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• BRIEF REPORTS •

Expression and localization of c-Fos and NOS in the central nerve system following esophageal acid stimulation in rats

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Abstract

AIM: To determine the distribution of neurons expressing c-Fos and nitric oxide synthase (NOS) in the central nerve system (CNS) following esophageal acid exposure, and to investigate the relationship between c-Fos and NOS.

METHODS: Twelve Wistar rats were randomly divided into two equal groups. Hydrochloric acid with pepsin was perfused in the lower part of the esophagus for 60 min. As a control, normal saline was used. Thirty minutes after the perfusion, the rats were killed and brains were removed and processed for c-Fos immunohistochemistry and NADPH-d histochemistry. Blood pressure (BP), heart rate (HR), and respiratory rate (RR) during the experimental procedures were recorded every 10 min.

RESULTS: There were no significant differences in BP, HR and RR between the two groups. c-Fos immunoreactivity was significantly increased in rats receiving acid plus pepsin perfusion in amygdala (AM), paraventricular nucleus (PVN), parabrachial nucleus (PBN), nucleus tractus solitarius and dorsal motor nucleus of vagus (NTS/DMV), nucleus ambiguous (NA), reticular nucleus of medulla (RNM) and area postrema (AP). NOS reactivity in this group was significantly increased in PVN, PBN, NTS/DMV, RNM and AP. c-Fos and NOS had significant correlation between PVN, PBN, NTS/DMV, RNM and AP.

CONCLUSION: Acid plus pepsin perfusion of the esophagus results in neural activation in areas of CNS, and NO is likely one of the neurotransmitters in some of these areas.

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INTRODUCTION

Reflux esophagitis (RE) is a common gastrointestinal motility disorder. Esophageal reflux occurs when gastric contents move in a retrograde direction into the esophagus, and esophagitis develops by prolonged exposure to gastric contents. This happens when the lower esophageal sphincter fails to provide an adequate mechanical barrier, when the esophageal peristaltic contractions fail to provide adequate clearance of the gastric contents, and/or when gastric contents exist for a prolonged time due to gastroparesis[1]. Esophageal motility is controlled by a variety of factors of which the nerve system is the most important one. Locally, motility disorder caused by esophagitis is usually due to the decreased release of acetylcholine[2,3], signal transduction failure[4], and/or decreased intracellular Ca^{2+}[5]. In CNS, little has been known about the distribution of activated neurons after esophageal acid exposure[6].

It is reported that *c-fos* is the most well characterized IEGs (immediate early genes) in neurons; the *c-fos* message is induced within minutes of stimuli and the protein is expressed within 1-3 h[7-10]. The expression of *c-fos* in CNS is considered to be a marker of neuronal activity following an appropriate stimulus, and the site of central expression of c-Fos in response to a stimulus is used as a means of elucidating the course of the response[11-16]. Nitric oxide (NO) acts as an intercellular messenger in CNS. As a highly diffusible and short-lived gas, NO is always studied by means of nitric oxide synthase (NOS)[17]. Studies have shown that NOS-containing neurons are identical to those selectively stained for NADPH diaphorase[18]. The present study was designed to determine the distribution of neurons expressing c-Fos and NOS in CNS following esophageal acid exposure, and to investigate the relationship between c-Fos and NOS.

MATERIALS AND METHODS

Animals

Twelve male Wistar rats weighing 220-260 g were housed in standard home cages under conditions of controlled illumination (12:12 h light/dark cycle), humidity, and temperature (18-26 ℃) for at least 7 d prior to the experimental procedure. They were fed a standard rat diet and tap water. The animals were deprived of food but not water 12-16 h before each experiment. They were randomly divided into two equal groups. All procedures were approved by the Committee for Animal Care and Usage for Research and Education of the Peking University.

Methods

Rats were anaesthetized with an intraperitoneal injection of urethane (1.0 g/kg). After a rat reached a complete state of anesthesia, the abdominal wall and gastric wall were incised, and a drainage cannula was inserted in the gastric cardia to collect run-off solution from the esophagus. The anesthetized rat, strapped supine to an animal board, was then positioned with its head elevated at a slight angle (20-30°). A single lumen clear vinyl tube (ID 0.05 mm, *A* 0.8 mm) was passed by mouth into the esophagus. The tip of the cannula was located 3 cm above the esophagogastric junction. The cannula was then positioned and connected to a continuous perfusion pump (Medical Equipment Ltd. Zhejiang University, Hangzhou, China). A solution containing hydrochloric acid (HCl 0.1 mol/L) and pepsin (2 000-4 000 U/mL) (pH 1.5) was perfused continuously at a rate of 10 mL/h for 60 min. As a control, normal saline was used. Blood pressure (BP), heart rate (HR) and respiratory rate

(RR) during the experimental procedures were recorded every 10 min. After perfusion, the rat was left undisturbed for another 30 min before being deeply anesthetized with urethane (1.5 g/kg i.p.). The animal then was transcardially perfused with 9 g/L saline followed by 40 g/L paraformaldehyde in 0.1 mol/L phosphate buffer saline (PBS, pH 7.3). The brain was removed and postfixed in the same fixative overnight and cryoprotected by immersion in 200 g/L sucrose for 72 h. Coronal sections (40 μm) of the brain were cut in a cryostat. Every fourth section was used to reveal c-Fos immunoreactivity and NADPH-diaphorase (NADPH-d) staining, and the second set of sections was used as a control for the immunohistochemical reaction.

The sections were collected and rinsed in 0.01 mol/L PBS containing 3 g/L Triton X-100 (PBST). Then they were incubated at 37 ℃ for 2 h in a solution containing 1 mmol/L NADPH (Biomol, London, UK), 0.5 mmol/L nitroblue tetrazolium (Biomol), Tris-HCl 50 mmol/L, and Triton X-100 2 g/L. After a rinse in PBST, sections were placed into a 50 g/L goat serum for 30 min at room temperature (RT), and incubated overnight at RT in primary antibody c-Fos (1:200, Santa Cruz Biotechnology, California, USA). After washing for 15 min with PBST, the sections were incubated in biotinylated anti-rabbit IgG (Zymed, South San Francisco, Canada) diluted 1:300 in PBST at RT for 2 h, and then incubated in peroxidase-conjugated streptavidin (1:300 dilution, Zymed) for 2 h at RT. The immunoreactivity was visualized by incubating with 0.05 mol/L Tris-HCl buffer containing 0.1 g/L 3,3'-diaminobenzidine, and 0.3 mL/L H_2O_2 for 10-20 min at RT. The stained sections were mounted on APES-coated glass slides, dehydrated and coverslipped.

Statistical analysis

BP, HR and RR recorded every 10 min during the 90-min experimental procedures were averaged per animal and then per experimental group, respectively. The distribution of c-Fos and NADPH-d positive cells was detected under a microscope (Olympus, Tokyo, Japan), and the cells were counted on LEICA Q550CW system (Leica Microsystems Imaging Solutions Ltd, Wetzlar, Germany). The numbers of cells containing c-Fos immunoreactivity and NADPH-d were counted unilaterally in specific nuclei in several sections; 5 sections for amygdala (AM), nucleus tractus solitarius and dorsal motor nucleus of vagus (NTS/DMV), nucleus ambiguous (NA) and reticular nucleus of medulla (RNM) and 4 sections for paraventricular nucleus (PVN), supraoptic nucleus (SON), parabrachial nucleus (PBN) and area postrema (AP). The average number of c-Fos or NADPH-d positive neurons per section for each rat was calculated, respectively, by dividing the total number of c-Fos or NADPH-d positive cells obtained from all sections by the number of sections taken for each brain nucleus. Data were expressed as mean±SD of the respective brain areas. Statistical analyses were performed by SPSS 12.0 using the *t*-test, and a *P* value of less than 0.05 was considered statistically significant. The relationship between c-Fos and NADPH-d positive cells was performed by the correlation analysis.

RESULTS

BP, HR and RR to acid-pepsin perfusion

Esophageal acid perfusion did not change BP (18.03±1.07 *vs* 17.26±0.62 kPa, F=2.663, P=0.134), HR (275.30±14.43 *vs* 265.00±22.12 beats/min, F=1.343, P=0.273), and RR (92.00±10.41 *vs* 94.56±9.46 breathes/min, F=0.078, P=0.785) compared with control group.

c-Fos and NADPH-d staining in CNS

The c-Fos positive cell nuclei of activated cells showed the characteristic dark brown staining of oxidized DAB. In both groups of rats, c-Fos expression was observed in several brain regions. In telencephalon and diencephalon, c-Fos positive cells were mainly located in AM (Figure 1A, B), PVN (Figure 1C, D), SON and the numbers of the former two areas increased significantly in the acid-pepsin perfusion group (Table 1). Esophageal exposure to acid and pepsin also stimulated a significantly greater number of c-Fos-labeled neurons in areas of brain stem including PBN, NTS/DMV, NA (Figure 2A, D), RNM and AP (Table 1). NADPH-d activity was visualized as a vibrant blue color within perikarya, dendrites and axons. Acid-pepsin perfusion significantly increased the numbers of NADPH-d stained cells in PVN (Figure 1C, D), PBN, NTS/DMV, RNM and AP (Table 1). There were some coexistence of Fos

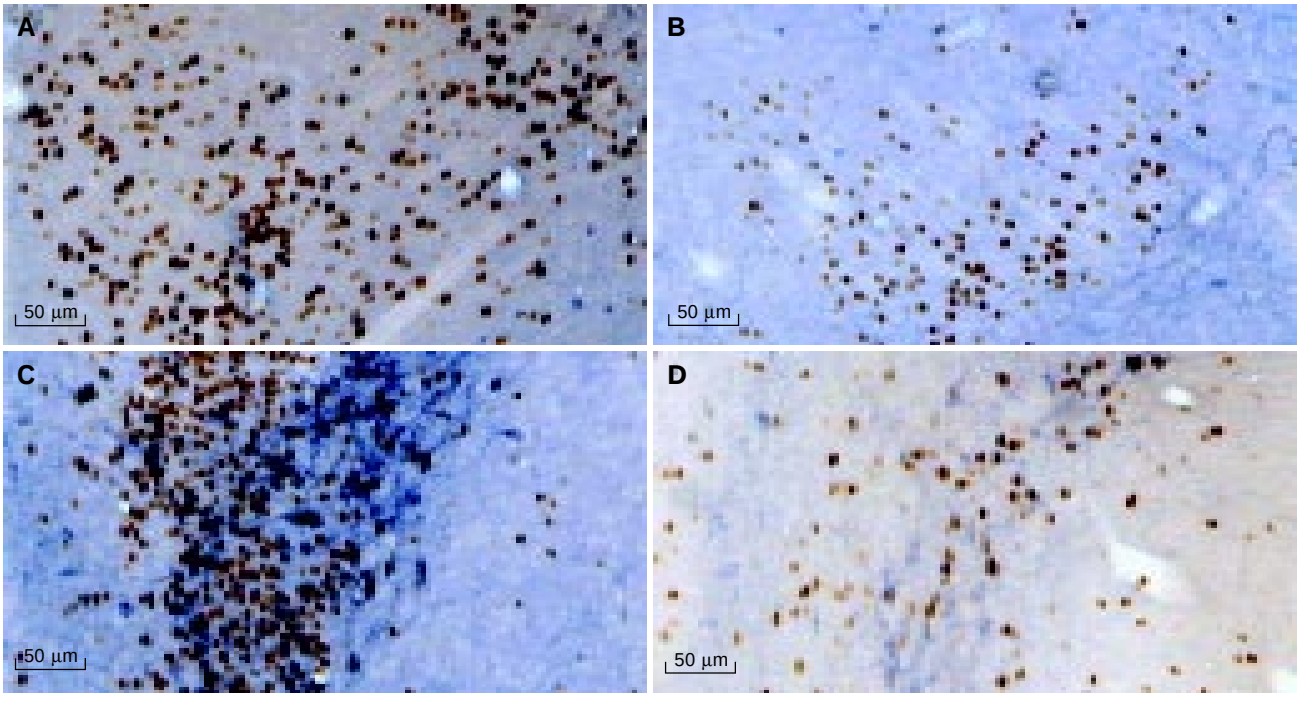

Figure 1 Photomicrographs showing c-Fos and NOS positive neurons in amygdale (A and B), paraventricular nucleus (C and D). A and C were taken from rats with acid-pepsin perfusion, while B and D were taken from rats with saline perfusion. (3V: the third ventricle).

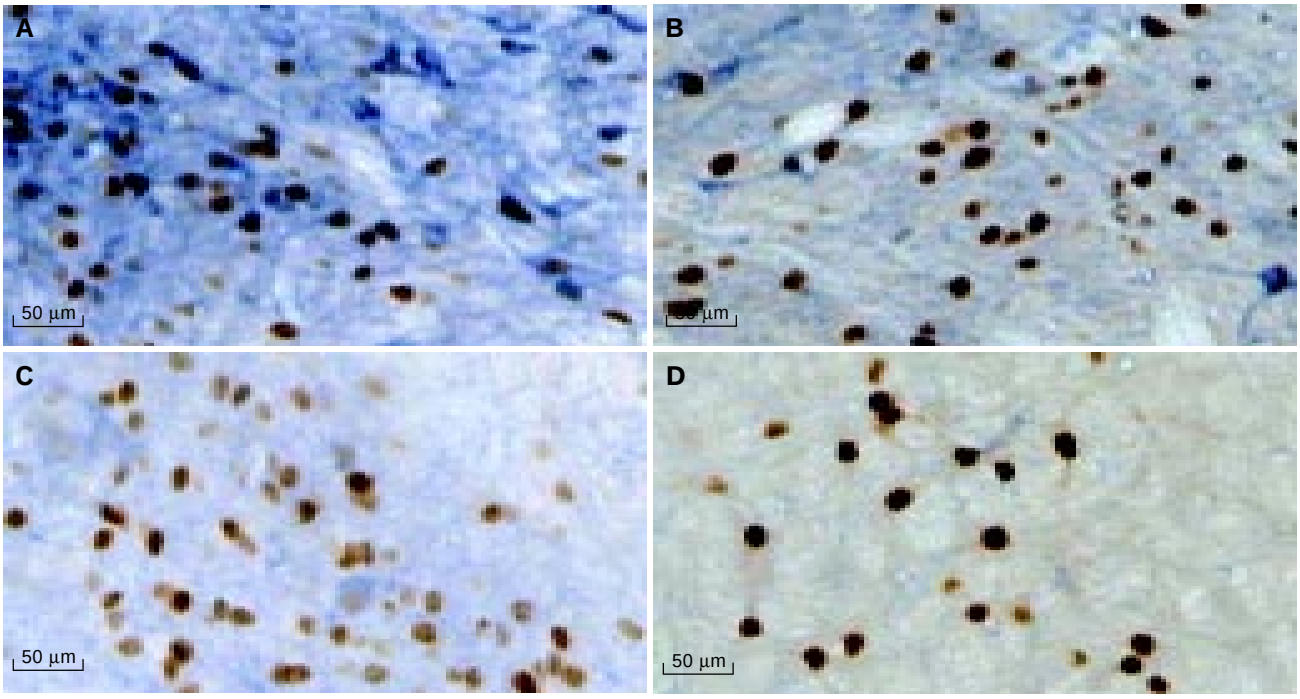

Figure 2 Photomicrographs showing c-Fos and NOS positive neurons in nucleus tractus solitarius (A and B), nucleus ambiguous (C and D). A and C were taken from rats with acid-pepsin perfusion, while B and D were taken from rats with saline perfusion.

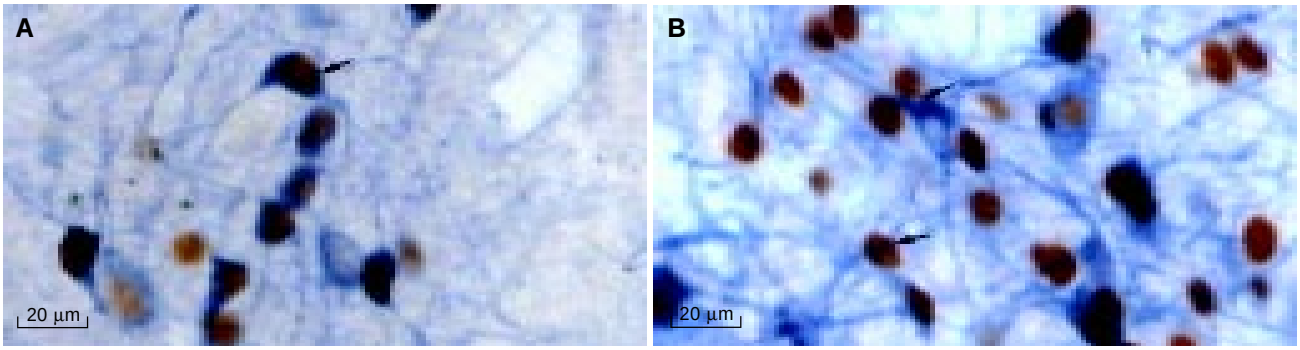

Figure 3 Photomicrographs showing the coexistence of c-Fos and NADPH-d positive staining, i.e. colocalization (left) and close proximity (right).

Table 1 Effects of esophageal acid-pepsin perfusion on c-Fos and NOS expression in brain nuclei, as determined by the average of number of c-Fos or NADPH-d positive neurons / section

Nuclei	Acid-pepsin perfusion			Saline perfusion		
	c-Fos	NOS	c-Fos&NOS	c-Fos	NOS	c-Fos&NOS
AM	341.3±13.7[b]	8.0±2.0	1.7±0.7	166.2±2.7	6.5±0.5	1.9±0.4
PVN	551.1±11.6[b]	151.8±48.5[b]	127.6±34.1[b]	232.2±12.9	66.9±1.5	64.1±4.4
SON	181.0±3.5	96.2±2.4	66.0±7.0	183.3±5.8	95.3±4.2	64.9±2.1
PBN	103.0±4.1[b]	17.1±1.8[b]	2.9±1.0[b]	79.7±2.6	3.4±0.6	1.1±0.5
NTS / DMV	161.1±6.9[b]	48.8±6.8[b]	32.3±4.7[b]	75.0±0.8	23.7±0.7	8.4±1.5
NA	42.7±0.8[b]	2.1±0.4	1.0±0.2	25.0±1.5	2.0±0.6	1.0±0.2
RNM	77.4±7.6[b]	15.1±1.5[b]	7.6±1.1[b]	32.9±0.4	5.1±0.5	1.9±0.3
AP	190.1±11.1[b]	6.0±2.3[b]	2.3±1.1[a]	107.2±2.1	1.9±0.6	0.9±0.3

Data are expressed as mean±SD. [a]$P<0.05$, acid-pepsin perfusion *vs* saline perfusion. [b]$P<0.01$, acid-pepsin perfusion *vs* saline perfusion. c-Fos, c-Fos positive neurons; NOS, NADPH-d positive neurons.

and NADPH-d positive staining (Figure 3). The coexistence included colocalization that was visualized as blue-stained perikarya (NADPH-d activity) containing a clearly visible dark brown nucleus (c-Fos protein), and close proximity that was visualized as c-Fos positive nucleus being within neuronal processes of NADPH-d, and that was the presence of NADPH-d positive staining within 3 μm from c-Fos-positive nucleus. Both of them have been adopted as a criterion of close proximity[19,20]. The coexisting cells were mainly observed in PVN, SON and NTS/DMV.

Correlation between c-Fos and NADPH-d positive cells

There was a high correlation between c-Fos and NADPH-d positive cells in PVN, PBN, NTS/DMV, RNM and AP. The correlation coefficient (r) was 0.805, 0.943, 0.923, 0.947, 0.869 (all $P<0.01$) respectively. There was no correlation between c-Fos and NADPH-d expression in AM, SON, and NA.

DISCUSSION

Acid, in combination with pepsin, was chosen to be the stimulant in this rat model of gastroesophageal reflux. This combination has been shown to cause esophagitis in experimental models[21,22].

The nerve supply to esophagus is composed of extrinsic and intrinsic components. The extrinsic innervation is mainly through the autonomic nervous system, which is divided into sympathetic and parasympathetic components. The parasympathetic innervation of esophagus is supplied by the vagus nerves. Three types of vagal afferent fibers are classified on the basis of their sensitivity to mechanical stimulation: those responding to mucosal stroking (mucosal receptors), those responding to circular tension (tension receptors) and those responding to mucosal stroking and circular tension (tension/mucosal receptors)[23]. Sensory afferents from the esophagus usually travel to NTS. DMV, which contains preganglionic motor neurons, has efferent fibers. The dorsal vagal complex (DVC) comprising NTS and DMV is the center of the integration of vagal control of esophagus[24,25]. Exposing the subdiaphragmatic vagus nerves (SDV) to horseradish peroxidase (HRP), Norgren *et al.* found that retrogradely labeled neurons occurred within NA and the reticular formation caudal to NA, and DMV whereas anterograde HRP reaction product occurred in NTS and AP[26]. Besides, connections of NTS with the medullary reticular formation and AP existed[27]. They were reported to take part in some visceral reflexes. In the present study, c-Fos positive neurons were seen in NTS, DMV, NA, RNM, and AP. In comparison with the controls, the number was greater in the acid-pepsin group. In this context, the present results confirm those reports mentioned above. During the esophageal exposure to acid, a cascade of chemoreceptors that lie along the passage is stimulated. Some of these signals are carried by vagal afferents to NTS in brainstem. From there, visceral information is disseminated to various brain sites, where it affects regulatory functions by engaging endocrine, autonomic, and some other effector mechanisms. But how all these different pathways interconnect within subnuclei is still unknown. It has been reported that PBN is related to noxious information from the visceral organs[14]. Esophageal acid exposure also induces high density of c-Fos expression in PBN.

A significantly increased number of c-Fos positive nuclei was observed in AM and PVN. Although many of c-Fos staining cells were seen in SON, there was no significant increase in this area in response to acid-pepsin perfusion. PVN is immediate beneath the ependyma of the third ventricle. The afferent connections of PVN are from hippocampal formation, septal nuclei, locus ceruleus, AM, and NTS. The efferent connections appear, in part, to be reciprocal to the afferent systems. The AM has reciprocal connections with locus ceruleus, substantia nigra, NTS, DMV, PBN, reticular formation, and nuclei of the hypothalamus. The present study showed that only some of those areas expressed c-Fos immunoreactivity, which suggests that those activated neurons are related to esophageal innervation. In order to exclude the potential contribution of the pressor response to the induction of c-Fos in NTS and other nuclei, BP, HR, and RR were recorded during the experimental procedures. There were no significant changes in BP, HR, and RR between the two groups.

It has been reported that NOS exists in neurons of DVC. The premotoneurons in NTS express NOS, and NO acting in the NA takes part in the esophageal peristalsis[28]. The present study showed that many NADPH-d positive neurons were seen in PBN, NTS/DMV, RNM, and that some were seen in NA and AP. This suggests that NO release may modulate characteristics of the activated neurons in these nuclei that are evoked by esophageal acid stimulus. It has been reported that NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME), reduces the spontaneous discharge rate of the NTS neurons *in vivo* and *in vitro*, which confirms that NO has the excitatory effect on NTS[29]. L-NAME also reduces the c-Fos expression in DVC, suggesting that c-Fos expression is, in part, related to NO release in DVC. Little has been known about the neurotransmitters in telencephalon and diencephalon. In the present study, many NADPH-d positive cells were observed in PVN and SON, but only few were found in AM.

The present study observed the coexistence of c-Fos and NADPH-d positive staining. It is possible that the neuronal cells containing NOS are activated during esophageal acid exposure, which may cause NO release to themselves or to other brain regions in modulating the esophageal reflux.

In conclusion, acid-pepsin exposure to lower part of the esophagus stimulates the mucosal receptors, which in turn activates the neurons of NTS through vagal afferent fibers, and finally the neurons in DMV and NA to modulate the esophageal peristalsis. The possible nuclei involved in these procedures are AM, PVN, PBN, RNM, and AP. Double labeled staining of c-Fos and NADPH-d suggests that NO is one of the neurotransmitters in PVN, PBN, NTS/DMV, RNM and AP.

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