Triggering of transient LES relaxations in ferrets: role of sympathetic pathways and effects of baclofen

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Staunton, Esther, Scott D. Smid, John Dent, and L. Ashley Blackshaw. Triggering of transient LES relaxations in ferrets: role of sympathetic pathways and effects of baclofen. Am J Physiol Gastrointest Liver Physiol 279: G157–G162, 2000.—Activation of gastric vagal mechanoreceptors by distention is thought to be the trigger for transient lower esophageal sphincter relaxations (TLESR), which lead to gastroesophageal reflux. The contribution of higher-threshold gastric splanchnic mechanoreceptors is uninvestigated. GABA<sub>B</sub> receptor agonists, including baclofen, potently reduce triggering of TLESR by low-level gastric distention. We aimed to determine first whether this effect of baclofen is maintained at high-level distention and second the role of splanchnic pathways in triggering TLESR. Micromanometric/pH studies in conscious ferrets showed that intragastric glucose infusion (25 ml) increased triggering of TLESR and reflux. Both were significantly reduced by baclofen (7 μmol/kg ip) (P < 0.05). When 40 ml of air was added to the glucose infusion, more TLESR occurred than with glucose alone (P < 0.01). These were also reduced by baclofen (P < 0.001). TLESR after glucose/air infusion were assessed before and after splanchnectomy (2–4, 9–11, and 23–25 days), which revealed no change. Baclofen inhibits TLESR after both low- and high-level gastric distention. Splanchnic pathways do not contribute to increased triggering of TLESR by high-level gastric distention.

Transient lower esophageal sphincter relaxations; gastroesophageal reflux; splanchnic nerves; GABA<sub>B</sub> receptors; ferret

Transient lower esophageal sphincter (LES) relaxations are the major cause of gastroesophageal reflux in humans, dogs, and ferrets (4, 8, 13). In all three species, triggering of transient LES relaxations is increased after gastric distention. Activation of GABA<sub>B</sub> receptors has been demonstrated to reduce consider-ably the occurrence of transient LES relaxations and, consequently, reflux after meals or gastric distention. This effect has important therapeutic potential in development of treatments for gastroesophageal reflux disease (5, 11, 12).

The vagus nerve has been shown to be an essential pathway in the triggering of transient LES relaxations (13); however, it is not known whether it carries the afferent or efferent limb of the pathway or both. We have demonstrated inhibition of mechanotransduction in low-threshold gastroesophageal vagal mechanoreceptors by GABA<sub>B</sub> receptors (17), suggesting that this may be a site of action of GABA<sub>B</sub> receptor agonists in reducing transient LES relaxations. However, there is an alternative pathway to the vagus in the splanchnic nerves, the role of which remains unexplored. There is functional evidence for both vagosplanchnic and splanchnovagal reflexes to the upper gastrointestinal tract (10, 18). Electrophysiological evidence shows that there is a subpopulation of vagal preganglionic efferent neurons that continue to respond to gastric distention after removal of vagal afferent input (9, 18), providing direct evidence that spinal afferent pathways project to the dorsal vagal complex, where coordination of transient LES relaxation is believed to take place (14). The sparse literature on direct electrophysiological recordings from gastric splanchnic afferents suggests that they respond at higher thresholds than vagal afferents (see Ref. 7).

This study aims to determine the role of high-threshold gastric splanchnic afferents in the triggering of transient LES relaxations. We have established that there is a potent effect of GABA<sub>B</sub> receptor agonists on triggering of transient LES relaxations at low levels of gastric distention; a second aim of this study was to ascertain whether or not this effect is maintained at higher levels.

Materials and Methods

Animals and Experimental Protocol

The procedures performed in this study were approved by the Animal Ethics Committee of the Institute of Medical and Veterinary Science, Adelaide. Experiments were performed on seven adult female ferrets (weight range 0.55–0.75 kg) obtained from the Institute for Medical and Veterinary Science. They were acclimatized to handling and laboratory conditions soon after weaning and were trained to wear a harness. Chronic lateral cervical mucosa-to-skin esophagostomies were constructed when the ferrets reached adulthood. Surgery was performed under isoflurane (2–4% inhalation) anesthesia. Manometric studies were begun at least a month after surgery.

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Recording Methods

Manometry was performed with a micromanometric assembly (2.0-mm OD; Dentsleeve, Adelaide, Australia) that consisted of a reverse-perfused sleeve sensor to monitor LES pressure, three side holes located 2, 5, and 7.5 cm proximal to the sleeve for esophageal pressure, and a side hole located distal to the sleeve for gastric pressure measurement. Esophageal side holes were perfused with degassed distilled water at 0.02 ml/min, and the sleeve sensor and gastric side hole were perfused at 0.04 ml/min. Gastric infusion was via a central channel (0.75-mm ID) distal to the sleeve. A pH electrode (0.8-mm OD; Synectics Medical, Stockholm, Sweden) was attached 10 mm above the proximal end of the sleeve sensor with Parafilm and was referenced to a Ag-AgCl electrode. The combined pH/manometric assembly was introduced via the esophagostomy and held in place by a Neoprene harness around the animal's upper thorax and neck. Swallows were detected with a microphone that was attached to the collar and positioned over the hyoid bone. The recording chamber comprised a plastic cylinder mounted on a freely rotating platform that allowed monitoring of animals in their normal postprandial body position (see Ref. 4 for details). After preamplification (Synectics Polygraf), manometric and pH outputs were acquired to disk and analyzed off-line with Labview-based software. Audio signals from the throat microphones were converted to a software time marker and integrated with other analog inputs.

Experimental Protocol

Animals were fasted overnight before studies. On the day of study, 60 min before gastric infusions, an intraperitoneal injection of saline (1 ml/kg) or baclofen (7 μmol/kg) was given. The pH/manometric assembly was then introduced, and the ferret was placed into the recording chamber and allowed to adapt to study conditions for 10 min before data acquisition was commenced. A gastric load was then administered after a 30-min baseline period. In one series of experiments, after 30 min of baseline data acquisition, a load of 25 ml of 10% D-glucose (pH 3.5) was introduced directly into the stomach via the 0.75-mm-diameter central channel of the manometric assembly over 2 min. In a second series of experiments, this initial load was immediately followed by 2 ml/min air for 10 min, then a rest period of 10 min, and finally another, similar infusion of air. The reasons for using this pattern of administration of air were first to avoid a noxious or pathological stimulus, which may occur if 40 ml of air were delivered all in one infusion, and second to replace air that would be vented via transient LES relaxations soon after the initial infusion, as we found in a previous study (4). Thus we could achieve a maintained stimulus for transient LES relaxations. Each ferret was studied four times, twice with saline injection (control-glucose and control-glucose plus air) and twice with baclofen injection (baclofen-glucose and baclofen-glucose plus air) with at least 1 wk between each randomized study.

After the completion of the above studies, another control-glucose plus air study was performed. At the beginning of the subsequent week a laparotomy was performed under isoflurane (2–4% inhalation) anesthesia, and the splanchnic nerves were divided at the phrenoesophageal ligament by removal of ~1 cm of the left and right splanchnic nerves adjacent to the LES. The laparotomy was closed, and the animals were allowed to recover before being studied again 2–4 days, 9–11 days, and 23–25 days after splanchnectomy. The animals showed no adverse effects in the postoperative period, maintained their body weight, and had normal food and fluid intake. The completeness of splanchnic nerve section was verified by inspection of the operative site post mortem after the full series of studies, which confirmed no regrowth of the nerves.

In Vitro Experiments

In addition to confirmation of splanchnic nerve section by gross inspection, we also confirmed this by functional assessment of spinal denervation of the LES. Circular strips of LES muscle were taken from two animals according to methods described in detail elsewhere (19) immediately after euthanasia and placed in 10-ml water-jacketed organ baths containing carbogenated Krebs solution at 37°C of the following composition (in mM): 118 NaCl, 25 NaHCO3, 4.6 KCl, 1.2 MgSO4, 1.3 NaH2PO4, 11 glucose, and 2.5 CaCl2. One end of the tissue was fastened to a support, and the other was attached to an isometric force transducer (FT03; Grass, Quincy, MA). Each strip was placed under an initial tension of 2 g and left to equilibrate for 60 min. Responses of the LES strips to substance P (10−8−10−6 M) and capsaicin (10−8−10−7 M) were measured. Administration of the capsaicin vehicle (see Drugs) alone in an equivalent dilution did not alter basal LES tone. Our previous data indicate that capsaicin causes a profound LES relaxation by releasing substance P from splanchnic afferent collaterals (2, 3, 19, 20). Therefore, in successfully denervated LES, no response to capsaicin should persist.

Data Analysis

Manometry. Basal LES pressure was referenced to mean intragastric pressure and taken as the visual mean of end-expiratory pressure after exclusion of the effects of movement artefacts, straining, swallowing, and transient LES relaxation. Transient LES relaxations were defined as rapid (>1 mmHg/s) drops in LES pressure to 2 mmHg or less above gastric pressure for >5 s, with no associated swallow signal. Swallowing was defined as drops in LES pressure occurring within 2 s before or after a swallow was detected. A drift was scored when there was a slow (<1 mmHg/s) decrease in LES pressure to <2 mmHg above gastric pressure that remained at its nadir for >10 s. Acid reflux was scored when intragastric pH dropped to at least pH 4 for >5 s.

Statistics. Data were normally distributed (Kolmogorov-Smirnov test) and so were expressed as means ± SE. Differences between two data groups were assessed using Student’s paired t-test. Differences between three or more data groups were assessed using repeated measures ANOVA with a Fisher’s protected least significant differences post hoc test.

Drugs

Substance P was obtained from Auspep (Melbourne, Australia), capsaicin from Sigma-Aldrich (Sydney, Australia), and baclofen from Research Biochemicals International (Natick, MA). All drugs were dissolved in normal saline, except for capsaicin, which was dissolved in saline, ethanol, and Tween-80 (8:1:1 vol/vol/vol).

RESULTS

Effect of Different Gastric Loads

No transient LES relaxations or reflux episodes occurred in the preinfusion period in any of the protocols followed. Significantly more transient LES relaxations occurred in the 30-min postinfusion period following combined glucose and air infusion than after glucose alone (P < 0.05, Fig. 1A). This was reflected in an
increase in the occurrence of gastroesophageal acid reflux episodes (Fig. 1B), although this was not statistically significant. Basal LES pressure showed a significant increase in the postinfusion period after the glucose/air infusion vs. the preinfusion period, whereas after glucose alone there was no change in LES pressure (Fig. 2A). These changes were comparable with those in mean intragastric pressure. Although no significant increase in intragastric pressure occurred after either type of infusion, the value after the combined infusion was significantly greater than after glucose alone ($P < 0.05$, Fig. 2B). Peak intragastric pressure was reached within 2 min of the onset of air infusion. This tended to be higher than that at the same time after glucose alone ($11.0 \pm 0.9$ vs. $6.9 \pm 0.4$ mmHg; $P = 0.08$).

**Effect of Baclofen**

As shown previously (5), baclofen was effective in reducing the number of transient LES relaxations after glucose infusion. When the intragastric load was increased by additional air infusion, the effect of baclofen was still present and was in fact proportionally larger, because it completely abolished the occurrence of transient LES relaxations (Fig. 1A). This effect was associated with the expected inhibition of reflux episodes (Fig. 1B). Basal LES pressure after glucose infusion was unaffected both in baclofen and control studies (Fig. 2A). However, baclofen switched the increase in basal LES pressure seen in control studies after glucose/air infusion to a decrease (Fig. 2A). Intragastric pressure was unchanged by either of the infusions in baclofen studies (Fig. 2B).

**Effect of Chronic Splanchnic Nerve Section**

Bilateral chronic splanchnic nerve section (referred to as splanchnectomy) had no effect on glucose/air-induced transient LES relaxations or gastroesophageal acid reflux episodes at any of the times after the operation (from 2 to 25 days; Fig. 3). The number of reflux episodes was reduced after splanchnectomy (Fig. 3B), but this was not statistically significant ($P < 0.1$). This observation suggests that more transient LES relax-
ations were associated with gas than with fluid reflux, as would be expected with an infusion of mainly air.

Basal LES pressure increased after the combined infusion preoperatively and at all stages postoperatively (Fig. 4A). The absolute value of postinfusion basal LES pressure was significantly higher when measured 2–4 days after splanchnectomy compared with preoperatively, but this difference was not maintained. Mean intragastric pressure did not increase significantly after gastric infusion of glucose plus air in controls or after splanchnic nerve section. Postinfusion intragastric pressure was lower after splanchnectomy, this being significant 23–25 days after splanchnectomy (Fig. 4B).

Responses of LES Muscle Strips After Splanchnic Nerve Section

Controls from these studies were taken from previously published data (19, 20). These showed potent reproducible relaxatory responses to capsaicin (10–6 M), which are shown in Fig. 5A (data from Ref. 19). These responses and those to substance P were antagonized by the selective neurokinin-1 receptor antagonist CP-99994 (19). In LES strips from splanchnectomized ferrets, no response to capsaicin (10–6–10–5 M) could be evoked, even though strips responded with profound relaxation to substance P (10–8–10–6 M; Fig. 5B), demonstrating a selective removal of the source of substance P but not its receptor.

DISCUSSION

The data presented confirm our previous observation of a potent inhibition by GABA_B agonists of the triggering of transient LES relaxations (5). This was observed not only after low-level gastric distention, as in our previous study, but also after higher-level gastric distention, achieved with additional air infusion. The triggering of transient LES relaxations by higher-level distention did not involve a major contribution from spinal afferent pathways, as shown by the lack of effect...
of chronic section of the greater splanchnic nerves, which provide the majority of the spinal afferent innervation of the stomach (1, 6).

Spinal pathways are considered to signal higher-intensity stimuli than vagal pathways (7). Spinal neurons receiving input from abdominal viscera respond to intraluminal pressure increases of 10 mmHg or greater (15). This level therefore represents the threshold for activation of ascending spinal pathways, and we aimed to reach this level of gastric distention in this study without reaching the noxious range, which is ~15 mmHg in humans (16). We have already demonstrated in anesthetized ferrets that spinal pathways are activated by gastric distention in the 10–15 mmHg range, because the responses of vagal preganglionic neurons to gastric distention persisted after bilateral cervical vagotomy (18). These responses demonstrate the existence of a spinovagal reflex pathway. In addition to this pathway, we also showed that there is a vagospinal pathway to the LES (10). The splanchnic nerves have a profound inhibitory influence on the ferret LES when electrically stimulated (3). Because of this evidence that afferent and efferent pathways in the splanchnic nerves are involved in reflex control of the LES, we chose to investigate their involvement in the triggering of transient LES relaxations in this study. We found no effect on triggering of transient LES relaxations or reflux episodes after splanchnic nerve section either acutely or chronically. This indicates that an alternative pathway to the splanchnic nerves is involved. A study in conscious dogs demonstrated that cold blockade of the vagal nerve trunks abolished transient LES relaxations in response to distention of the stomach with air (13) and concluded that the vagus nerves are important in carrying the afferent and/or efferent components of the pathway for transient LES relaxations. The results of our study support this conclusion and add further evidence that the vagus nerves mediate both afferent and efferent pathways involved in triggering of transient LES relaxations.

It may be argued that there are alternative pathways connecting the spinal cord with the stomach and LES other than the greater splanchnic nerves and that these may have mediated the triggering of transient LES relaxations after splanchnectomy. However, our in vitro data show that the response to capsaicin was eradicated by chronic splanchnic nerve section. We have shown previously that capsaicin activates inhibitory pathways in the LES via release of substance P derived from extrinsic afferent fibers (2, 3, 19, 20). The abolition of the capsaicin response would render unlikely the contribution of other spinal afferent pathways to this region.

In a previous study, we showed that GABAB receptor agonists, including baclofen (at the same dose as in this study), potently inhibited the occurrence of transient LES relaxations and reflux after a 25-ml intragastric load of glucose (5). This relatively low-level stimulus triggered relatively few events in control studies, so the full potential for inhibition by GABAB receptor agonists may have been obscured. In the present study, the volume of the intragastric load was increased using 40 ml of air, which resulted in approximately double the number of transient LES relaxations compared with glucose alone. The effect of baclofen on this increased number of transient LES relaxations was to completely abolish them, showing that the potency of baclofen had indeed been underestimated in our previous study. This effect was associated with a near-complete blockade of reflux episodes. Although the triggering of transient LES relaxations by glucose alone may have been mediated via both mechano- and

Fig. 5. In vitro strain gauge recordings of tension in circular LES strips taken from an unoperated animal (A) and those 4 wk after splanchnectomy (B). In the unoperated preparation, capsaicin (10⁻⁶ M) evoked potent relaxatory responses on three occasions. In a LES strip from a splanchnectomized ferret, no response to capsaicin (10⁻⁶–10⁻⁵ M) could be evoked, even though strips responded with profound relaxation to substance P administered cumulatively (10⁻⁸–10⁻⁶ M).
REFERENCES