Enhancement of spontaneous baroreflex by antisense c-fos oligonucleotide treatment in the NTS of the rat

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Chan, Julie Y. H., Cheng-Dean Shih, and Samuel H. H. Chan. Enhancement of spontaneous baroreflex by antisense c-fos oligonucleotide treatment in the NTS of the rat. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2200–H2208, 1997.—We evaluated the hypothesis that basal Fos protein at the nucleus tractus solitarii (NTS), the primary terminal site for baroreceptor afferents, exerts a tonic inhibitory modulation on the spontaneous baroreceptor reflex (BRR) control machinery, which is responsible for beat-to-beat regulation of resting systemic arterial pressure (SAP). In adult male Sprague-Dawley rats anesthetized and maintained with pentobarbital sodium, microinjection bilaterally into the caudal NTS of a 15-mer antisense oligonucleotide that targets against the initiation codon of c-fos mRNA (5′-129 to 143-3′) significantly enhanced the spontaneous BRR response, as determined by transfer function analysis of SAP and heart rate signals. The same treatment also diminished baseline Fos-like immunoreactivity in the absence of acute cardiovascular perturbation. Control treatments with artificial cerebrospinal fluid, sense cDNA, or antisense oligonucleotides that either target against a different site of the c-fos mRNA (5′-135 to 149-3′) or with three mismatched nucleotides in the antisense sequence, were ineffective. These observations support the notion that, under minimal cardiovascular perturbation, basal expression of Fos protein in the NTS may represent an early step in the cascade of intracellular events that leads to long-term inhibitory modulation of beat-to-beat baroreflex control of blood pressure.

nucleus tractus solitarii; c-fos gene; auto- and cross-spectral analysis; transfer function analysis; Fos-like immunoreactivity

BASpE ON THE NOTIO (12, 21, 27, 28) that the proto-
oncogene c-fos plays a role in the responses of postmit-
otic neurons to trans-synaptic stimulation, immunohis-
stochemical detection of Fos-like protein has been used to
identify neuronal groups and trace pathways that
subserve specific physiological functions (27). A major
focus in recent studies on the anatomic and temporal
specificity of Fos expression after cardiovascular pertur-
bation is the caudal nucleus tractus solitarii (NTS), the
principal terminal site of the primary baroreceptor
afferents (7). Experimental activation of the barorecep-
tors (4, 9, 19, 23, 24, 29) reportedly increases the level of
inducible c-fos at the NTS. The physiological role of Fos
protein expressed in the NTS in central cardiovascular
regulation, however, has not been fully characterized.

The development (8) and application of antisense
oligonucleotides complementary to strategically chosen
sequences within the c-fos mRNA (6, 14–16) provided
an opportunity to decipher the functional role of Fos
protein in systemic physiology. In the area of central
cardiovascular regulation, Suzuki et al. (30) reported a
decrease in arterial pressure 6 h after injection of an
antisense c-fos cDNA into the rostral ventrolateral medulla. Our laboratory demonstrated recently (29) that microinjection of an antisense c-fos oligonucleotide bilaterally to the caudal NTS enhances the baroreceptor reflex (BRR) and reduces Fos expression at the NTS evoked by scheduled and transient pressor responses. We interpret these data to suggest that the inducible c-fos gene in the NTS may exert an inhibitory modulation on BRR responses evoked by cardiovascular perturbations.

The expression of immediate early genes, including
c-fos, is low in quiescent cells but is rapidly induced
within minutes of extracellular stimulation (28). Thus
a basic design in studies that evaluate the functional
roles of Fos protein invariably engages a physiological
or pharmacological intervention. One important ques-
tion that requires immediate attention is whether Fos
protein also plays a systemic physiological role during
minimal external disturbances. In this regard, the
spontaneous BRR, which is responsible for beat-to-beat
maintenance of resting systemic arterial pressure in
the absence of evoked cardiovascular perturbation (1,
11, 22), provides a suitable test model. In particular,
recent advances in computer applications allowed for
the evaluation of spontaneous BRR responses in the
form of, for example, transfer function analysis of
simultaneous fluctuations in resting systemic arterial
pressure and heart rate (2, 10, 18, 22, 25, 26, 32).

The specific purpose of this study is to test the
hypothesis that Fos protein at the NTS exerts a tonic
inhibitory modulation on the spontaneous BRR control
machinery. Our experimental strategy was to effect, in
rats with minimal perturbation of the cardiovascular
system, a functional blockade of Fos expression at the
NTS by an antisense oligonucleotide designed to target
a region of the c-fos mRNA that flanks the initiation
codon. Temporal changes in spontaneous BRR re-
response, as determined by transfer function analysis of
systemic arterial pressure and heart rate, were evalu-
ated along with immunohistochemical staining for Fos-
like immunoreactivity.

MATERIALS AND METHODS

Animals. Specific pathogen-free adult male Sprague-
Dawley rats (250–278 g) were obtained from the Experimen-
tal Animal Center of the National Science Council, Taiwan.
They were anesthetized initially with an intraperitoneal
injection of pentobarbital sodium (40 mg/kg). Preparatory
surgery included intubation of the trachea and cannulation of
the right femoral artery and both femoral veins. The head of
the animal was thereafter placed in a stereotaxic headholder
(Kopf 1404), with the rest of the body positioned on a heating
pad and elevated to a suitable position. During the experi-
ment, animals were allowed to breathe spontaneously with room air. Anesthetic maintenance was provided by intravenous infusion of pentobarbital sodium at 15–20 mg·kg⁻¹·h⁻¹. This management scheme was found (32) to provide stable anesthesia while preserving the capability of cardiovascular regulation, including the BRR response. Incision sites were also infiltrated with 0.25% bupivacaine hydrochloride, a long-duration local anesthetic. All data were collected from animals with a maintained rectal temperature of 37°C, with a steady mean systemic arterial pressure >90 mmHg throughout the experiment.

Phosphorothioate oligonucleotides. Four 15-mer oligonucleotides (6, 8, 14–16, 29), which were phosphorothioated in all positions (Quality Systems, Taipei, Taiwan), were used in this study. None of them showed significant complementarity to any other gene sequence in the GenBank database (14, 15).

The key antisense oligonucleotide (AS1) was designed to target against a region of the c-fos mRNA that flanks the initiation codon (5'-129 to 143-3'; 5'-GAA-CAT-CAT-GGT-GTG-3'). Three other oligonucleotides were used as our treatment controls. These included a sense c-fos oligonucleotide that is complementary to the antisense sequence (5'-ACG-ACC-ATG-ATG-CTC-3'), but shows no discernable complementarity to any part of the c-fos mRNA. We also used an antisense DNA (AS2) that targets against the initiation codon and a different portion of the coding sequence (5'-135 to 149-3'; 5'-ACC-CGA-GAA-CAT-CAT-3') of the c-fos mRNA as well as another antisense c-fos oligonucleotide (AS3) with three mismatched nucleotides (5'-GTA-CAC-CAT-GGT-TGT-3') in the AS1 sequence.

Microinjection of oligonucleotides. Bilateral microinjection of sense or antisense c-fos oligonucleotide into the caudal NTS was carried out stereotaxically with a glass micropipette (50–70 µm tip) connected to a 0.5-µl Hamilton microsyringe. The stereotaxic coordinates used were as follows: 0.35 to 0.9 mm below the surface of the fourth ventricle, 0 to −0.5 mm from, and 0.35 to 0.5 mm lateral to, the obex. As in our previous studies (5, 29), the volume of microinjection was limited to 20 nl on each side to restrict the extent of diffusion and minimize the confounding effect of volume artifact. All oligonucleotides were diluted in artificial cerebrospinal fluid (aCSF) at pH 7.4. Apart from using sense and AS2 or AS3 c-fos antisense oligonucleotides as the treatment control, we also included two additional control groups in our study. First, animals that were surgically prepared, placed in the stereotaxic headholder without subsequent experimental treatments, and maintained by intravenous infusion of pentobarbital sodium for 180 min served as the sham control. Second, animals that received microinjection of aCSF into the bilateral NTS served as the volume and vehicle control.

Recording and analysis of systemic arterial pressure, heart rate, and spontaneous BRR response. We routinely followed for 180 min the effect of each c-fos oligonucleotide on systemic arterial pressure (SAP), heart rate (HR), and spontaneous BRR response. The procedures for recording and analysis were similar to those in our previous studies (17, 32) and were based on computer algorithms that our laboratory developed for auto- and cross-spectral analysis of SAP and HR signals. SAP was measured from the right femoral artery via a PE-50 catheter filled with heparinized saline (200 U/ml). The arterial catheter was connected to a pressure transducer (Statham P23 ID) and in turn to a pressure processor amplifier (Gould G-13-4615-52). The SAP signals were relayed to a 12-bit analog-to-digital converter (Advantech PCL818) that was connected to a computer at a sampling rate of 2,048 Hz. HR was estimated continuously and instantaneously from the digitized SAP signals. The computer we used was a general-purpose personal computer (IBM-PC compatible) equipped with an Intel 80486 microprocessor and 4 MB of random access memory. A dot-matrix printer was used to output the results graphically or numerically.

The power density of individual spectral components of SAP and HR signals was simultaneously estimated based on fast Fourier transform, displayed, and printed in an online and real-time manner. For each 896-s recording period, 55 data sets (1,024 points/set) of SAP and HR signals, overlapping by 50%, were processed. The average periodogram was generated by averaging the autospectra of each data set. Cross-spectral analysis was generated from the same 55 data sets of SAP and HR signals, which led to the estimation of magnitude-squared coherence function and magnitude of transfer function. The value of coherence ranges from 0 to 1 and provides an assessment of the linear relationship at each frequency and of the statistical reliability of transfer function. For this purpose, a coherence = 0.5 was considered to be statistically significant (10, 18, 32). The magnitude of SAP-HR transfer function was used as the index for spontaneous BRR response (2, 10, 18, 22, 26, 32). For the purpose of this study, values determined at the frequency range for the high-frequency spectral component (0.8–2.4 Hz) were used. Because this spectral component is related to respiratory movements (32) and animals were breathing spontaneously, measurements of the magnitude of SAP-HR transfer were usually obtained within the range of 0.9–1.9 Hz.

Immunohistochemical staining. At the conclusion of the experiment, each animal was perfused intracardially with warm heparinized isotonic saline, followed by ice-cold paraformaldehyde in phosphate-buffered saline at pH 7.2. The brain stem was removed and postfixed by submersion in the latter solution overnight at 4°C, followed by cryoprotection with 30% sucrose in 0.1 M phosphate-buffered saline at 4°C. Free-floating frozen transverse sections (20 µm) of the medulla oblongata were processed for Fos-like immunoreactivity (Fos-LI) based on our modification (29) of the peroxidase-antiperoxidase method, using a polyclonal sheep anti-Fos antisemur raised against Fos protein (1:4,000; Cambridge Research Biochemicals OA-11-824). Visualization of the immunoreactive product included incubation in sheep peroxidase conjugated against rabbit IgG (1:160, DAKO, Copenhagen Research), followed by reaction with glucose oxidase and diaminobenzidine using a nickel/cobalt intensification procedure.

Sections from rats that received various treatments were processed together for Fos-LI. In control experiments, sections were incubated without the anti-Fos antisemur or substituting Fos antisemur with normal sheep serum. No specific immunoreactivity was observed in these control sections when they were processed together with the experimental tissues. After immunohistochemical processing, sections were mounted onto slides covered with gelatin and chromium potassium sulfate and air dried. To localize the distribution of Fos-LI with reference to anatomic structures, sections were counterstained with 1% neutral red. The same sections were used to verify the location of the tip of the micropipette used for microinjection. Evans blue (1%) was added to all microinjection solutions to facilitate this verification.

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Tissue sections were examined under bright-field microscopy (Olympus BH-2) to localize Fos-LI in the NTS. The criterion for identification of Fos-LI neurons was a distinctly stained nucleus (23, 29). Nuclei within the confines of the NTS thus identified were counted bilaterally in each section with the aid of a camera lucida drawing attachment. The caudal medulla oblongata was divided into eight levels, at 200-µm intervals, between 1 mm caudal and 0.4 mm rostral.
to the obex. Five sections selected randomly from each level were counted separately by two individuals, and the mean number of the Fos-positive cells for each level of the NTS was tabulated. Photomicrographs were taken with a Nikon inverted microscope (Diaphot 300) equipped with Nomarski optics.

Statistical analysis. All values are expressed as means ± SE. The temporal effect of treatments on the spontaneous BRR response was assessed by two-way analysis of variance (ANOVA) with repeated measures. This was followed by the Scheffe’s multiple-range test for a posteriori comparison of means at corresponding time intervals. The number of Fos-positive neurons at each level of the NTS under various treatments was compared using one-way ANOVA. This was followed by the Scheffe’s multiple-range test for a posteriori multiple comparison of means. In both analyses, \( P < 0.05 \) was considered to be statistically significant.

RESULTS

Effect of AS1 antisense c-fos oligonucleotide treatment on spontaneous BRR response, SAP, and HR. Bilateral microinjection of AS1 antisense c-fos oligonucleotide (20 pmol) into the caudal NTS discernibly (\( F_{5,25} = 12.95, P < 0.01 \)) enhanced the spontaneous BRR response, as determined by the magnitude of transfer function between SAP and HR signals (Fig. 1). The trend of enhancement began at 90 min, became significant at 120 min, and lasted until at least 180 min postinjection (Fig. 2A). No appreciable alteration in SAP (\( F_{5,25} = 0.76, P > 0.05 \)) or HR (\( F_{5,25} = 1.52, P > 0.05 \)), however, was noted during the 180 min after application of AS1 antisense c-fos cDNA bilaterally into the NTS (Fig. 3).

Effect of AS1 antisense c-fos oligonucleotide treatment on baseline Fos-LI in NTS. Microinjection of AS1 c-fos oligonucleotide (20 pmol) bilaterally into the caudal NTS also reduced baseline Fos-LI at the NTS (Figs. 4C and 5C), detected 180 min later, to a level that was significantly lower (\( F_{5,24} = 16.39, P < 0.01 \)) than all other treatment groups (Fig. 6A). In particular, the number of Fos-positive neurons around the level of, and immediately caudal to, the obex was discernibly (\( P < 0.05 \)) less than that in sham-control animals. It should be mentioned that Fos-LI was still demonstrated in the caudal (Fig. 4D) and rostral ventrolateral medulla in the AS1-treated animals. This ascertained that the decrease in Fos-LI at the NTS after treatment with this antisense oligonucleotide was not due to false-negative reaction.

Effects of control c-fos oligonucleotides on spontaneous BRR response, SAP, and HR. We verified the specificity of the observed biological activity of AS1 antisense c-fos cDNA by evaluating the effects of three control sequences of oligonucleotide. Treatment with microinjection bilaterally into the caudal NTS of the sense c-fos oligonucleotide (Figs. 1 and 2B), similar to sham- and aCSF-control animals (Fig. 2A), resulted in no discernible alteration in the magnitude of SAP-HR transfer function. Comparable observations were obtained from treatment with an antisense cDNA that

![Fig. 1. Illustrative examples of average periodograms of systemic arterial pressure (BPSD) and heart rate (HPSD) signals and their cross-spectrograms showing coherence (COHER) and magnitude of transfer function, taken 30 min before (control) and 180 min after microinjection bilaterally into caudal nucleus tractus solitarii (NTS) (20 pmol) of a sense oligonucleotide or an antisense cDNA that targets against the initiation codon (AS1) or a different region (AS2) of c-fos mRNA, or with three mismatched nucleotides in AS1 sequence (AS3). A coherence function ≥0.5 is considered to be statistically significant. Note that corresponding range of frequencies (0.9–1.9 Hz) in transfer function, which represents spontaneous baroreceptor reflex response, is denoted by a heavy line.]
either targets against a different site (5'-135 to 149-3') of the c-fos mRNA (AS2; 20 pmol) (Figs. 1 and 2B) or with three mismatched nucleotides in the AS1 sequence (AS3; 20 pmol) (Fig. 2B). Bilateral microinjection into the caudal NTS of the sense or AS2 or AS3 antisense c-fos oligonucleotide also produced minimal effect on SAP or HR during the 180-min observation period (Fig. 3). Similarly, microinjection bilaterally into the caudal NTS of AS2 antisense cDNA (20 pmol) resulted in findings (Figs. 4E, 5E, and 6B) that were reminiscent of those obtained from treatment with the sense oligonucleotide. Similar observations (Figs. 4F, 5F, and 6B) were made with AS3 antisense c-fos oligonucleotide (20 pmol) and aCSF (Figs. 4A, 5B, and 6A).

Effect of control c-fos oligonucleotides on baseline Fos-LI in NTS. In sham-control animals, the NTS neurons that expressed Fos-LI were bilaterally represented (Fig. 5A), with no apparent topographic distribution (Fig. 6A). Because these animals received only surgical preparation, were placed in the stereotaxic headholder, and were maintained under pentobarbital anesthesia, this level of Fos-LI in the NTS was taken as the baseline in the present study.

A significant increase in Fos-LI over sham-controls was observed (Fig. 4B) in the NTS of animals that received microinjection of the sense c-fos oligonucleotide (20 pmol), extending from the rostral extent of the pyramidal decussation to the level of area postrema (Fig. 6B). Within the NTS, Fos-positive cells were bilaterally represented and were found mainly in the medial, dorsomedial, and commissural subnuclei (Fig. 5D). Treatments with microinjection bilaterally into the caudal NTS of AS2 antisense cDNA (20 pmol) resulted in findings (Figs. 4E, 5E, and 6B) that were reminiscent of those obtained from treatment with the sense oligonucleotide. Similar observations (Figs. 4F, 5F, and 6B) were made with AS3 antisense c-fos oligonucleotide (20 pmol) and aCSF (Figs. 4A, 5B, and 6A).

Fig. 2. Time course changes in magnitude of transfer function between systemic arterial pressure and heart rate signals, which represents spontaneous baroreceptor reflex response, in sham-control animals (A) and in rats that received bilateral microinjection into caudal NTS of artificial cerebrospinal fluid (aCSF; 20 nl), a sense oligonucleotide (20 pmol; B) or an antisense cDNA that targets against the initiation codon (AS1, 20 pmol; A) or a different region of c-fos mRNA (AS2, 20 pmol; B), or with 3 mismatched nucleotides in AS1 sequence (AS3, 20 pmol; B). Values are means ± SE; n = 5 or 6 animals per experimental group. Significant difference (F5,25 = 12.95, P < 0.01) detected among treatment groups in 2-way analysis of variance (ANOVA) with repeated measures. *P < 0.05 vs. sham-control group in Scheffe’s multiple-range analysis.

Effect of control c-fos oligonucleotides on baseline Fos-LI in NTS. In sham-control animals, the NTS neurons that expressed Fos-LI were bilaterally represented (Fig. 5A), with no apparent topographic distribution (Fig. 6A). Because these animals received only surgical preparation, were placed in the stereotaxic headholder, and were maintained under pentobarbital anesthesia, this level of Fos-LI in the NTS was taken as the baseline in the present study.

A significant increase in Fos-LI over sham-controls was observed (Fig. 4B) in the NTS of animals that received microinjection of the sense c-fos oligonucleotide (20 pmol), extending from the rostral extent of the pyramidal decussation to the level of area postrema (Fig. 6B). Within the NTS, Fos-positive cells were bilaterally represented and were found mainly in the medial, dorsomedial, and commissural subnuclei (Fig. 5D). Treatments with microinjection bilaterally into the caudal NTS of AS2 antisense cDNA (20 pmol) resulted in findings (Figs. 4E, 5E, and 6B) that were reminiscent of those obtained from treatment with the sense oligonucleotide. Similar observations (Figs. 4F, 5F, and 6B) were made with AS3 antisense c-fos oligonucleotide (20 pmol) and aCSF (Figs. 4A, 5B, and 6A).

Fig. 3. Time course changes in mean systemic arterial pressure (MSAP, A) or heart rate (HR, B) in sham-control animals and in rats that received bilateral microinjection into caudal NTS of aCSF, a sense oligonucleotide (20 pmol) or an antisense cDNA that targets against the initiation codon (AS1, 20 pmol) or a different region of c-fos mRNA (AS2, 20 pmol), or with 3 mismatched nucleotides in AS1 sequence (AS3, 20 pmol). Values are means ± SE; n = 5 or 6 animals per experimental group. No significant difference was detected between groups (P > 0.05) in 2-way ANOVA with repeated measures for MSAP (F5,25 = 0.76) or HR (F5,25 = 1.52).
Fig. 4. Representative photomicrographs taken under Nomarski optics showing distribution of Fos-like immunoreactivity (Fos-LI) in caudal NTS, at 0.2–0.4 mm posterior to obex, of animals that received bilateral microinjection into caudal NTS of aCSF (A), a sense oligonucleotide (20 pmol; B), an antisense cDNA that targets against the initiation codon (AS1, 20 pmol; C) or a different region (AS2, 20 pmol; E) of c-fos mRNA, or with 3 mismatched nucleotides in AS1 sequence (AS3, 20 pmol; F). D: same brain stem section as in C; demonstrates presence of Fos-LI at caudal ventrolateral medulla. AP, area postrema; NTS, nucleus tractus solitarii; ts, tractus solitarii; X, nucleus dorsalis nervi vagi. Calibration bar = 50 µm.
Microinjection sites. Histological verifications revealed that the above findings were obtained from animals in which the core of the microinjection sites was localized in the caudal NTS, at 0 to \(-0.6\) mm from the obex. Microinjection of the same amount of aCSF, or AS1, AS2, AS3 antisense or sense c-fos oligonucleotide, into more rostral part of the NTS (0.8 to 1.0 rostral to the obex) or areas adjacent to the NTS elicited no discernible alterations in Fos-LI in the caudal NTS, SAP-HR transfer function, SAP, or HR.

DISCUSSION

Based on a multidisciplinary approach that incorporated methodologies in molecular biology, computer applications, cardiovascular physiology, and immunohistochemistry, the present study provided the first demonstration of a tonic inhibitory modulatory role for Fos protein at the NTS in beat-to-beat cardiovascular regulation in animals under minimal perturbation of the circulatory system. Our data showed that antisense c-fos oligonucleotide treatment enhanced the spontaneous BRR response. We further showed that this enhancement was accompanied by a reduction in the baseline level of Fos protein at the NTS.

Activation of the baroreceptors induces Fos expression at the NTS in experimental animals (4, 9, 19, 23, 24). We reported recently (29) that the BRR and Fos-LI in the NTS evoked by transient pressor responses are, respectively, enhanced and reduced upon microinjection of AS1 antisense c-fos oligonucleotide into the caudal NTS. By demonstrating comparable results in animals with stable SAP and HR, the present observations extend the implied modulatory role of Fos protein at the NTS to spontaneous BRR regulation. Baroreceptors belong to the slowly adapting class of sensory transducers (13). Thus, in the absence of abrupt cardiovascular perturbations, beat-to-beat SAP and HR are maintained primarily by the spontaneous BRR, which

Fig. 5. Distribution of Fos-like immunoreactivity in 4 representative rostral-caudal sections of NTS in sham-control animals (A) and in rats that received bilateral microinjection into caudal NTS of aCSF (B), a sense oligonucleotide (20 pmol; D), an antisense cDNA that targets against the initiation codon (AS1, 20 pmol; C) or a different region (AS2, 20 pmol; E) of c-fos mRNA, or with 3 mismatched nucleotides in AS1 sequence (AS3, 20 pmol; F). Each dot represents one Fos-positive nucleus. Numbers indicate distance from obex. XII, hypoglossal nucleus.
in essence finely adjusts the natural fluctuations in these two hemodynamic parameters (1, 11, 22). Thus our present demonstration that Fos protein at the NTS exerts a tonic inhibitory modulation on the sensitivity of spontaneous BRR is of physiological significance. It indicates that Fos protein in the NTS participates actively in the cascade of intracellular events that are engaged in beat-to-beat modulation of baroreflex control of blood pressure.

The present study benefited from our capability to carry out on-line auto- and cross-spectral analysis of SAP and HR signals. Studies on animals and humans have demonstrated that the power spectrum of beat-to-beat changes in SAP or HR has a very specific pattern, which justifies its use as a quantitative probe (3, 20). By evaluating the complex relationship between the short-term rhythms in SAP and HR, cross-spectral analysis further offers a sophisticated and powerful tool in cardiovascular research (2, 10, 18, 22, 25, 26, 32). We previously demonstrated (32) that under the scheme of anesthetic maintenance we used in this study, the sensitivity of spontaneous BRR as represented by the magnitude of transfer function is comparable to that measured by the reflex bradycardia in response to evoked transient pressor responses. Nonetheless, the current method allowed us to evaluate, without physiological or pharmacological intervention of the cardiovascular system, the temporal changes in spontaneous BRR response after microinjection of AS1 antisense c-fos oligonucleotide bilaterally into the NTS. As a result, we were able to detect a time course of enhancement of spontaneous BRR response that was consistent with biochemical results that showed rapid uptake of antisense oligonucleotides by neurons in the vicinity of injection site within 15–30 min (31).

We reported previously (17, 18, 32) that substantial changes in the spectral components may take place in the face of minimally observable alterations in SAP or HR. On the basis of the heightened detection sensitivity of power spectral analysis, the same observation was again made in the present study. Microinjection of AS1 antisense c-fos oligonucleotide bilaterally into the NTS resulted in significant enhancement of spontaneous BRR response in the absence of discernible alterations in SAP or HR over 180 min. This revealed that basal Fos protein at the NTS participates actively in the subtle cascade of intracellular events that leads to long-term inhibitory modulation of BRR control of blood pressure.

A number of recent studies (6, 14–16, 30) reported that direct application of antisense c-fos oligonucleotides complementary to strategically chosen sequences within the target mRNA into specific regions of the brain produces discrete, reversible inactivation of c-fos gene in a highly selective manner. We demonstrated in the present study that microinjection into the caudal NTS of AS1 antisense oligonucleotide significantly enhanced the spontaneous BRR response and reduced the number of baseline Fos-positive NTS neurons. This signifies that the presence of a critical amount of expressed Fos protein at the NTS is pivotal to beat-to-beat baroreflex regulation of blood pressure. Because Fos protein has been suggested to function as a transcription factor in stimulus-transcription coupling (21), we speculate that the expression of c-fos gene in NTS neurons may lead to the induction of other genes that encode neurotransmitters and/or neuropeptides. These chemical signals may in turn exert a long-term inhibitory modulation on the sensitivity of BRR response. Previous work from our laboratory indicate that endogenous neuropeptides such as neurotensin (5) exert a tonic suppression on BRR response by acting on the caudal NTS. Baroreceptor activation results in the expression of Fos-LI in neurons in the NTS (19, 23, 24) that are also immunoreactive to tyrosine hydroxylase and/or phenylethanolamine-N-methyltransferase. Whether these chemical signals may underlie the long-term modulation of spontaneous BRR sensitivity by Fos
protein in NTS neurons, however, requires further elucidation.

The use of antisense oligonucleotide to block gene expression and to delineate potential roles of c-fos to specific stimulation in the central nervous system has attracted substantial enthusiasm. However, we must ascertain that the key antisense c-fos oligonucleotide depressed specifically the expression of the target gene. In this regard, the AS1 antisense c-fos oligonucleotide we used in this study is designed to target a region of the c-fos mRNA that flanks the initiation codon (5'-129 to 143-3'). Application of AS1 into specific regions of the brain produces discrete and reversible inactivation of c-fos gene in response to administration of amphetamine into the striatum (14, 15), reduces Fos protein without affecting another immediate early gene product, c-jun (16), and blunts Fos expression in the NTS evoked by repeated and scheduled transient pressor responses (29).

Sense oligonucleotides complementary to the antisense sequence (14, 16, 30), oligonucleotides of the same length as the antisense but targets a different site of the mRNA (8, 14), composed of a random mixture of all four nucleotides (14) or a mismatch of two or three base pairs (6), have all been used as controls to verify the biological activity of the antisense c-fos oligonucleotide. Our present results demonstrated that a sense oligonucleotide complimentary to the antisense c-fos sequence, and an antisense (AS2) cDNA that targets the initiation codon and portion of the coding sequence (5'-135 to 149-3') or with three mismatched base pairs (AS3) against the AS1 sequence, resulted in minimal alterations in SAP, HR, or spontaneous BRR response. Thus it appears that the blunting effect of AS1 antisense oligonucleotide was related to its complementarity with c-fos mRNA. It is also interesting to note that Chiasson et al. (6) reported that the three-base mismatch (AS3) does not reduce the effectiveness of the antisense c-fos oligonucleotide in their striatal model system. Thus interpretations of data obtained from antisense oligonucleotides with mismatched nucleotides must take into consideration the neural substrate and test system.

We did observe that microinjection bilaterally into the caudal NTS of aCSF, or sense or AS2 or AS3 antisense c-fos oligonucleotides, elicited a significant increase in Fos-LI over the sham controls. Because the rostrocaudal extent of this increase coincided with the location of microinjection sites, the possibility exists that the Fos expression may have been induced by the physical action of microinjection. However, the contribution of this confounding factor was considered small, since none of these control treatments resulted in significant alterations in the SAP-HR transfer function. On the other hand, AS1 antisense c-fos oligonucleotide applied similarly elicited a discernible reduction in baseline Fos-LI at the caudal NTS and an enhancement of spontaneous BRR. These results again support the notion that the presence of a critical amount of expressed Fos protein at the NTS is crucial to beat-to-beat baroreflex regulation of SAP. All oligonucleotides used in the present study were phosphorothioated in every position and are reportedly stable in brain tissues for at least 12 h (6, 14–16). The concern for toxicity of nuclease-resistant phosphorothioate oligonucleotides was deemed minimal for several reasons. 1) The four c-fos oligonucleotides elicited differential effects in animals in which the core of the microinjection sites was verified histologically to be localized in the caudal NTS. 2) Microinjection of the same amount of AS1 antisense c-fos oligonucleotide into more rostral part of the NTS (0.8 to 1.0 rostral to the obex) or areas adjacent to the NTS elicited no discernible effect on Fos-LI in caudal NTS or spontaneous BRR response. 3) A single injection of the phosphorothioate oligonucleotide into neural tissues in vivo does not appear to cause toxicity (6).

In conclusion, the present study demonstrated that functional blockade of Fos expression in the NTS with an antisense oligonucleotide that targets a region of the c-fos mRNA that flanks the initiation codon resulted in an enhancement of the spontaneous BRR response. The same treatment also diminished baseline Fos-LI in the absence of acute cardiovascular perturbation. These observations support the notion that basal expression of Fos protein in the NTS may represent an early step in the cascade of intracellular events that leads to long-term modulation of beat-to-beat baroreflex control of blood pressure.

We thank Dr. Terry B. J. Kuo for assistance with the computer algorithms for power spectral analysis.

This work was supported in part by National Science Council Grants NSC-86-2314-B075-001-M10 (to J. Y. H. Chan) and NSC-86-2314-B010-039-M10 (to S. H. H. Chan) and by National Health Research Institutes, Taiwan, Republic of China, Grant DOH-86-HR-510 (to S. H. H. Chan).

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Received 6 March 1997; accepted in final form 7 July 1997.

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