

Effects of granisetron and vagotomy on c-fos mRNA expression in the rat medulla oblongata as assessed by *in situ* hybridization

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ABSTRACT

Cancer chemotherapy-induced nausea and vomiting have been demonstrated to involve humoral as well as neuronal mechanisms. A leading role of serotonin (5-hydroxytryptamine, 5-HT) in these mechanisms is supported by inhibition of the emesis by 5-HT₃ receptor antagonists. We compared the effects of granisetron, a selective 5-HT₃ receptor antagonist, and vagotomy on c-fos mRNA expression in the nucleus of the solitary tract (NTS) and the area postrema (AP) of the rat caudal brainstem by means of *in situ* hybridization. The expression of c-fos mRNA in the NTS and AP was significantly elevated 2 h after cisplatin administration. The induction of c-fos expression by cisplatin in the NTS was significantly inhibited by pretreatment with granisetron. In contrast, the c-fos expression in the AP did not differ between the cisplatin group and the granisetron-treated cisplatin group. The degree of the induction of c-fos mRNA expression in both the AP and NTS was similar between the vagotomy and sham operation groups. Our results suggest that the expression of c-fos mRNA in the NTS may be specifically controlled by 5-HT₃ receptors and that nonspecific humoral factors, such as modulation of transcriptional activity, play an important role in c-fos expression in the AP after vagotomy.

Neuronal sites and signal substances involved in cancer chemotherapy-induced nausea and vomiting have been identified neurochemically (20). Serotonin type 3 receptor (5-HT₃ receptor) antagonists protect against emesis in experimental animal models as well as in cisplatin-treated patients with cancer (35). Autoradiographic studies of 5-HT in the central nervous system showed that binding sites with the strongest affinity for 5-HT₃ receptors are located in the nucleus of the solitary tract (NTS) and the area

postrema (AP) of the caudal brainstem (33).

However, considerable evidence supports the hypothesis that cytotoxic drug-induced emesis is mediated by local 5-HT release in the intestinal wall (4, 14, 15, 27, 28). 5-HT has a potent depolarizing action on the vagal afferent nerve (18, 34). It has been proposed that anticancer drugs cause 5-HT release from enterochromaffin (EC) cells to activate the 5-HT₃ receptors on the afferent vagal nerve fibers (1, 26). These afferent fibers may project to the dorsal vagal complex, which also expresses 5-HT₃ receptors on its central terminals (20, 33). The vomiting center receives input from the afferent vagal fibers to evoke an emetic reflex (1, 20). The vagus is the major nerve involved in the detection of emetic stimuli, and the abdominal vagus contains approximately 80–90% of the afferent fibers (3). Electrical

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stimulation of the abdominal vagal afferents is capable of inducing emesis (26), and abdominal vagotomy suppresses cisplatin-induced emesis (12, 17, 40). Recent evidence supports the notion that 5-HT₃ receptor blockade at the level of the peripheral terminals of the vagal afferent fibers protects experimental animals and cancer patients from cisplatin-induced emesis (2).

c-fos was proved to be a useful marker of neuronal activation because its expression is rapidly and intensely induced by such activation (31). c-fos expression as evaluated by *in situ* hybridization and immunohistochemistry is now widely used to map brain regions activated by various types of stimuli, including drugs (21, 32, 36, 39).

The existence of an integrative site that generates vomiting responses is still open to debate (5, 19, 25). Therefore, we compared the expression patterns of c-fos mRNA in the dorsal vagal complex, NTS and AP of rats after treatment with cisplatin alone or with cisplatin plus granisetron, a selective 5-HT₃ receptor antagonist, by means of *in situ* hybridization. We also examined the effect of vagotomy on the c-fos mRNA expression.

MATERIALS AND METHODS

Animals. Male rats (weighing 180–300 g) were housed in groups of 3 or 4 animals in a temperature-controlled room (23 ± 1°C) with a 12-h light/dark cycle (lights on from 8:00–20:00). Food and water were provided *ad libitum*. The rats were given a single dose of saline vehicle or cisplatin (10 mg/kg) intraperitoneally. Saline or granisetron (1 mg/kg i. p.; SmithKline Beecham, Tokyo, Japan) was administered 10 min before the injection of cisplatin. Rats given cisplatin do not vomit, but display a characteristic behavior, namely eating of non-nutritive substances such as kaolin. This behavior is known as pica and is useful for the evaluation of emesis (24, 38).

Dissection of abdominal vagus nerve. Six rats received bilateral abdominal vagus nerve lesions 4 days before cisplatin administration. The other animals underwent sham operations. To create abdominal vagus nerve lesions, animals were deeply anesthetized with ketamine (10 mg/kg i. p.). Under aseptic conditions, a bilateral (anterior and posterior nerves) vagal nerve excision was performed as described by Mackay and Andrews (22). The incisions were sutured and the animals were given penicillin. Postoperative recovery was uneventful in all tested

rats.

In situ hybridization. After the rats were sacrificed with an overdose of ketamine, the brains were quickly removed and frozen at –80°C. Sections (16 µm thick) of the caudal medulla oblongata, including the dorsal vagal complex, were cut in a cryostat and placed on glass slides. The tissue sections were fixed with paraformaldehyde (4%) for 15 min and were acetylated for 10 min with acetic anhydride (0.25%) in 0.1 M triethanolamine-HCl (pH 8.0).

Two non-overlapping antisense oligonucleotides were used for *in situ* hybridization. They were complementary to nucleotide residues 601–645 and 1001–1045 of rat c-fos mRNA (database accession no. X06769). Prehybridization was performed in a hybridization buffer containing 50% formamide, 0.1 M Tris-HCl buffer (pH 8.0), 0.6 M NaCl, 1 mM EDTA, 10% (w/v) dextran sulfate (Sigma, St. Louis, MO, USA), 4 × saline sodium citrate (SSC) (1 × SSC = 150 mM NaCl and 15 mM trisodium citrate), 0.25% sodium dodecyl sulfate, Denhardt's solution (0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, Sigma), and 200 µg/ml tRNA.

The synthesized oligonucleotides were labeled with [³⁵S]-dATP (10,000 cpm/µl) (NEN™; Life Science Products Inc., Boston, USA) using terminal deoxynucleotidyltransferase (Promega, WI, USA). Incubation in hybridization buffer was done at 42°C for 10 h. The sections were washed at room temperature for 30 min in 2 × SSC and twice at 55°C for 40 min each in 0.1 × SSC. The sections were exposed to X-ray films (Hyperfilm™-β max; Amersham, Buckinghamshire, UK) for 1 week and to autoradiographic emulsion (Type NTB2; Kodak, NY, USA) for 2 months. The specificity of hybridization was confirmed by observing consistent labeling using two non-overlapping antisense probes as well as by the disappearance of the signals when an excess dose of cold probes was added to the hybridization fluid (data not shown).

Statistical analysis. For quantitative analysis of X-ray films, densitometry was performed with a National Institutes of Health image analyzer. The results are expressed as % change as compared with the control group. The numbers of cells expressing c-fos mRNA were quantified by counting heavily labeled cells with a light microscope (DMIRB; Leica, Tokyo, Japan) under dark-field illumination. The size of the tissue area surveyed in each rat was about 0.03 mm². Data are expressed as means ± SE. The significance of differences between 2 groups

was assessed with the F-test followed by the Student's t-test. One-way analysis of variance (ANOVA) with Dunnett's multiple comparison procedure was used to compare 3 or more groups (control, granisetron-treated, and vagotomy groups) (41). A p value less than 0.05 was regarded as indicating statistical significance.

RESULTS

Time course of induction of c-fos mRNA expression by cisplatin

In situ hybridization analysis showed that cisplatin administration induced selective and intense expression of c-fos mRNA in the medulla oblongata 2–3 h after the treatment (Fig. 1). Under the light microscope, dark- and light-field images of hybridized sections showed c-fos mRNA-expressing cells in the AP and NTS after cisplatin administration (Fig. 2a, b). Heavy labeling was found both in small cells clustered in the AP and in large neurons dispersed in the NTS. Fig. 3 shows the time-course of the ex-

pression of c-fos mRNA in the NTS (as determined by the densitometric analysis of X-ray films) 1 to 6 h after cisplatin administration. The expression of c-fos mRNA in the NTS 2 to 6 h after cisplatin administration was significantly higher than the control

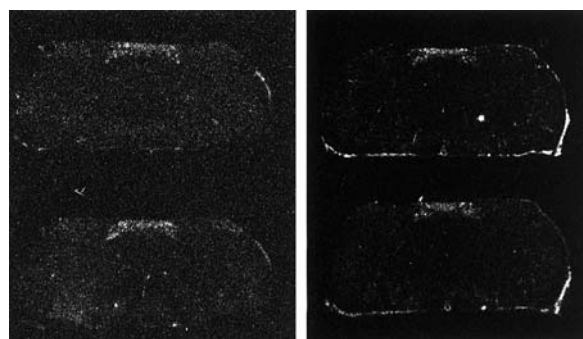


Fig. 1 X ray images showing *in situ* hybridization analysis of the expression of c-fos mRNA in the dorsal surface of the medulla oblongata in two rats. The expression sites correspond to the nucleus of the solitary tract and the area postrema.

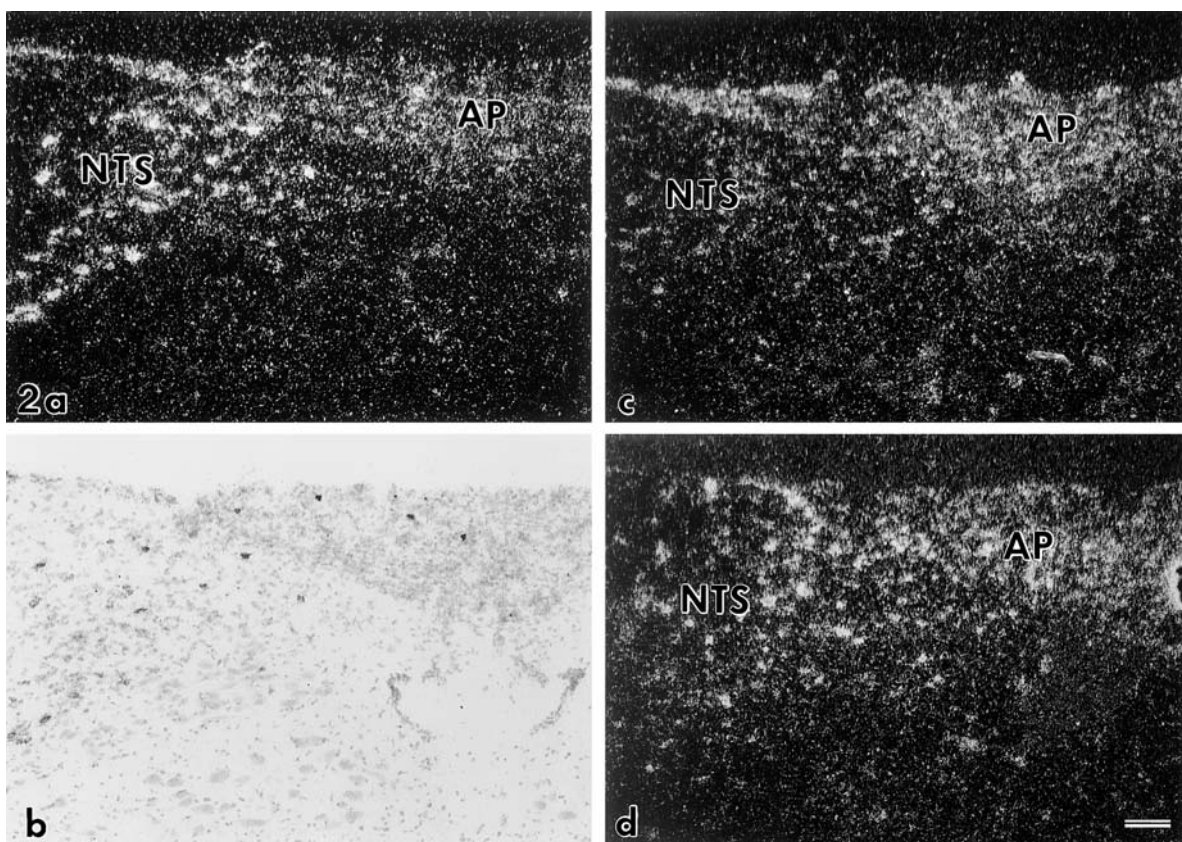


Fig. 2 Expression of c-fos mRNA induced by cisplatin in the rat medulla oblongata (2a, 2b) and the effect of granisetron (2c) or vagotomy (2d). The medulla oblongata was obtained 2 h after cisplatin administration. Fig. 2a and 2b are dark and bright field images of the same section. AP: area postrema, NTS: nucleus of the solitary tract

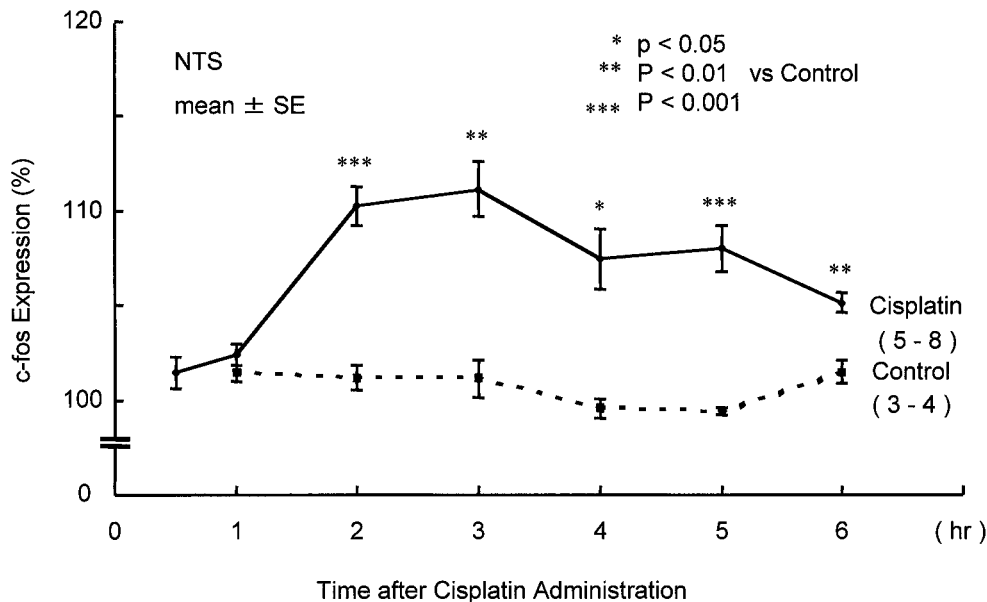


Fig. 3 Time course of the induction of c-fos expression by cisplatin in the nucleus of the solitary tract (NTS) quantitated by densitometry.

value. The c-fos expression peaked at 2–3 h after the administration of cisplatin. The increased c-fos expression was maintained up to 6 h after administration, but the expression level at 6 h was significantly lower than that at 2 h after treatment. The AP showed a similar pattern of c-fos mRNA expression to the NTS (data not shown).

Effect of granisetron, a selective 5-HT₃ receptor antagonist, on the induction of c-fos expression by cisplatin

We examined the effects of granisetron, a selective 5-HT₃ receptor antagonist, on c-fos mRNA expression in the AP and NTS 2 h after the administration of cisplatin. Fig. 2c shows c-fos mRNA expression in the medulla oblongata after treatment with cisplatin plus granisetron. The c-fos mRNA expression in the AP of rats receiving granisetron before cisplatin was similar to that of the cisplatin control, while in the NTS there were few intensely labeled cells. Densitometric analysis confirmed that c-fos mRNA expression in the NTS was significantly inhibited by granisetron (Fig. 4).

Effect of vagotomy on the induction of c-fos expression by cisplatin

When the effects of vagotomy on c-Fos induction in the medulla oblongata were examined 2 h after the administration of cisplatin, there was no appreciable difference from the sham operation group in

8 of 12 rats. The remaining rats showed a slight trend toward c-fos induction. Fig. 2a and d show the dark-field images of c-fos mRNA expression in the AP and NTS regions in the cisplatin group (Fig. 2a) and the vagotomy plus cisplatin group (Fig. 2d), respectively. Comparison of the two groups revealed no significant effect of vagotomy on c-fos mRNA expression in the AP or NTS.

DISCUSSION

Here we studied on the c-fos induction in the brain stem of rats given cisplatin to understand the involvement of the medulla oblongata in cisplatin-induced emesis, since c-fos protein plays a role in nuclear events (transcription) associated with signal transduction processes in a manner analogous to immediate-early genes (29). Our study showed that pretreatment with the 5-HT₃ receptor antagonist granisetron significantly inhibited cisplatin-induced c-fos mRNA expression in the NTS of the medulla oblongata. Abdominal afferent vagal nerves are thought to have direct input to the dorsal nuclei of vagal nerve fibers as well as the NTS and AP (20, 33). Suppression of the cisplatin-induced c-fos expression by granisetron probably may involve two mechanisms. The first is the reduced release of 5-HT from enterochromaffin cells, which stimulates abdominal afferent vagal fibers distributed in the intestinal wall. The second mechanism is the inhibited

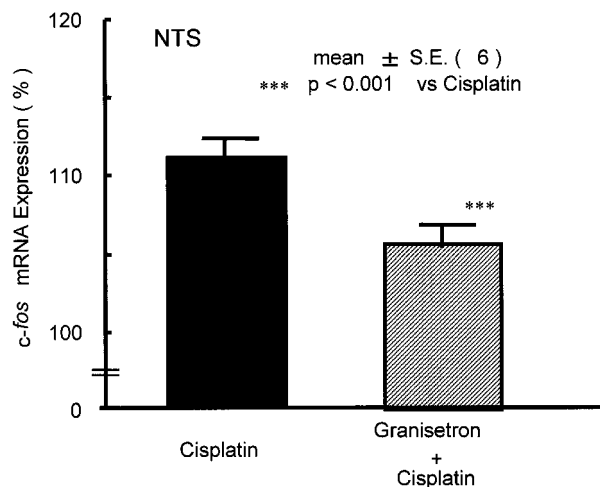


Fig. 4 Effect of granisetron on the induction of c-fos expression by cisplatin in the rat the nucleus of solitary tract (NTS) quantitated by densitometry.

input to the NTS, resulting from the blockade of 5-HT₃ receptors at peripheral terminals of the abdominal afferent vagal fibers. The granisetron-induced blockade of the vomiting signal input to NTS via 5-HT₃ receptors suggests that input from the AP to the NTS is blocked. However, cisplatin-induced c-fos mRNA expression in the AP was not affected by pretreatment with granisetron. Because AP also functions as a chemoreceptor trigger zone, humoral factors probably play an important role in the induction of c-fos mRNA expression by cisplatin in the AP. Pretreatment with granisetron had no discernible effect on c-fos mRNA expression induced by circulatory or systemic effects. It also appeared that 5-HT₃ receptors were not densely distributed in the AP. Input from the AP to the NTS, however, was also apparently inhibited by granisetron. These findings suggest that granisetron blocks input to the NTS in addition to acting on 5-HT₃ receptors on the peripheral terminals of the abdominal afferent vagal fibers (15, 16).

One of the most important methodological considerations for immunohistochemical analyses of c-fos is the time course of c-fos elevation and decay (10). After a seizure, the elevated c-fos levels in neurons generally decrease more slowly than those in cell culture systems (30). Neuronal c-fos levels after a seizure have been reported to return to nearly baseline values within 24 h, although the onset latency and time of maximal c-fos accumulation in neurons after seizures are region-dependent in the brain (9). In our study, however, the cisplatin-induced c-fos mRNA expression in the NTS and AP

was highest 2 and 3 h after cisplatin administration and returned to the baseline value 6 h later. Consistent with the pattern of c-fos mRNA expression seen in the present study, cisplatin-induced vomiting in the ferret starts 2 h after administration and continues for 6 h (11). In rats, which show pica, a type of emesis (23, 37), abdominal afferent vagal nerve activity increases 2 h after cisplatin treatment (13).

Miller *et al.* (25) clearly demonstrated that emetic drugs excite neurons in the AP. After removal of the AP, however, vomiting induced by 5-HT₃ receptor agonists or by electrical stimulation of the abdominal vagus nerve cannot be inhibited (25). These findings suggest that neuronal excitation in the AP does not play an essential role in the vomiting caused by 5-HT-mediated abdominal afferent vagal nerve stimulation and that activation of the AP evokes emesis via projections to the adjacent NTS. Vagotomy decreases the excitation threshold in the AP. Excitation is therefore transmitted to the NTS, but is too weak to induce emesis. The induction of c-fos at this time apparently depends on transcription-related differences after vagotomy. c-fos expression can be induced rapidly and transiently by a great variety of agents, such as 5-HT, acetylcholine, and humoral factors (8). Neurons in regions of the medulla oblongata, including the AP, are excited by gastrin, histamine, substance P, and enkephalin as well as 5-HT (6, 7). Cisplatin may have directly stimulated the release of these neurotransmitters in the AP or NTS. Alternatively, cisplatin may have facilitated neurotransmitter release in the intestine; these neurotransmitters then systemically reached the chemoreceptor trigger zone and induced c-fos expression in the AP.

In both the AP and NTS, c-fos mRNA expression was essentially unchanged between the cisplatin group and the cisplatin group receiving vagotomy. We previously reported that cisplatin-induced vomiting is inhibited in 80% of ferrets undergoing vagotomy (12, 26). 5-HT levels in the AP and NTS regions of cisplatin-administered animals were significantly increased as compared with those of saline-administered control animals. The 5-HT level of animals pretreated with 5-HT₃ receptor antagonist or vagotomy was significantly lower than that of the cisplatin-treated animals (26). Taken together, the findings of this previous study by us suggested that cytotoxic drugs induce emesis mainly through actions on the gastrointestinal tract. We have reported that vagotomy inhibited vomiting (12), but the present study did not find c-fos induction, implying that the relationship between c-fos induction and emetic

behavior is not direct. These results also suggest that abdominal vagotomy is effective for the inhibition of emetic stimuli which release 5-HT from the gut and stimulate the vagal afferent nerves. On the other hand, abdominal vagotomy may be ineffective for emesis in which humoral agents excite the AP.

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