined by the difference in binding observed on adjacent sections in the absence and presence of 1 μmol/liter porcine neuropeptide Y.

The sections were then washed in chilled Tris hydrochloride buffer four times for 4 min each, followed by a dip in chilled, double-distilled water. The sections were quickly air dried and then tightly juxtaposed against hydrogen-3 Hyperfilm (Amersham) sealed within Picker International cassettes. The boxes were stored at 4°C for 4 days before developing. Measurements of receptor concentration were carried out using the computer image analysis system (Jandel Scientific). Alternating sections were stained with thionin to facilitate anatomic identification.

In the autoradiographic experiments, the rat brains were sectioned and thaw mounted on slides without undergoing prior cryoprotection to avoid any interference of salt ions with our radioactive isotope, iodine-125 neuropeptide Y. Fresh sets of rats, other than those used for the microdialysis experiments, were taken for the experimental group and the sham group. This was done primarily to avoid desensitization of the neuropeptide Y receptors in the paraventricular nucleus area of the brain during the microdialysis experiments in which the concentration of neuropeptide Y was 10^{-8} mol/liter.

Statistical analysis. All data are presented as mean value ± SEM. Statistical differences between mean values of experimental and sham-operated control animals were analyzed using two-way analysis of variance with Tukey's multiple

Table 1. General Characteristics of Experimental Rats 2, 4 and 8 Weeks After Induction of Myocardial Infarction

Parameters	Week 2		Week 4		Week 8	
	Sham	Ligated	Sham	Ligated	Sham	Ligated
L vent. wt (g)	0.71 ± 0.06	0.85 ± 0.08*	0.84 ± 0.05	0.96 ± 0.04*	0.99 ± 0.09	1.26 ± 0.07*
R vent. wt (g)	0.20 ± 0.02	0.21 ± 0.04	0.23 ± 0.03	0.33 ± 0.04	0.24 ± 0.01	0.38 ± 0.05*
L vent. wt (10^{-3})/body wt	2.17 ± 0.21	2.66 ± 0.25	2.02 ± 0.18	2.46 ± 0.19*	2.41 ± 0.29	2.67 ± 0.26*
Scar wt (g)	ND	0.14 ± 0.02	ND	0.14 ± 0.01	ND	0.26 ± 0.03
Lung wet wt (g)	1.28 ± 0.18	1.21 ± 0.16	1.48 ± 0.12	1.66 ± 0.21	1.58 ± 0.17	1.88 ± 0.14*
Lung dry wt (g)	0.29 ± 0.03	0.28 ± 0.07	0.37 ± 0.07	0.38 ± 0.08	0.39 ± 0.04	0.42 ± 0.03
Lung wet wt/lung dry wt	4.36 ± 0.33	4.36 ± 0.43	4.10 ± 0.31	4.27 ± 0.29	3.93 ± 0.23	4.38 ± 0.21*

*$p < 0.05$. Data are expressed as mean ± SEM of six experiments in each group. Left ventricular (L vent.) weight (wt) indicated for experimental animals does not include scar tissue. ND = not detectable; R vent. = right ventricular.

comparison testing. Statistical significance for all the tests was set at $p < 0.05$.

Results

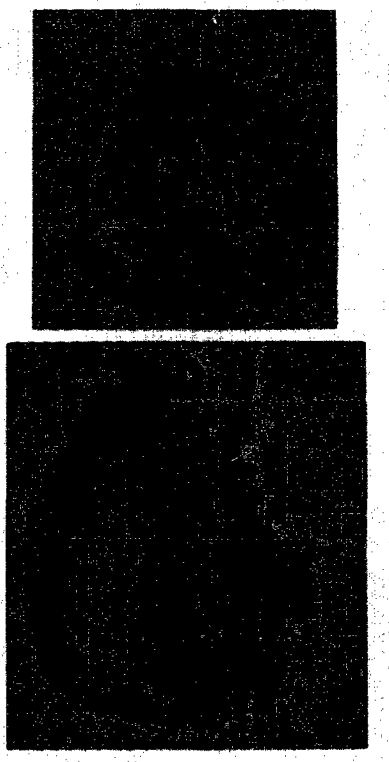
General characteristics of experimental animals. Table 1 summarizes the general characteristics of the experimental animals 2, 4 and 8 weeks after infarction. A study of the left ventricle revealed significant differences between experimental and sham-operated groups of animals. A study of the left ventricle 8 weeks after infarction revealed significant differences in weight between the experimental and sham groups. Evidence of cardiac hypertrophy after the initial myocardial infarction was observed by the increased mass of the remaining viable left as well as the right ventricular myocardium in 8-week old experimental animals. Furthermore, the left ventricular weight/body weight ratio was significantly higher at the 4- and 8-week stages in the experimental animals. However, no difference in the scar weights of the left ventricular free wall was seen among the 2-, 4- or 8-week old experimental animals. Congestion of lungs was evident by the increased wet and wet/dry lung weight ratio in the 8-week old experimental rats.

Histologic sections of the heart of 8-week old experimental animals showed hypertrophy and extensive collagen tissue deposition (Fig. 2A, B), which reflected an increase in trend from 2 to 4 to 8 weeks after infarction.

Microdialysis in vitro. Relative recovery rates of the microdialysis probes were performed every day before the experiments to determine the probe's ability to recover catecholamine from the brain. The relative recovery rate of the probes ranged from 10% to 15%. Probes were perfused with neuropeptide Y to assess the possible effects of the drug on the ability of the probe to recover norepinephrine. However, no differences were found in the recovery of norepinephrine in the dialysate before and after the perfusion time of 1 h.

Microdialysis in vivo. Significant differences in baseline norepinephrine levels from the paraventricular nucleus were observed between experimental and sham-operated animals in all the three groups. Baseline of norepinephrine 2 weeks after infarction showed a significant increase of threefold ($p < 0.05$) (Fig. 3), 4 weeks showed an increase of more than fourfold ($p < 0.02$) (Fig. 3) and 8 weeks showed an increase of more than ninefold ($p < 0.001$) (Fig. 3), when compared with their respective sham-operated animals.

Figure 2. Photomicrographs representing cross sections of the heart stained with Martius scarlet blue in sham-operated control (**A**) and experimental rats (**B**) 8 weeks after ligation of the coronary artery. Increase in the size of the heart is clearly demonstrated in the section of the heart from the experimental animal. The arrow pointing to the blue portion shows the extent of deposition of collagen fiber in the experimental animal.



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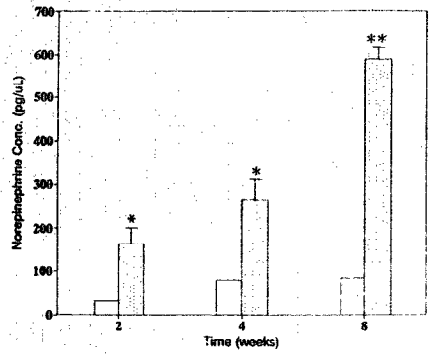


Figure 3. Baseline norepinephrine levels in the paraventricular nucleus of sham control and experimental animals 2, 4 and 8 weeks after coronary artery ligation. Released concentration (Conc.) values of norepinephrine (pg/μl of sample) are presented as the mean value ± SEM of six animals in each group. Baseline samples were collected 2 h after implantation of the microdialysis probe. *$p < 0.05$, **$p < 0.001$, compared with the sham-operated control animals. **Open bars** = sham; **dotted bars** = experimental.

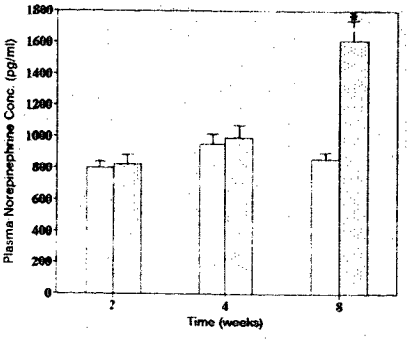
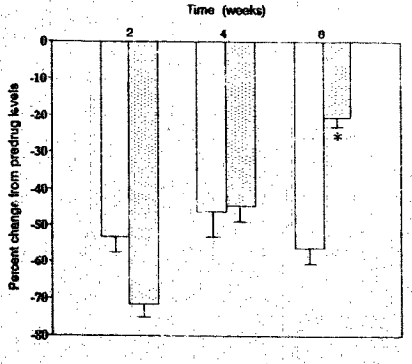


Figure 5. Plasma norepinephrine concentration (Conc.) in sham control and experimental animals 2, 4 and 8 weeks after coronary artery ligation. The concentration of norepinephrine in plasma (pg/ml of sample) is represented as the mean value ± SEM of six animals in each group. *$p < 0.05$, compared with the sham-operated control animals.

After the administration of neuropeptide Y at a concentration of 10^{-8} mol/liter, norepinephrine levels of the paraventricular nucleus in all the sham-operated control groups decreased by 50% to 60% when compared with the predrug baseline levels obtained before neuropeptide Y infusion (Fig. 4). Reduction was 70% (Fig. 4) in 2-week old experimental animals and ~50% (Fig. 4) in 4-week old experimental animals. A significant alteration was observed in 8-week old experimental animals in which the level of norepinephrine in the paraventricular nucleus changed by only 20% from the preneuropeptide Y level ($p < 0.05$) (Fig. 4).

To verify whether the inhibitory effect of neuropeptide Y was dose dependent, we performed experiments using other concentrations, such as 10^{-7} and 10^{-10} mol/liter. The results showed similar findings as those observed with the 10^{-8} mol/liter concentration (Fig. 5). Thus, most of the experiments were performed with 10^{-8} mol/liter concentration of neuropeptide Y. Preliminary results were also carried out to show that the release of norepinephrine by neuropeptide Y was specific and not artifactual, as other peptides such as metenkephalin failed to inhibit norepinephrine release.

Figure 4. Effect of neuropeptide Y (10^{-8} mol/liter) on the in vivo release of endogenous norepinephrine in the paraventricular nucleus of sham control and experimental animals 2, 4 and 8 weeks after coronary artery ligation. Norepinephrine release is expressed as the percent change from the predrug baseline levels. **Open bars** = sham; **dotted bars** = experimental.



Plasma norepinephrine analysis. Plasma levels of norepinephrine in the 2- and 4-week old experimental groups showed no differences in the level of norepinephrine with their sham-operated group; however, the 8-week old experimental group showed a 100% increase in the amount of norepinephrine ($p < 0.05$) over the sham group.

Neuropeptide Y receptor study. Figure 6B and C shows autoradiograms of neuropeptide Y receptors in the paraventricular nucleus area of sham and experimental rats. Nonspecific binding was determined as the binding observed in the presence of 1.0 μmol/liter porcine neuropeptide Y (Fig. 6A). The densitometric measurements of these autoradiograms demonstrated a significant decrease in the number of neuropeptide Y receptors in the paraventricular nucleus of experimental rats as compared with the sham group, as measured by optical density units (Fig. 7).

Discussion

Congestive heart failure secondary to myocardial infarction of the left ventricle has been reported to occur in rats after surgical ligation of the left coronary artery (21). This model has been widely accepted for studying the pathophysiologic course of this disease, and our animals with large, healed infarcts showed signs of pulmonary edema and cardiac hypertrophy. This was particularly evident in 8-week old rats after infarction.

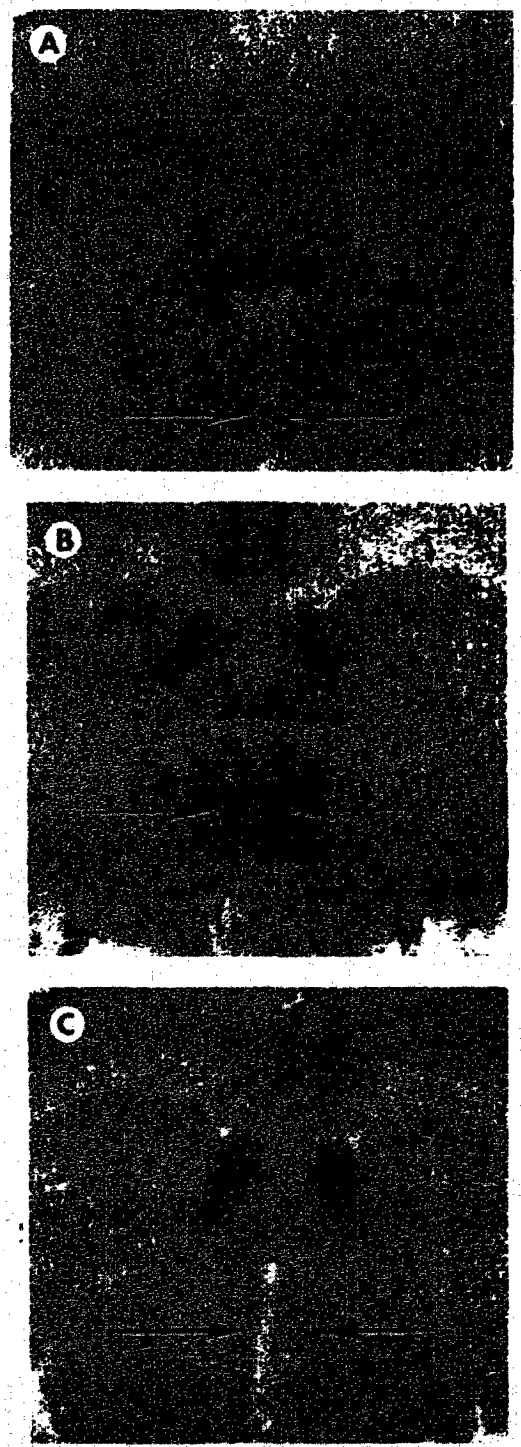


Figure 6. Autoradiographs of the paraventricular nucleus of sham control and experimental animals. The arrows point to the paraventricular nucleus region of the brain. **A,** Nonspecific binding in the presence of 1.0 μmol/liter porcine neuropeptide Y. **B,** Iodine-125 neuropeptide Y binding in the sham-operated control rats. **C,** Iodine-125 neuropeptide Y binding in the experimental rats 8 weeks after coronary artery ligation.

Although additional hemodynamic data are required to establish congestive heart failure in our model, the animals did, indeed have a large myocardial infarction with an early sign of congestive heart failure.

Microdialysis in vivo. Our results showed that the baseline norepinephrine level in the paraventricular nucleus was elevated several times over in the experimental animals of all the groups. These results were similar to our earlier findings, where increased release of endogenous norepinephrine from the paraventricular nucleus was found in aortic-banded and spontaneous hypertensive rats (10,25). Neuropeptide Y has been postulated to act as a neuromodulator in the central and peripheral sympathetic nervous systems (10,12). Exposing the paraventricular nucleus of sham-operated animals of all three groups to neuropeptide Y at a concentration of 10^{-8} mol/liter resulted in a decreased amount of norepinephrine in the microdialysate by 50% to 60%. Because neuropeptide Y did not affect the norepinephrine recovery rates of the probes, these changes are most likely the result of neuropeptide Y acting at the synaptic interface. This is further substantiated by the fact that neuropeptide Y is a known presynaptic inhibitor of norepinephrine release (9). In the central nervous system, neuropeptide Y is mainly colocalized with norepinephrine in postganglionic sympathetic neurons and seems to potentiate postsynaptic responses to the amine transmitter while presynaptically inhibiting its release (8). However, when neuropeptide Y at the same concentration was infused into the paraventricular nucleus of the 8-week old group of experimental animals, a reduction of only 20% in norepinephrine value was observed. The mechanisms responsible for this lack of effect of neuropeptide Y on catecholamines are not known; however, it has been reported that a decreased inhibitory effect of neuropeptide Y on norepinephrine release induces increased sympathetic nerve activity in the hypothalamus (12).

Plasma norepinephrine analysis. Although there are many reports indicating increased sympathetic activity after the development of congestive heart failure, the exact cause of heightened sympathetic drive remains an interesting point. Because the paraventricular nucleus is an important integrative area for both autonomic and cardiovascular control (26), an elevation in extracellular norepinephrine levels in the paraventricular nucleus may then be correlated with elevated central sympathetic activity (25). Enhanced central noradrenergic nerve activity has been suggested to be involved in the development and maintenance of congestive heart failure. Because sympathetic activity is increased in congestive heart failure models, alterations in the norepinephrine levels in the

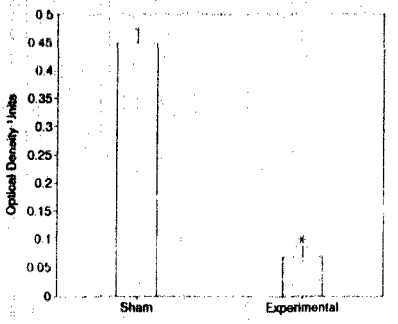


Figure 7. Histogram showing the number of neuropeptide Y receptors in optical density units. Specific binding was determined as the difference in binding observed on adjacent sections in the absence and presence of 1.0 μmol/liter porcine neuropeptide Y. Data are represented as the mean value ± SEM of six animals in each group, compared with the sham control animals. *$p < 0.05$.

paraventricular nucleus may be associated with this increase in sympathetic drive. This augmented sympathetic drive was demonstrated by our plasma analysis of catecholamine levels. The 8-week old experimental group had an almost 100% increase in plasma norepinephrine, as compared with all other groups.

Neuropeptide Y receptor study. It may be pointed out that the decreased ability of neuropeptide Y to act on the presynaptic site in the paraventricular nucleus at an advanced stage of congestive heart failure portrays a series of causal factors for this abnormality. Keeping in mind the role of receptors, our autoradiography experiment clearly shows a decrease in neuropeptide Y receptor density, which may be partially responsible for the ineffectiveness of neuropeptide Y in inhibiting norepinephrine presynaptically. Compared with the sham group, the number of neuropeptide Y receptors found in the paraventricular nucleus was significantly less in the experimental group of rats when analyzed statistically. This study was further supported by our previous experiments, which show a similar decrease in neuropeptide Y receptors in the paraventricular nucleus area of the aortic-banded rats (27).

Because neuropeptide Y is known to coexist with norepinephrine both centrally and peripherally and there is an increase in sympathetic activity during congestive heart failure, a downregulation of neuropeptide Y receptor kinetics could be expected in response to an increased catecholamine level and release. This downregulation of neuropeptide Y receptors after myocardial infarction may aggravate the release of norepinephrine and myocardial dysfunction. Studies have also shown a lower level of neuropeptide Y immunoreactivity in the hypertensive brain with respect to normotensive rats, where sympathetic activity is greater (11). Taken together, decreasing the sympathoinhibitory influence of neuropeptide Y in the paraventricular nucleus, through which a decrease in neuropeptide Y receptors is a possible mechanism, may reciprocally increase sympathetic activity in congestive heart failure.

Conclusions. The findings of the present study suggest the inability of neuropeptide Y to affect the presynaptic inhibition of norepinephrine release in the paraventricular nucleus, as evidenced by the unaltered norepinephrine levels 8 weeks after infarction, thus strengthening the hypothesis that the chain of events that initiates congestive heart failure may involve neuropeptide Y at the level of the paraventricular nucleus. Increased sympathetic activity may be caused by the changes in action of neuropeptide Y at the level of the paraventricular nucleus. Further studies to define the association between neuropeptide Y and congestive heart failure are clearly needed.

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