STOMACH EROSION AND ULCERS are one of the three symptoms of stress syndrome described by Selye (25). Clinical observations indicate that various kinds of stress such as shock, burns, sepsis, and severe trauma as well as severe emotional distress are closely related to acute upper gastrointestinal erosions and ulcerations (stress erosions and ulcerations). Water immersion and restraint of rats are commonly used for studying stress-induced gastrointestinal erosion and ulcers (29). Involvement of central nervous system components such as the limbic system, hypothalamus, and brain stem nuclei have been considered (10). Mucosal ischemia and enhanced backdiffusion of hydrogen ions have been proposed as major local mechanisms for these stress-induced injuries (33). Previous studies, using physiological techniques, have so far failed to reveal the molecular mechanisms responsible for the pathological changes induced by stress or for repairing of tissues damaged in stress. Major questions we have raised here are 1) Which cells are involved in the stress response? and 2) What kinds of molecular alterations are induced by stress? These questions are important for understanding the adaptive mechanisms of the organism in response to stress and injury.

Protooncogenes such as c-fos and c-jun are rapidly induced by growth factors or other stimuli to couple transmembrane signaling to cellular growth and transcriptional control mechanisms. These protooncogenes are also called immediate-early genes (IEG) because their rapid and transient transcriptional induction does not require de novo protein synthesis (19, 24). These protooncogenes are very important at the cellular level of the stress response because these genes act like a molecular switch. Fos- and Jun-family proteins form heterodimers (Fos-Jun) or homodimers (Jun-Jun) (activator protein 1; AP-1), which may elicit expression of target genes and contribute to the cellular response to the primary stimuli.

Expression of IEG in cells in response to a stimulus indicates that 1) the cells have been activated by the stimulus and 2) the cells have initiated the modulation of gene expression necessary for a particular cellular response. In the central nervous system, IEG expression in response to several kinds of stimuli has also been reported. In particular, expression of IEG in the hypothalamic-pituitary-adrenal axis, sympathetic pathway, and limbic system in response to emotional stress has been demonstrated using in situ hybridization (ISH) histochemistry (5, 6, 26, 28). Compared with Northern blotting, ISH can visualize not only the temporal but also the spatial patterns of mRNA expression in individual cells even though the number of cells is limited. Expression of IEG in response to stress is not limited to the nervous system (27). In fact, we previously demonstrated the expression of c-fos and c-jun mRNA and their protein products in the myocardium and the smooth muscle layer of the coronary arteries in response to immobilization stress (32). On the basis of these findings we applied ISH histochemistry of IEG to the investigation of the stress response in the gastrointestinal tract.

In this study, we have demonstrated for the first time the temporally and spatially restricted expression of IEG in the epithelial cells and smooth muscle cells of the stomach and duodenal walls and blood vessels in response to immersion-restraint stress. In the pit and isthmus regions of gastric epithelial cells or in the crypt region of duodenal epithelial cells, the colocalization of mRNA for c-fos or nerve growth factor-induced gene NGFI-A and immunoreactivity for proliferating cell nuclear antigen (PCNA) (4, 36) were examined to determine whether these IEG are involved in the proliferation of epithelial cells.
MATERIALS AND METHODS

Tissue preparation. Male Wistar rats, 6 wk old, were purchased from Kiwa Laboratory Animals (Wakayama, Japan) and housed in a temperature-controlled environment. Experiments were performed after allowing the rats free access to food and water for 1 wk. The animals were fasted for 24 h before the stress. We restrained the animals by wrapping them with iron net and then immersing them vertically to the level of the xiphoid process in a water bath maintained at 20°C. Five animals remaining undisturbed in their home cages served as unstressed controls. For the detection of IEG mRNA by ISH, the rats were decapitated at 15, 30, 60, and 90 min from the start of stress (n = 5 rats at each time point) under ether anesthesia. In a second series, rats were subjected to the stress for 90 min, the body was then wiped dry, and the rats were returned to their home cages. They were then killed by decapitation at 30 min, 90 min, and 3 h after the end of water immersion-restraint stress (n = 5 rats at each time point). The stomach and duodenum were rapidly removed and immediately frozen using powdered dry ice within 1 min after decapitation. All animal manipulations were approved by the Wakayama Medical College Animal Care and Use Committee. The frozen tissues were stored at −80°C until being sectioned.

Frozen sections of 10 µm in thickness were cut in a cryostat and thaw mounted onto silane-coated slides. They were fixed in 4% paraformaldehyde-0.1 M phosphate buffer pH 7.4 for 15 min at room temperature, rinsed in 2 × standard saline citrate (SSC), and dehydrated by passing through 70, 80, 90, and 100% ethanol. After drying, the slides were stored at −80°C until being hybridized.

For the detection of c-fos protein by immunohistochemistry, at 60 min after the onset of water immersion-restraint stress and at 30 min, 90 min, and 24 h after the end of water immersion-restraint stress for 90 min, the animals were deeply anesthetized with an excess dose of pentobarbital sodium and perfused transcardially with saline followed by 4% paraformaldehyde-0.1 M phosphate buffer pH 7.4 and the stomach was removed (n = 3 rats at each time point). Three animals not subjected to stress served as controls. The specimens were postfixed overnight and cryoprotected in PBS containing 30% sucrose. The stomach and duodenum were immediately frozen on powdered dry ice. Frozen sections (10 µm thick) were cut in a cryostat, thaw mounted onto silane-coated slides, and stored at −80°C until being processed for immunohistochemistry.

In situ hybridization. Oligonucleotide probes were synthesized using an Applied Biosystem 381A DNA synthesizer and then purified using high-performance liquid chromatography. The probes for the detection of c-fos, c-jun, and NGFI-A mRNA were complementary to the nucleotides spanning amino acids 1–15 of rat c-fos protein (7), the last 20 amino acids of the predicted c-jun (1), and amino acids 2–16 of rat NGFI-A protein (20), respectively. A computer-assisted homology search revealed no identical sequences in any genes in the data base (DNASIS, Hitachi, Tokyo). The probes were labeled with 32P-dATP using terminal deoxynucleotidyltransferase (Toyobo, Osaka, Japan). The specific activity of each probe was 5–10 × 106 counts per minute (cpm)/µg. Excess (×50) amounts of cold probes completely eliminated the hybridization signals for the respective mRNA. Before hybridization, tissue sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 15 min, rinsed two times in 2× SSC (pH 7.2), and dehydrated in a graded ethanol series. Sections were hybridized overnight at 37°C in 100 µl of buffer containing 4 × SSC, 50% formamide, 0.12 M phosphate buffer, 1× Denhardt’s solution, 0.2% sodium dodeyl sulfate, 250 µg/ml yeast tRNA, 10% dextran sulfate, and 100 mM dithiothreitol with 106 cpm of labeled probe per slide. After hybridization, the sections were washed four times for 20 min at 55°C in 1× SSC, immersed briefly in distilled water, and dehydrated with a graded ethanol series and then dried. Film autoradiography was performed using a Biomaging-analyzer BAS2000 (Fuji Film). The slides were next coated with Ilford k-5 emulsion diluted 1:2 with water for autoradiography and then exposed for 4 wk at 4°C. Slides were developed in D-19 (Kodak), and the sections were counterstained with hematoxylin-eosin or 1% neutral red for morphological examination. All slides for each probe were processed simultaneously.

Immunohistochemistry. The sections were incubated with primary anti-Fos serum (rabbit, Ab-5, Oncogene Science), diluted 1:500–1,000 with 0.1 M PBS containing 5% normal goat serum and 0.3% Triton X-100, for 72 h at 4°C. After being washed in PBS, they were incubated with the secondary antibody (biotinylated goat anti-rabbit antiserum; Vector) diluted 1:200 in PBS for 1 h at room temperature. After a brief rinse with PBS, they were incubated with 0.3% H2O2 in methanol for 30 min to quench endogenous peroxidase activity. After being rinsed twice with PBS, they were reacted with avidin-biotin-peroxidase complex (Vector) for 1 h. After being washed in 0.1 M Tris·HCl-buffered saline (pH 7.5), they were incubated in 0.05% 3,3‘-diaminobenzidine solution containing nickel ammonium sulfate (0.2%) for 5–10 min.

Immunohistochemistry for PCNA was performed in fresh frozen sections. Frozen sections (6 µm thick) were cut in a cryostat and thaw mounted onto silane-coated slides. They were fixed in 4% paraformaldehyde-0.1 M phosphate buffer pH 7.4 for 2 min at room temperature, rinsed in 2× SSC, and fixed again in 100% ethanol for 10 min, and air dried. The sections were incubated with primary anti-PCNA monoclonal antibody (mouse, DAKO), diluted 1:50–100 with 0.1 M PBS containing 5% normal goat serum and 0.3% Triton X-100, for 72 h at 4°C. After being washed in PBS, they were incubated with the secondary antibody (biotinylated rabbit anti-mouse IgG; Vector) diluted 1:200 in PBS for 1 h at room temperature. After a brief rinse with PBS, they were incubated with 0.3% H2O2 in methanol for 30 min to quench endogenous peroxidase activity. After being rinsed twice with PBS, they were reacted with avidin-biotin-HRP complex (Vector) for 1 h. After being washed in 0.1 M Tris·HCl-buffered saline (pH 7.5), they were incubated in 0.05% dianimobenzidine solution for 5–10 min.

RESULTS

Mucosal changes in the stomach were not observed at 15–30 min after the onset of water immersion-restraint stress. However, mild mucosal erosions and bloody clots on the surface of the stomach were observed between 60 min from the onset of stress and 90 min from the end of stress. These changes were not observed 3 h after the end of stress.

There were very few signals for c-fos, c-jun, and NGFI-A mRNA or for Fos-like immunoreactivities in the stomach of unstressed control rats. Water immersion-restraint stress induced c-fos and c-jun mRNA from 60 min and NGFI-A mRNA from 15 min in the stomach (Fig. 1). The levels of these mRNA were increased during the stress and reached a maximum at 30 min after 90 min of stress (Fig. 1); they returned to
the control level at 90–180 min after the end of stress (Fig. 1). Signals for c-fos and NGFI-A mRNA were observed in the epithelial cells localized particularly in the pit and isthmus regions and the smooth muscle layer of small blood vessels and the gastric wall (Fig. 2). The expression pattern of NGFI-A mRNA was almost spatially and temporally similar to that of c-fos mRNA, but the signals for NGFI-A mRNA were more intense than those for c-fos mRNA. A low level of signals for c-jun mRNA was observed in the epithelial cells in

Fig. 1. Film autoradiography showing time course for expression of c-fos (A and B), nerve growth factor-induced gene (NGFI-A) (C and D), and c-jun (E and F) mRNA in stomach (A, C, and E) and duodenum (B, D, and F). Axial sections of stomach and duodenum were taken from an unstressed animal (0) and animals subjected to water immersion-restraint stress for 15, 30, 60, 90 min. p30, p90, p180, minutes after 90 min of water immersion-restraint stress. Note that water immersion-restraint stress induced c-fos and c-jun mRNA from 60 min in stomach and from 30 min in duodenum and NGFI-A mRNA from 15 min in stomach and from 30 min in duodenum. Levels of these mRNA were increased during stress period and reached a maximum at 30 min after 90 min of stress (p30); they were decreased at 90–180 min after end of stress.

Fig. 2. Bright-field photomicrographs showing signals for c-fos (A and B), NGFI-A (C and D), and c-jun (E and F) mRNA in stomach. Numbers indicate minutes from start of stress. Note that signals for c-fos and NGFI-A mRNA are localized particularly in pit and isthmus regions (A and C) and smooth muscle layer of gastric wall (B and D). Few signals for c-jun mRNA were observed in epithelial cells, whereas those in smooth muscle layer of small blood vessels were markedly increased in response to stress (F). BV, blood vessels; FS, forestomach; M, mucosa; ME, muscularis externa. Bar: 125 (A, C, and E), 200 (B and D), and 50 (F) µm.
response to stress, whereas the level was clearly increased in the smooth muscle layers of small blood vessels (from 30 min) and of the gastric wall (from 60 min) after the onset of stress (Figs. 1 and 2). These mRNA signals were widely observed throughout the stomach including the cardia, body, and pyloric antrum. In addition, the intensity of signals for these mRNA was not correlated with the severity of mucosal damages at 30 min after 90 min of stress. There were no mRNA signals for these IEG in the stratified squamous epithelium of forestomach (Fig. 2) and esophagus (data not shown). Strong immunoreactivity for PCNA was observed in the gastric epithelial cells of the isthmus regions (Fig. 3). As shown in Fig. 4, most of the PCNA-immunopositive epithelial cells seem to express c-fos mRNA in response to stress. Fos-like immunoreactivities were observed in the same regions in accordance with their mRNA from 60 min after the onset of stress (data not shown). The levels reached a maximum at 30 min after the end of 90-min stress (Figs. 5 and 6). Signals for IEG mRNA and immunoreactivities were essentially undetectable beyond 24 h after the stress (data not shown).

In the epithelial cells and Brunner’s glands of the duodenum, mRNA for c-fos, c-jun, and NGFI-A (data not shown) and Fos-like immunoreactivities (Fig. 7) are constitutively expressed. However, in the smooth muscle layer of small blood vessels and duodenal wall of unstressed controls, there were few signals for c-fos, c-jun, or NGFI-A mRNA or for Fos-like immunoreactivities (data not shown). Stress for 30 min upregulated the mRNA levels for c-fos and NGFI-A in the epithelial cells of the duodenum and induced the expressions of c-fos, c-jun, and NGFI-A mRNA de novo in the smooth muscle layer of small blood vessels and duodenal wall (Figs. 1 and 8). Stress-induced upregulation of c-fos mRNA was mainly observed in the villus epithelium (Fig. 8). In contrast, NGFI-A mRNA was mainly upregulated in the crypt epithelium (Fig. 8), where the colocalization of mRNA for NGFI-A and immunoreactivity for PCNA was observed (Figs. 3 and 4).

Mononuclear cells scattered in the lamina propria of the stomach and duodenum express mRNA for c-fos (Fig. 8A) and c-jun. Immunoreactivities for Fos were also observed in the corresponding cells in the lamina propria (Fig. 7E). However, stress did not influence the mRNA levels for c-fos and c-jun in these cells.

**DISCUSSION**

This is the first histological report showing the expression of IEG in the stomach and duodenum of rats in response to water immersion-restraint stress. Immunohistochemistry showed that Fos protein was subsequently synthesized in the epithelial cells and the smooth muscle layer of small blood vessels and gastric and duodenal walls after water immersion-restraint stress. These genes and protein were scarcely detectable in tissues from unstressed animals under the experimental conditions employed. The most important aspect of the present findings is the spatially and temporally characteristic expression patterns of IEG in the stomach and duodenum. Distribution of IEG mRNA and its protein product in the stomach and duodenum was not homogeneous but rather restricted to the smooth muscle cells of the small blood vessels and gastric wall and the epithelial cells, especially those localized in the pit and isthmus regions of the stomach. In the duodenum, c-fos mRNA was mainly upregulated in the villus epithelium and NGFI-A mRNA was mainly upregulated in the crypt epithelium.

Expression of IEG in response to water immersion-restraint stress was extensive in the smooth muscle cells of blood vessels and gastrointestinal walls. Smooth muscle cells in the gastrointestinal tract and vascular smooth muscle cells have excitation-depolarization characteristics similar to those of neuronal cells, suggesting
that the expression of IEG reflects the activation and presumably a hypercontractility of smooth muscle cells around the blood vessels and gastrointestinal wall. This idea is consistent with the physiological observation that water immersion-restraint stress resulted in increased gastric motility and arterial vasomotion in the gastric wall (34). Gastric mucosal ischemia and the acute gastric mucosal lesions have also been reported in patients with head injury (17) and in burn stress in the rat (16). The role of the autonomic nervous system in the stress response has been well documented, with respect to both the central nervous system centers that participate in the response and the effects on target organs of autonomic activation. For example, electrical stimulation in the paraventricular hypothalamus resulted in gastrointestinal ulceration by influencing medullary vagal preganglionic neurons (9). In addition, vasoconstrictive neuropeptides (vasoressin, angiotensin II), vasodilative neuropeptides (vasoactive intestinal peptide, somatostatin, calcitonin gene related peptide, substance P, etc), which are released from myenteric neurons, and locally released metabolic products and cytokines (histamine, serotonin, bradykinin, prostaglandins, etc) all participate in altering the regulation of the microcirculation of the gastrointestinal tract in response to stress (33). It is postulated that the decrease in gastric regional blood flow in water immersion-restraint stress is caused by a lowering of body temperature via decreasing cardiac output (2).

Wang and Johnson (37) observed the expression of c-fos and c-myc mRNA with Northern blot analysis in the stomach in response to stress. Bar: 50 (A and C), 25 (B and D) \( \mu m \).

Fig. 4. Bright-field photomicrographs showing PCNA-immunoreactive nuclei (A and B) and signals for c-fos mRNA in stomach (C) and NGFI-A mRNA in duodenum (D) in 2 consecutive sections (A and C, B and D) taken from animals killed after 60 min of water immersion-restraint stress. Symbols indicate corresponding areas in 2 consecutive sections. Note that most PCNA-immunopositive epithelial cells seem to express c-fos or NGFI-A mRNA in response to stress. Bar: 50 (A and C), 25 (B and D) \( \mu m \).

Fig. 5. Photomicrographs showing Fos-immunoreactive (IR) nuclei in stomach from control animals (0; A and B) and animals killed at 30 min after end of 90-min water immersion-restraint stress (p30; C and D). Note that Fos-immunoreactive nuclei were observed in epithelial cells localized particularly in pit and isthmus regions (C and D) and smooth muscle layer of small blood vessels and gastric wall (D). Bar, 100 \( \mu m \).
stress. They estimated the level of c-fos mRNA after 2 h of stress and concluded that the expression of c-fos was in response to mucosal damage and healing. In support of this conclusion was the fact that biosynthesis of polyamines, which are growth factors for mucosal cells, was stimulated sequentially with the expression of IEG and that an inhibitor of polyamine synthesis decreased both the expression of IEG and mucosal healing. In the present study we also observed a rapid induction of IEG in the gastrointestinal tract of stressed animals. Water
immersion-restraint stress induced c-fos (from 60 min) and NGFI-A (from 15 min) in the epithelial cells, with localization to the pit and isthmus regions. PCNA immunoreactivity and c-fos or NGFI-A mRNA are colocalized in the epithelial cells of the isthmus regions, suggesting that stress-induced c-fos and NGFI-A might be involved in the proliferation of mucosal epithelial cells (stem cell of mucosal cells) (15). However, histological changes in the mucosa were not apparent at 15–30 min after the onset of water immersion-restraint stress. Mucosal erosions and blood clots on the surface of the stomach were first observed 60 min after the onset of stress. Therefore, the induction of IEG, at least NGFI-A mRNA preceded the mucosal lesions. In addition, the intensity of signals for these mRNA was not correlated with the severity of mucosal damage. We considered that induction of IEG could not have been the consequence of lesions. We speculate that these genes might accelerate the proliferation of mucosal epithelial cells independently of the formation of mucosal lesions. Transient ischemia and recirculation induce the expression of c-fos, c-jun, and NGFI-A mRNA within 30 min in the small intestine (23). We considered that disturbance of gastric mucosal blood flow and formation of oxygen-derived free radicals (14, 22) may be responsible for the expression of IEG in the epithelial cells. Prostacyclin, with potent antiplatelet and vasodilating activities, could significantly attenuate the induction of IEG (Saika, Ueyama, and Semba, unpublished observation).

Expression of IEG in the intestine was also reported in response to feedings. Refeeding after fasting upregulated the expression of c-fos, jun-B, and NGFI-A in the jejunum and ileum (12) and of c-fos and c-jun mRNA in the duodenum, jejunum, and ileum (11). Those studies considered the role of these genes in regulating intestinal growth and differentiation; however, they did not show the localization of IEG. Small intestinal epithelium is composed of a continuously renewable population of cells that originate in the crypts and migrate up the villus tip where they are extruded into the lumen (18). In our stress model, c-fos mRNA was mainly upregulated in the villus epithelium and NGFI-A mRNA was mainly upregulated in the crypt epithelium. Compared with stomach, NGFI-A but not c-fos might be involved in intestinal epithelial proliferation in response to stress. Co-localization of mRNA for NGFI-A and immunoreactivity for PCNA in the crypt epithelium may support this idea. Interestingly, expression of c-fos in gastric myenteric neurons was reported in response to the stretching of the stomach wall that accompanies feeding (8). In our stress model, few signals for IEG were observed in myenteric neurons.

Various kinds of adaptive reactions to stress arise when an organism is confronted by environmental challenges. Many genes other than IEG are also induced or upregulated at the late phase in response to mucosal injury. It is reported that heat shock protein HSP72 was increased at 6 h after water immersion stress in rat gastric mucosa (38). Mucosal injury caused by indomethacin induced the expression of c-myc at 3 h and of c-Ha-ras at 6–12 h after treatment (13). Several kinds of growth factors that show mitogenic activity for mucosal epithelial cells were also involved in repairing the injury. For example, activity and gene expression of ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis were increased in response to mucosal lesions (22, 23, 30). Expression of c-met mRNA, a functional receptor for hepatocyte growth factor, was also increased 6–48 h after the mucosal injury induced by HCl administration (31).

Growth factors also induce the expression of IEG in gastrointestinal mucosal epithelial cells. For example, transforming growth factor-α increased the rate of thymidine incorporation, the activity of mitogen-activated protein kinase, S6 kinase, and expression of c-fos and c-myc (21) and of c-jun and c-myc (3) in the intestinal epithelial cell line IEC-6. Epidermal growth factor, insulin, and dibutyryl cAMP stimulated the proliferation of cells derived from gastric fundus and induced the expression of c-fos and c-myc (35). Accordingly, there seems to exist a complex interaction between IEG and other late genes, especially growth factors, in stress-induced molecular events in the gastrointestinal tract. Production of IEG activates the transcription of an array of genes, which underlie long-term plastic and adaptive changes in the tissue in response to environmental challenge. A complex of Fos and Jun is a potent transcription factor, which binds to
the AP-1 site of various target genes. However, we do not know which genes are actually affected by the IEG expressed in the present stress model. To prove a functional relationship between IEG and transcriptional factors and downstream genes, further studies will be required. For example, differential RNA display might be a useful method to identify early- and late-onset genes activated by emotional stress, and this project is an ongoing project in our laboratory.

Expression of mRNA for c-fos and c-jun as well as Fos-like immunoreactivities were also observed in mononuclear cells scattered in the lamina propria of stomach and duodenum. Morphologically, these cells are considered to be lymphoid cells, suggesting to us that expression of c-fos and c-jun in these cells might influence the mucosal immune system.

In conclusion, water immersion-restraint stress in rats induced rapid expression of IEG in the smooth muscle cells of blood vessels and walls of the gastrointestinal system and in subsets of the epithelial cells. The former might indicate the hyperexcitation spasm of smooth muscle cells, and the latter might be associated with proliferation of epithelial cells in response to stress.

The authors are grateful to Edith D. Hendley (Dept. of Molecular Physiology and Biophysics, University of Vermont, Burlington, VT) for helpful comments and careful reading of the manuscript.

This work was supported by a grant from J apan Foundation of Cardiovascular Research (Tokyo, J apan), a Young Investigator’s Award (Wakayama Prefectural Government, Wakayama, J apan), and a Grant-in-Aid for Scientific Research from Ministry of Education, Science and Culture of Japan (no. 09670740) (all to T. Ueyama). And a Grant-in-Aid for Scientific Research from Ministry of Education, Science and Culture of Japan (no. 09670740) (all to T. Ueyama).

Received 27 February 1998; accepted in final form 28 April 1998.

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