Published in final edited form as:

Auton Neurosci. 2011 December 7; 165(2): 178–190. doi:10.1016/j.autneu.2011.07.009.

Neostigmine-induced contraction and nitric oxide-induced relaxation of isolated ileum from STZ diabetic guinea pigs

Joseph Cellini, Anne Marie Zaura Jukic, and Kathy J. LePard

Department of Physiology, Chicago College of Osteopathic Medicine, Midwestern University, Downers Grove, IL USA

Abstract

Both delayed gastrointestinal transit and autonomic neuropathy have been documented in patients with diabetes mellitus. The mechanism of neostigmine, an agent that mimics release of acetylcholine from autonomic neurons by prokinetic agents, to contract smooth muscle, despite dysfunctional enteric neural pathways, was determined using isolated ilea from STZ-treated and control guinea pigs. Both bethanechol- and neostigmine-induced contractions were stronger in diabetic ileum. Bethanechol-induced contractions of control but not diabetic ileum were increased by low dose scopolamine suggesting reduced activation of presynaptic muscarinic autoreceptors in diabetic ileum. The muscarinic receptor antagonist 4-DAMP strongly, but the nicotinic receptor antagonist hexamethonium only weakly, reduced neostigmine-induced contractions of control and diabetic ilea. The amount of acetylcholine, inferred from tissue choline content, was increased in diabetic ileum. Nicotinic neural and noncholinergic postjunctional smooth muscle receptors contributed more strongly to neostigmine-induced contractions in diabetic than control ileum. Relaxation of diabetic ileum by exogenous nitric oxide generated from sodium nitroprusside was comparable to control ileum, but smooth muscle relaxation by L-arginine using neuronal nitric oxide synthase to generate nitric oxide was weaker in diabetic ileum with evidence for a role for inducible nitric oxide synthase. Despite autonomic neuropathy, neostigmine strongly contracted ileum from diabetic animals but by a different mechanism including stronger activation of postjunctional muscarinic receptors, greater synaptic acetylcholine, stronger activation of noncholinergic excitatory pathways, and weaker activation of inhibitory pathways. A selective medication targeting a specific neural pathway may more effectively treat disordered gastrointestinal transit in patients with diabetes mellitus.

Keywords

muscarinic autoreceptors; iNOS; acetylcholinesterase inhibitor

Introduction

Small intestinal contractions mix luminal contents to promote digestion of nutrients by digestive enzymes and to expose nutrients to the apical surface of enterocytes for absorption

© 2011 Elsevier B.V. All rights reserved.

Corresponding Author: Kathy J. LePard, PhD, Associate Professor of Physiology, Chicago College of Osteopathic Medicine, Midwestern University, 555 West 31st Street, Downers Grove, IL 60515, USA, Telephone: 630-515-6391, FAX: 630-515-7245, klepar@midwestern.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

and propel luminal contents towards the large intestine. Small intestinal motility can modulate the rate of gastric emptying. Arrival of nutrients to the distal small intestine delayed gastric emptying by the ileal brake (Brown et al., 1992; Holgate and Read, 1985; Maljaars et al., 2008; Spiller et al., 1984). In turn, gastric distension by a meal enhanced the flow of luminal contents from the ileum into the cecum (Kerlin and Phillips, 1983). This bidirectional coordination between stomach and ileum is mediated by vagal autonomic reflexes and gastrointestinal (GI) hormones (Maljaars et al., 2008).

Gastrointestinal motor abnormalities in patients with diabetes mellitus (DM) are often attributed to autonomic neuropathy (Feldman and Schiller, 1983; Scarpello and Sladen, 1978; Vinik et al., 2003; Yang et al., 1984). Extrinsic autonomic reflexes controlling GI function were impaired in patients with DM. Gastric acid secretion in response to sham feeding, a vagal reflex, was reduced in patients with DM (Feldman et al., 1979). Increased colonic motility in response to gastric distension, the parasympathetic gastrocolonic reflex, was absent in patients with DM reporting severe constipation (Battle et al., 1980; Battle et al., 1983). Diabetic diarrhea has been associated with autonomic neuropathy (Ellenberg, 1964; McNally et al., 1969; Scarpello et al., 1976; Whalen et al., 1969) with predominantly reports of slowed intestinal transit (Hodges et al., 1947; Iber et al., 1993; Scarpello et al., 1976; Whalen et al., 1969), but occasionally reports of accelerated intestinal transit (Muri, 1953; Vinnik et al., 1962). Hence, inappropriate postprandial intestinal motility may be the consequence of inadequate activation of extrinsic parasympathetic reflex pathways by disordered gastric motility (Kumar et al., 2008; Rosztoczy et al., 2004; Samsom et al., 1995; Samsom et al., 1996; Samsom et al., 1998; Troncon et al., 1998) or by abnormal gastric emptying (Maggs et al., 2008; Sarosiek et al., 2010), and may result in inappropriate gastric emptying through an ineffective ileal brake reflex as observed in streptozotocin (STZ) rats (Martin et al., 2004).

Some agents used to treat slowed GI transit in patients increase release of endogenous acetylcholine (ACh) from autonomic neurons (Hasler, 2007; Park and Camilleri, 2006; Patrick and Epstein, 2008; Rayner and Horowitz, 2005; Sanger and Alpers, 2008). However, in patients with dysfunctional enteric motor pathways due to neuropathy, agents that modulate the activity of enteric neural pathways may not effectively normalize GI transit (Gershon, 2004). Pathology of enteric neurons has been documented in some DM patients with diarrhea (Bennett et al., 1956; Ellenberg, 1964; Vinnik et al., 1962; Whalen et al., 1969; Yoshida et al., 1988) or gastric dysfunction (Harberson et al., 2010; He et al., 2001; Grover et al., 2011; Pasricha et al., 2008) suggesting altered activity of intrinsic enteric pathways. Since, ACh released from both extrinsic and intrinsic autonomic neurons can activate both enteric excitatory and inhibitory pathways to generate smooth muscle pressure gradients required for normal GI transit, we hypothesized that despite documented autonomic neuropathy of the vagus nerve (Kniel et al., 1986; Regalia et al., 2002; Robertson and Sima, 1980) and enteric neurons (Chandrasekharan and Srinivasan, 2007) as well as smooth muscle myopathy (Ordog, 2008) in animals models of DM, nonselective cholinergic activation of enteric excitatory and inhibitory pathways by ACh using neostigmine, an inhibitor of acetylcholinesterase (AChE), would normalize intestinal motility. Contributions of excitatory and inhibitory enteric neural pathways to neostigmine-induced contractions were evaluated and compared by assessing neuronal ACh content, direct smooth muscle contraction and relaxation to cholinergic agonists and nitric oxide (NO), and participation of enteric nicotinic and muscarinic receptors using isolated ileum from control and diabetic animals.

In animal models of DM, GI dysfunction may result from either a low concentration of effective plasma insulin, abnormal plasma c-peptide concentration, or elevated plasma glucose. This study uses a STZ-induced model of type 1 DM in guinea pigs to capitalize on

the wealth of information on ileal enteric neurochemical coding and motor circuits published for guinea pig (Bornstein et al., 2004; Brookes, 2001; Costa et al., 1996; Furness and Costa, 1987). Similar to rodent models, insulin and c-peptide were very low in STZ guinea pigs (Gorray et al., 1986; Howell et al., 1971; Junod et al., 1969; Lundquist et al., 1975). However, hyperglycemia and ketosis were milder in STZ guinea pigs (Elliott and Pogson, 1977; Gorray et al., 1986; Hootman et al., 1998; Johnson, Jr., 1978; Peterssen et al., 1970; Schlosser et al., 1984; Schlosser et al., 1987) as compared to STZ rats (Hawkins et al., 1986; Junod et al., 1969). Hence, to focus on the contribution of insulin deficiency to autonomic neuropathy and motor dysfunction, the effect of neostigmine on ileal motility was determined in STZ-treated guinea pigs.

Materials and Methods

Induction of diabetes by streptozotocin

Diabetes was induced in male Hartley guinea pigs (n=11, 200–300 g, Charles River Laboratories, Portage, MI) by a single intraperitoneal (ip) injection of STZ (pancreatic beta cell toxin, 50 mg/mL, 280 mg/kg, Sigma, St. Louis, MO) dissolved in citrate buffer (0.05 M, pH 4.5) (Schlosser et al., 1984). Control animals (n=11) were given a single ip injection of citrate buffer. Animals were euthanized by CO₂ narcosis 5–6 wks after injection. Urine was tested for glucose using uristix (Siemens Healthcare Diagnostics Inc, Tarrytown, NY). Ileum was removed and placed in warmed, oxygenated Krebs solution of the following composition (mM): NaCl, 117; KCl, 4.7; CaCl₂, 2.5; MgCl₂, 1.2; NaH₂PO₄, 1.2; NaHCO₃, 25; glucose, 11. Kidneys were removed and weighed to determine the ratio of final body weight to kidney weight, an indicator of type 1 diabetes mellitus in guinea pigs (LePard, 2005; Schlosser et al., 1984). These protocols were submitted to and approved by the Downers Grove Midwestern University IACUC.

Preparations for motility recordings

Two adjacent, whole-thickness, longitudinal strips were cut using razor blades from one rectangular piece of ileum opened along the mesenteric border. Suture loops (4-0 suture) were used to mount tissues onto hooks. Tissues were lowered into a 10 mL organ bath (8 channel Myobath, World Precision Instrument, Sarasota, FL) or 40 mL conical tubes continuously bubbled with 95% O₂/5% CO₂ and maintained at 37°C by a heater/circulating bath (DC10, Haake Thermo Electron Corporation, Karlsruhe, Germany). Contractile responses were recorded using isometric force transducers connected either to an 8-channel Grass paper chart recorder or to an analogue-to-digital converter (PowerLab 8SP, ADInstruments, Colorado Springs, CO) then saved to a computer hard drive.

At the start of each experiment, strips were washed 6×10 min with normal Krebs and resting tension was adjusted to approximately 1.0 g. For ilea to be used for an experiment, a peak nerve-stimulated contraction (6V) or KCl (40 mM)-induced contraction of ≥ 1g was required. Tissues were washed 3×10 min between treatments. For some tissues, KCl treatment was repeated at the end of the experimental protocol. Tissues were weighed at the end of the experiment.

Cholinergic Contractions

Tissues were treated with the muscarinic receptor agonist bethanechol (1–1000 μM, Sigma) for 2 min. To confirm muscarinic receptors mediated bethanechol-induced contractions, tissues were pretreated with scopolamine (3–3000 μM, Sigma), or the muscarinic M₃ receptor antagonist 4-diphenylacetoxy-N-(2-chloroethyl)piperidine hydrochloride (1 μM 4-DAMP, Sigma) (Sawyer and Ehlert, 1998) for 2–5 min, then treated with a moderate or maximal dose of bethanechol (10 or 100 μM, respectively) for 2 min. Cholinergic

contractions to endogenous Ach were recorded for 5 min after treatment with neostigmine (0.05–50 μ M, Sigma), an inhibitor of AchE.

Roles of cholinergic receptors in neostigmine-induced contractions

In evaluating the individual roles of nicotinic and muscarinic receptors in mediating neostigmine-induced contractions, tissues were pretreated for 2 min with either vehicle (water), 4-DAMP, or the nicotinic neural receptor antagonist hexamethonium (C6, Sigma), then treated with neostigmine (1 μ M) for 5 min. Both 4-DAMP and hexamethonium were evaluated in all tissues.

Role of enteric nervous system in neostigmine-induced contractions

Tissues were pretreated for 2 min with the fast sodium channel blocker tetrodotoxin (TTX, 3 μ M, Sigma), then treated with increasing doses of neostigmine (0.1–10 μ M).

Histamine dose-response

Experiments were performed to determine the dose of histamine (0.03–30 μ M, Sigma) required to provide stable, sustained contractions for study of relaxation. For these experiments, Krebs solution contained 3–6 μ M scopolamine to minimize phasic contractions. Ilea were treated with increasing doses of histamine for 10 min. In separate experiments, tissues were treated 4 times with 30 μ M histamine for up to 12 min to confirm that histamine-induced contractions were reproducible and stable.

Nitric-oxide-induced relaxation

Tissues were precontracted with histamine (30 μ M), then after 5 min treated with increasing doses of either L-arginine (0.1–100 mM, Sigma) or sodium nitroprusside (SNP, 0.3–300 μ M, Sigma) for 3 min. The role of neuronal (n) nitric oxide synthase (NOS) was evaluated by precontracting tissues with histamine (30 μ M) for 3 min, then pretreating ilea with *N*_ω-nitro-L-arginine (L-NNA, 0.3–30 μ M, Sigma) for 2 min, before treatment with either L-arginine (10 mM) or SNP (100 μ M) for 3 min. The nadir of relaxation was determined. Recovery of ilea from relaxation was evaluated 3 min after L-arginine or SNP treatment.

Analysis of smooth muscle contractions

Results were evaluated either visually from the chart recordings (g contraction) or by computer software program (Chart 5 v 5.5.4, ADInstruments, Colorado Springs, CO). Computer analysis identified prominent peaks over 2 min for calculation of frequency, and basal tone was calculated by average force over 2 min. Basal tone and frequency of contraction were determined in the absence and presence of 4-DAMP or hexamethonium. Computer analysis calculated area under the curve (AUC, g*s) for a maximum of 5 min. Area under the curve was determined for all contractions in the absence or presence of bethanechol or neostigmine as well as for contractions elicited by bethanechol or neostigmine after pretreatment with 4-DAMP, hexamethonium, or TTX. In addition, data were expressed as either percent inhibition or percent change with positive and negative values representing an increase and decrease, respectively. For bethanechol dose-response curves, curve fitting was performed using 3 parameter sigmoid curve fitting (Sigma Plot 10, Systat Software, Inc.) with r^2 of 0.7643 to 0.9788 and EC₅₀ derived from curve fits. For neostigmine, histamine, L-arginine, and SNP dose-response curves, curve fitting was performed using 3 or 4 parameter logistic standard curve fitting, whichever resulted in the best fit, (Sigma Plot 10) with r^2 of 0.8428 to 0.9997 and EC₅₀ derived from curve fits. Data were expressed as peak initial or latter sustained contraction (g), contraction (g) per tissue wet weight (g), or area (g*s) per tissue wet weight (g). The minimum and maximum

effective doses were defined as the lowest doses at which the drug response was significantly different than baseline or was not significantly different than higher doses.

Choline tissue content

After ileal motility preparations were made, separate pieces of ileum were cut then frozen at -20°C . After thawing on ice, each piece was weighed then ground in normal Krebs (final volume 2 mL) using a manual grinding tube (Fisher Scientific). The slurry was transferred into a microcentrifuge tube, then centrifuged for 4 minutes at $14,000\text{ min}^{-1}$. Supernatant from each microcentrifuge tube was aspirated, avoiding the pellet, and placed in a clean centrifuge tube then frozen at -20°C until analysis for choline.

Tissue supernatant was assayed for choline content by enzymatic assay using a chemiluminometer (Model 20 photometer, Mt. View, CA). A reaction mixture was made consisting of 7.5 mL normal Krebs, 100 μL of 250 U/mL choline oxidase, 50 μL of 2 mg/mL horseradish peroxidase, 100 μL of 1 μM luminol (Sigma), and 80 μL of 1000 U/mL cholinesterase. Tissue supernatant (100 μL) was vortexed with 150 μL of reaction mixture for 60 s. After a 5 s delay, the light value was read from the chemiluminometer. The choline concentration in each 100 μL sample was determined using standard curves to choline (10–1000 nM). Data were expressed as nM choline/100 μL /mg tissue. The correlation coefficients (r^2) for each standard curve were ≥ 0.98 .

Statistics

All data were expressed as mean $\pm$ SEM. The n represents the number of tissue preparations. Data were statistically analyzed using SPSS (version 18.0, Chicago, IL). Outliers of control and diabetic groups were determined using a boxplot graph and removed prior to comparisons using the Student's t-test or factorial ANOVA. Depending on the analysis, independent factors for ANOVA included 1) control or diabetic, 2) drug (vehicle, 4-DAMP, hexamethonium, TTX, neostigmine, L-arginine, SNP, L-NNA), 3) dose, 4) neostigmine exposure (single, multiple), 5) presence or absence of TTX, or 6) repetitions of histamine. Both main effects of one independent factor and interactions of multiple independent factors were evaluated and Sidak correction was used for post-hoc analysis. A value of $p < 0.05$ was considered statistically significant. Statistically significant differences between control and diabetic animals were denoted using *, whereas a statistically significant difference between drug doses or treatments within the control or diabetic group was denoted using † or ‡ .

Results

Animal model

At the time of the experiment 5–6 weeks after STZ injection, diabetic animals weighed less than control animals [g: control (n=11), 570 ± 17 ; diabetic (n=11), $309 \pm 19^*$; $*p < 0.05$]. Diabetic animals had a higher kidney weight to body weight ratio [$\text{g/g} \times 10^{-3}$: control (n=11), 3.75 ± 0.07 ; diabetic (n=11), $6.02 \pm 0.31^*$; $*p < 0.05$]. At the time of sacrifice, glucose was detected in the urine of most diabetic (8/10) but no control (0/11) guinea pigs.

Basal motility

Ilea from control and diabetic animals had similar frequencies of basal contraction [Hz: control (n=8), 0.29 ± 0.02 ; diabetic (n=11), 0.29 ± 0.02]. Neither 4-DAMP nor hexamethonium alone significantly altered the frequency of basal contractions of ilea from control or diabetic animals as compared to vehicle (Table 1) [Interaction: control/diabetic*drug; $F(3,67)=0.964$, $p=0.415$]. In contrast, pretreatment with 4-DAMP equally reduced, but hexamethonium equally increased, the force generated by ileal tissues as compared to vehicle (Table 1) [Main effect: drug; $F(3,68)=31.568$, $p < 0.05$]. Contractions to KCl at the start of the

experiment were weaker in diabetic as compared to control ileum [contraction (g)/tissue wt (g): control (n=15), 73.0 ± 5.5 ; diabetic (n=16), $51.8 \pm 4.7^*$; $*p < 0.05$] with similar decreases observed at the conclusion of experiments [% decrease: control (n=24), 11 ± 4 ; diabetic (n=32), 15 ± 5 ; $p = 0.485$].

Bethanechol: initial contractions

The rapid, initial contraction of longitudinal smooth muscle in response to muscarinic receptor activation requires extracellular calcium (Murthy, 2006). Initial peak contractions elicited by bethanechol (1–1000 μM) were similar in control and diabetic ilea with maximal force observed at 100 μM [contraction (g): control (n=38), 1.7 ± 0.1 ; diabetic (n=32), 1.6 ± 0.1] and a trend for the contraction at 1000 μM bethanechol to be stronger in diabetic ileum [contraction (g): control (n=22), 1.3 ± 0.1 ; diabetic (n=24), 1.8 ± 0.1] [Interaction: control/diabetic*dose; $F(3,248) = 2.299$, $p = 0.078$]. The EC_{50} doses for bethanechol-induced initial peak contractions were similar [control, $1.01 \pm 2.58 \mu\text{M}$; diabetic, $3.06 \pm 1.81 \mu\text{M}$]. Scopolamine was equally effective at reducing the bethanechol (100 μM)-induced initial peak contraction of control and diabetic ilea (Fig. 1) [Interaction: control/diabetic*dose; $F(4,206) = 0.798$; $p = 0.528$] with 3 μM scopolamine weakly increasing, and 300 and 3000 μM strongly decreasing, bethanechol-induced initial peak contractions (Fig. 1) [Main effect: dose; $F(4,206) = 97.169$; $p < 0.05$].

Bethanechol: sustained contractions

The latter, sustained contraction of longitudinal smooth muscle in response to muscarinic receptor activation requires G protein-mediated activation of RhoA (Murthy, 2006). Latter sustained contractions elicited by bethanechol (1–1000 μM) were similar at all doses in ilea from control and diabetic animals [bethanechol 100 μM contraction (g): control (n=38), 1.6 ± 0.1 ; diabetic (n=32), 1.8 ± 0.1] [Interaction: control/diabetic*dose; $F(3,248) = 0.142$, $p = 0.934$]. The EC_{50} doses for bethanechol-induced sustained contractions were similar [control, $2.52 \pm 4.59 \mu\text{M}$; diabetic, $3.21 \pm 3.79 \mu\text{M}$]. Scopolamine (30 μM) weakly enhanced the maximal bethanechol (100 μM)-induced sustained contraction of control ileum by $16 \pm 4\%$ (n=16), as compared to $2 \pm 4\%$ for diabetic ileum (n=24) (Fig. 1) [Interaction: control/diabetic*dose; $F(3,128) = 3.098$; $p < 0.05$]. Scopolamine at 300 and 3000 μM strongly decreased bethanechol (100 μM)-induced sustained contractions of both control and diabetic ilea (Fig. 1) [Interaction: control/diabetic*dose; $F(4,206) = 2.667$; $p < 0.05$].

Bethanechol: area of contractions

The total force generated by intestinal smooth muscle, the area of bethanechol (10 μM)-induced contraction, was greater in diabetic than control ileum, but was equally reduced by 4-DAMP in both control and diabetic ilea (Table 2) [Interaction: control/diabetic*drug; $F(1,50) = 5.475$, $p < 0.05$].

Neostigmine-induced contractions

In the absence of nerve stimulation, sufficient Ach accumulated after neostigmine treatment to increase force production by isolated ilea (Fig. 2). Increasing concentrations of neostigmine (0.05–50 μM) dose-dependently increased area of contraction of ilea from control and diabetic animals (Fig. 3A) [Interaction: control/diabetic*dose; $F(5,543) = 3.176$, $p < 0.05$]. The minimum effective dose of neostigmine was lower in diabetic (0.1 μM) as compared to control (0.3 μM) ileum. The EC_{50} doses of neostigmine were similar for control and diabetic ilea [control, $0.20 \pm 0.06 \mu\text{M}$; diabetic, $0.23 \pm 0.03 \mu\text{M}$]. Neostigmine-induced contractions were stronger in diabetic as compared to control ileum at 0.3, 0.5, and 1 μM (Fig. 3A). Once maximal contraction to neostigmine was achieved in control ileum, higher doses of neostigmine reduced force production (Fig. 3A).

Contraction of tissues to a single dose of neostigmine

Previous studies showed that for some gastric tissues, maximal neostigmine-induced contractions were weaker if tissues had been previously exposed to lower neostigmine doses (Cellini et al., 2010). In these studies, ileal contractions to 1 μ M neostigmine were similar regardless of prior neostigmine exposure (0.05–0.5 μ M) for both control ileum and diabetic ileum (Table 2) [Interaction: control/diabetic*neostigmine exposure; $F(1,86)=0.88$, $p=0.767$]. Again, neostigmine-induced contractions were stronger in ileum from diabetic as compared to control animals [Main effect: control/diabetic; $F(1,86)=7.008$, $p<0.05$].

Role of muscarinic receptors in neostigmine-induced contraction

In the presence of 4-DAMP, only noncholinergic neurotransmitters, not Ach, released by enteric excitatory motor neurons could directly activate smooth muscle receptors. In addition, Ach released by vagal efferent nerves or enteric interneurons could activate enteric noncholinergic excitatory and inhibitory motor neurons through nicotinic cholinergic receptors to indirectly promote an increase or decrease, respectively, in smooth muscle force production.

In the presence of 4-DAMP (1 μ M), the area of neostigmine-induced contraction was greater in ileum from diabetic as compared to control animals, but both were similar to basal force production (Fig. 2 and Fig. 4) [Interaction: control/diabetic*drug; $F(2, 140)=7.763$, $p<0.05$]. When data were expressed as percent change, 4-DAMP was equally effective at reducing areas of neostigmine-induced contraction of control and diabetic ilea [Interaction: control/diabetic*drug; $F(2,74)=0.907$, $p=0.408$] [% decrease: control ($n=10$), 86 ± 4 ; diabetic ($n=17$), 75 ± 4].

Role of nicotinic receptors in neostigmine-induced contraction

With nicotinic receptors blocked by hexamethonium, Ach activated muscarinic receptors located postjunctionally on smooth muscle and presynaptically on excitatory and inhibitory enteric neurons to alter force production. The low dose of hexamethonium (100 μ M) was equally effective at reducing areas of neostigmine-induced contractions of both control and diabetic ilea with no further reduction observed by a higher dose of hexamethonium (300 μ M) (Table 3) [Main effect: dose; $F(2,98)=7.464$, $p<0.05$]. Overall, hexamethonium (100 and 300 μ M) equally reduced neostigmine-induced contractions by $17\pm9\%$ in control ileum ($n=20$) and $24\pm5\%$ in diabetic ileum ($n=33$) [Main effect: control/diabetic; $F(1,49)=0.526$, $p=0.472$].

In the presence of 300 μ M hexamethonium, the area of neostigmine-induced contraction was greater in ileum from diabetic as compared to control animals and both were greater than basal force production (Fig. 2 and Fig. 4) [Interaction: control/diabetic*drug; $F(2, 140)=7.763$, $p<0.05$].

Role of neuronal fast sodium channels in neostigmine-induced contraction

Since hexamethonium only weakly reduced neostigmine-induced contractions of both control and diabetic ilea (Fig. 4), the role of enteric neurotransmission was further investigated using TTX, a fast sodium channel blocker which significantly reduces neuronal action potential propagation. Tissues were pretreated with TTX (3 μ M), then exposed to increasing concentrations of neostigmine (0.1–10 μ M). Notably, neostigmine (1 μ M) - induced contractions evoked in the presence of TTX were similar in control and diabetic ilea (Fig. 3B) [Interaction: control/diabetic*dose; $F(3,322)=2.210$; $p=0.087$], which contrasted with neostigmine dose-response data shown in Fig. 3A [Interaction: control/diabetic*dose; $F(5,543)=3.176$, $p<0.05$].

For both control (Fig. 3C) and diabetic (Fig. 3D) ilea, in the presence of TTX, low dose (0.1 μ M) neostigmine contractions were weaker, but high dose (1 μ M) neostigmine contractions were stronger, than in the absence of TTX, and increasing the dose of neostigmine from 0.5 to 1.0 μ M significantly increased the strength of contractions [Interaction: dose *presence/absence of TTX; $F(3,322)=17.099$, $p<0.05$]. A main effect of control/diabetic was observed as all neostigmine-induced contractions in both the absence and presence of TTX were stronger in diabetic as compared to control ileum (Fig. 2) [area/tissue wt (g): control (n=111), 11.3 ± 0.6 ; diabetic (n=100), $13.0\pm0.8^*$; $*p<0.05$] [Main effect: control/diabetic; $F(1,322)=12.178$, $p<0.05$]. Stronger neostigmine-induced contractions of diabetic ileum suggest Ach-induced generation of NO by NOS may be reduced in diabetic ileum. Hence, the ability of NO to relax smooth muscle was investigated using the NO donor SNP and the NOS substrate L-arginine.

Histamine-induced contractions

Overall, initial peak histamine (0.03–30 μ M)-induced contractions were weaker in diabetic as compared to control ileum (Fig. 5) (Table 4) [Main effect: control/diabetic; $F(1,141)=5.085$; $p<0.05$] with maximal initial contractions observed at 3 μ M histamine for both control and diabetic ilea [Main effect: dose; $F(4,141)=128.616$; $p<0.05$]. The EC_{50} dose was 0.33 ± 0.12 μ M for control ileum and 0.21 ± 0.04 μ M for diabetic ileum. In addition, sustained histamine (3–30 μ M)-induced contractions were weaker in diabetic as compared to control ileum (Table 3) [Main effect: control/diabetic; $F(1,49)=17.578$; $p<0.05$]. Sustained contractions to histamine (30 μ M) persisted for 9.1 ± 0.7 min (range 6.0–13.75) in control ileum and 6.0 ± 0.4 min (range 3.25–8.5 min) in diabetic ileum with less than 0.2 gram deviation providing consistent, stable contractions for study of NO-mediated relaxations. Repetitive histamine (30 μ M)-induced contractions showed similar run-down for both control and diabetic ilea [Interaction: control/diabetic*repetitions; $F(3,268)=0.169$; $p=0.917$] allowing for repetitive drug treatments to investigate smooth muscle relaxation.

Effect of L-NNA

The role of nNOS was investigated using the nNOS inhibitor L-NNA. Pretreatment with L-NNA (0.3–30 μ M) alone produced a dose-dependent contraction of control, but not diabetic, ileum (Fig. 6) [Interaction: control/diabetic*dose; $F(3,247)=5.457$; $p<0.05$].

L-Arginine-induced immediate relaxation

Since activation of muscarinic (Kortezova et al., 2004; Olgart and Iversen, 1999) and nicotinic receptors (Curro et al., 2008; Takahashi, 2003) on enteric neurons by endogenous Ach can promote relaxation by increasing neuronal generation of NO by NOS, the ability of endogenous NOS to generate NO from exogenous L-arginine was determined. L-arginine (10, 30, 100 mM) was less effective at inducing immediate relaxation of diabetic ileum with the minimum effective dose lower for diabetic ileum (3 mM) than control ileum (10 mM) (Fig. 7A) [Interaction: control/diabetic*dose; $F(7,352)=8.073$; $p<0.05$]. The EC_{50} dose was 15.3 ± 0.8 mM for control ileum and 21.3 ± 2.5 mM for diabetic ileum. Overall, pretreatment with L-NNA (0.3–30 μ M) significantly increased L-arginine (10 mM)-induced relaxation of diabetic ileum by $57\pm5\%$ (n=92) with little effect on control ileum ($20\pm6\%$, n=64) (Fig. 7B) [Main effect: control/diabetic; $F(1,150)=25.290$, $*p<0.05$]. Overall, the magnitude of the L-arginine (10 mM)-induced relaxation in the presence of L-NNA (0.3–30 μ M) was greater in diabetic as compared to control ileum [immediate relaxation (g)/tissue wt (g): control (n=88), 16.5 ± 0.8 ; diabetic (n=123), $21.3\pm0.9^*$; $*p<0.05$] [Main effect: control/diabetic; $F(1,203)=17.473$, $*p<0.05$] with a minimum dose of 3 μ M L-NNA for augmenting L-arginine-induced relaxations [Main effect: dose; $F(3,203)=5.636$, $p<0.05$].

Recovery from L-arginine-induced relaxation

Recovery of ileum from L-arginine-induced (3–100 mM) relaxation was more complete in control than diabetic ileum (Fig. 8A) [% recovery: control (n=88), 109±8; diabetic (n=112), 71±6*; *p<0.05] [Main effect: control/diabetic; F(1,192)=14.138; *p<0.05]. After 3 min, neither control nor diabetic ilea had recovered from treatment with the highest dose of L-arginine (100 mM) (Fig. 8A) [Main effect: dose; F(3,192)=34.748, p<0.05]. Pretreatment with L-NNA (0.3–30 μM) dose-dependently improved recovery from L-arginine (10 mM) -induced relaxation of control and diabetic ilea with maximal recovery observed at 3 μM L-NNA (Fig. 8B) [Main effect: dose; F(3,202)=42.060, p<0.05]. Overall, in the presence of L-NNA, recovery was complete in both control and diabetic ilea with control ileum producing greater force than diabetic ileum (Fig. 8B) [% recovery: control (n=87), 140±6; diabetic (n=123), 112±4*; *p<0.05] [Main effect: control/diabetic; F(1,202)=21.872; *p<0.05].

SNP-induced immediate relaxation

To evaluate NO-induced smooth muscle relaxation independent of NOS activity, ilea were treated with the NO donor sodium nitroprusside (SNP). SNP (0.3–300 μM)-induced immediate relaxation of diabetic ileum was 8% stronger at 10 μM, but 10% weaker at 300 μM, as compared to control ileum (Fig. 7C) with similar minimum and maximum effective doses of 1.0 μM and 100 μM, respectively for both control and diabetic ilea [Interaction: control/diabetic*dose; F(7,302)=3.693; p<0.05]. The EC₅₀ dose was 4.3±0.4 μM for control ileum and 2.5±1.0 μM for diabetic ileum. Pretreatment with L-NNA (0.3–30 μM) did not alter SNP (100 μM) -induced immediate relaxation of ilea from control or diabetic animals (Fig. 7D) [Main effect: dose; F(2,76)=0.033, p=0.967].

Recovery from SNP-induced relaxation

Recovery of control and diabetic ilea from SNP (0.3–300 μM)-induced relaxation was similar (Fig. 8C) [Main effect: control/diabetic; F(1,216)=0.828; p=0.364]. At 30 μM SNP, recovery was maximal for both control and diabetic ilea at 53±3% (n=46) (Fig. 8C) [Main effect: dose; F(6,216)=18.802, p<0.05]. Overall, in the presence of L-NNA (3–30 μM), recovery from SNP (100 μM)-induced relaxation was more complete in control than diabetic ileum [% recovery: control (n=42), 59±3; diabetic (n=42), 44±2*; *p<0.05] [Main effect: control/diabetic; F(1,78)=17.897; p<0.05], with 30 μM L-NNA improving recovery of control and diabetic ilea (Fig. 8D) [Main effect: dose; F(2,78)=4.737; p<0.05].

Tissue Choline

The amount of choline in ileum was significantly increased in diabetic as compared to control animals [nM choline/100 μL/mg tissue: control (n=45), 112.6±14.5; diabetic (n=26), 179.3±17.8*; *p<0.05]

Discussion

In these studies, neostigmine, an AchE inhibitor that increases endogenous Ach levels, was used to mimic the general mechanism of action of many prokinetic drugs. In the presence of neostigmine, sufficient endogenous Ach accumulated at synaptic junctions to activate cholinergic receptors producing strong contraction of ilea. Despite neuropathy of autonomic neurons (LePard, 2005), neostigmine-induced contractions of diabetic ileum were stronger than control ileum (Figs. 2A, 3). Many mechanisms may contribute to this observation including 1) more effective postjunctional muscarinic receptor activation by Ach, 2) slower enzymatic degradation of extracellular Ach, 3) increased amount of Ach in the synaptic space, 4) reduced activation of presynaptic muscarinic inhibitory autoreceptors by Ach, 5) greater contribution of noncholinergic excitatory neurotransmitters to smooth muscle

contraction, and 6) decreased contribution of inhibitory neurotransmitters to smooth muscle relaxation. Overall, data suggest that along with stronger activation of postjunctional muscarinic receptors by abundant enteric Ach, stronger neostigmine-induced contractions can be attributed to greater nicotinic activation of noncholinergic excitatory pathways along with weaker generation of NO after nicotinic activation of inhibitory pathways (Fig. 9).

Activation of postjunctional muscarinic receptors

Bethanechol, a muscarinic receptor agonist resistant to enzymatic breakdown by endogenous AchE, contracts intestinal smooth muscle by activating smooth muscle muscarinic receptors. Bethanechol-induced contractions were stronger in diabetic as compared to control ileum due to stronger, calcium-dependent, initial contractions (Fig. 9). Bethanechol activated muscarinic M3 receptors to contract smooth muscle as 4-DAMP and scopolamine prevented bethanechol-induced contractions. Using small intestine from STZ rats, bethanechol-induced maximal contractions of whole thickness segments were similar in strength (Mathison and Davison, 1988; Nowak et al., 1990) but maximal contractions of longitudinal muscle strips with attached myenteric plexus were stronger than control (Carrier and Aronstam, 1990). Different degrees of myopathy (Fig. 9), such as smooth muscle stiffness (Jorgensen et al., 2001) or disruptions in interstitial cells of Cajal networks (Ordog, 2008) as suggested by weaker KCl contractions, as well as different types of tissue preparations (Chandrasekharan and Srinivasan, 2007) and different methods of expression of contractile response (Chandrasekharan and Srinivasan, 2007; Zandecki et al., 2008), may contribute to these reported differences in bethanechol-induced contractions. Area of contraction is a useful index of smooth muscle force production as it includes initial peak, sustained, and maximal contractions.

Activation of cholinergic receptors by endogenous Ach was evaluated using neostigmine, an inhibitor of AchE. Neostigmine slows the breakdown of Ach in the neuronal synapse and at postjunctional smooth muscle muscarinic receptors. Accumulation of Ach in the neuronal synapse was not essential for strong neostigmine-induced contractions; activation of nicotinic neural receptors on enteric motor neurons only weakly contributed to neostigmine-induced contractions of control and diabetic ilea (Fig. 4), which probably reflected the sparse vagal efferent innervation of this small intestinal region (Berthoud et al., 1990; Berthoud et al., 1991). In the presence of hexamethonium and neostigmine, Ach accumulated at the neuromuscular junction, thereby prolonging activation of smooth muscle muscarinic receptors and increasing force production. Accumulation of Ach at the neuroeffector junction was essential for strong neostigmine-induced contractions of control and diabetic ilea. Since neostigmine-induced contractions were almost completely prevented by pretreatment with a muscarinic receptor antagonist (Fig. 4), stronger neostigmine-induced contractions of diabetic ileum were due, in part, to enhanced smooth muscle muscarinic receptor activation as demonstrated by bethanechol.

Rate of enzymatic degradation of Ach

Another possible cause of stronger neostigmine-induced contractions of diabetic ileum may be greater accumulation of endogenous Ach in the synaptic space due to its slowed breakdown by cholinesterases. The activity of AchE was increased (Lincoln et al., 1984) or unchanged (Lucas and Sardar, 1991) in ileum from STZ rats and the activity of butyrylcholinesterase, another cholinesterase, was increased in diabetic rat heart (Dave and Katyare, 2002). Carbachol, like endogenous Ach, activates both muscarinic and nicotinic receptors; but in contrast to endogenous Ach, carbachol is resistant to breakdown by cholinesterases. In STZ rats, maximal ileal contractions to physostigmine, another reversible AchE inhibitor, were unchanged (Nowak et al., 1990) but maximal ileal contractions to carbachol were increased (Anjaneyulu and Ramarao, 2002; Carrier and Aronstam, 1990;

Talubmook et al., 2003) suggesting that the rate of endogenous Ach breakdown may be increased in diabetic ileum. Cholinesterase activity itself was not measured, but if it were indeed increased in ileum from STZ guinea pigs, then smooth muscle muscarinic receptor activation would be reduced, and stronger neostigmine-induced contractions of diabetic ileum would be due to more efficient amplification of signal transduction pathways in smooth muscle (Forrest et al., 2005).

Amount of Ach in synaptic space: Ach content

Another possible cause of stronger neostigmine-induced contractions of diabetic ileum may be greater accumulation of endogenous Ach in the synaptic space due to increased Ach tissue content. The amount of Ach substrate available for enzymatic breakdown, as inferred from tissue choline, was substantially increased in diabetic as compared to control ileum. Diabetes-induced increased in choline content of ileum may reflect the reported increased density of choline acetyltransferase (ChAT)-ir tertiary nerve fibers (LePard, 2005) and an increased capacity to synthesize Ach due to increased ChAT activity in enteric neurons (Lincoln et al., 1984). Since vagal efferent innervation of the ileum was sparse (Berthoud et al., 1990; Berthoud et al., 1991), choline content more accurately reflected Ach in enteric neurons. Hence, in diabetic ileum, more endogenous Ach from enteric neurons may be available to activate cholinergic receptors contributing to stronger neostigmine-induced contractions (Fig. 9).

Amount of Ach in synaptic space: inhibitory autoreceptors

Acetylcholine released in the absence of nerve stimulation (Kilbinger and Wessler, 1980b) or accumulating in the synaptic space after treatment with an AchE inhibitor (Kilbinger and Wessler, 1980a) can activate presynaptic muscarinic autoreceptors. Since basal tone was reduced by 4-DAMP (Table 1) and a low dose of neostigmine promoted smooth muscle contraction (Fig. 2), Ach was released at the neuroeffector junction in the absence of cholinergic receptor activation. Since scopolamine alone increased Ach release from guinea pig ileum (Kilbinger and Wesler, 1980b) by blocking presynaptic muscarinic autoreceptors on enteric cholinergic neurons (Dziedzic and Kilbinger, 1978; Kilbinger, 1977; Kilbinger and Wesler, 1980a), it is likely that blocking presynaptic autoreceptors with low dose scopolamine enhanced endogenous Ach release resulting in activation of additional postjunctional muscarinic receptors, thereby augmenting bethanechol-induced initial contractions (Fig. 1). The ability of presynaptic inhibitory muscarinic autoreceptors to suppress Ach release from autonomic neurons was altered in ileum from STZ rats (Coulson et al., 2002). In diabetic ileum, presynaptic muscarinic autoreceptors may not be as effective at curbing endogenous Ach release, as scopolamine did not increase bethanechol-induced sustained contractions as in control ileum (Fig. 1). Therefore, ineffective feedback to curb Ach accumulation may contribute to stronger neostigmine-induced contractions of diabetic ileum (Fig. 9).

Contribution of enteric noncholinergic excitatory pathways

Cholinergic activation of noncholinergic excitatory pathways contributed to neostigmine-induced contractions. For both control and diabetic ilea, neostigmine (0.1 μ M)-induced contractions in the presence of TTX were weaker than in the absence of TTX (Fig. 3) reflecting decreased activation of enteric excitatory pathways. For both control and diabetic ilea, neostigmine-induced contractions in the presence of 4-DAMP were not weaker than basal contractions (Fig. 4), despite the contribution of muscarinic receptors to basal force production, suggesting that noncholinergic, excitatory substances were contributing to smooth muscle contractions under these conditions. In diabetic as compared to control ileum, stronger 4-DAMP-resistant, neostigmine-induced contractions were observed (Fig. 4)

suggesting stronger nicotinic receptor activation of noncholinergic excitatory pathways (Fig. 9).

Contribution of enteric inhibitory pathways

Enteric inhibitory pathways tonically suppressed motility of control and diabetic ilea. Basal tone was equally increased after hexamethonium in both control and diabetic ilea (Table 1) suggesting that enteric inhibitory pathways were tonically active at rest to suppress basal tone. Neostigmine would result in activation of enteric inhibitory pathways as activation of nicotinic cholinergic receptors on enteric motor neurons increased NO generation by an L-NNA-dependent mechanism (Patel et al., 2008). Indeed, minimizing enteric neural transmission using TTX resulted in stronger neostigmine (1 μ M)-induced contractions of control and diabetic ilea (Fig. 3), reflecting the contribution of enteric inhibitory pathways to neostigmine-induced contractions. When cholinergic activation of inhibitory pathways was prevented using hexamethonium, neostigmine-induced contractions of diabetic ileum were stronger than control ileum (Fig. 4). Hence, data suggest that activation of enteric inhibitory pathways as a result of neostigmine treatment was reduced in diabetic as compared to control ileum (Fig. 9).

Nitric oxide-induced smooth muscle relaxation

In diabetic ileum, diminished contribution of enteric inhibitory pathways to smooth muscle motility may reflect an ineffectiveness of NO, an inhibitory neurotransmitter of enteric neurons, to relax smooth muscle. Since NO is a neurotransmitter not only of enteric inhibitory motor neurons innervating GI smooth muscle, but also of myenteric interneurons (Reiche et al., 2000), selective targeting to suppress NO production in only enteric inhibitory motor neurons, but not in enteric interneurons, was not feasible. Therefore, NO-induced relaxation of control and diabetic ilea was evaluated in this study using exogenous L-arginine and the NO donor SNP.

The ability of the NO donor, SNP, to relax ileum was either minimally altered (Fig. 7C) or unchanged in diabetic animals (Kaputlu et al., 1999; Martinez-Cuesta et al., 1995). In contrast, relaxation of intestine in response to nitroglycerin was weaker in diabetic as compared to control animals (Zandecki et al., 2008). Since nitroglycerin, but not SNP, required enzymatic transformation prior to NO release (Harrison and Bates, 1993; Miller and Megson, 2007), reduced nitroglycerin-induced relaxation may more strongly reflect sluggish enzymatic transformation in diabetic tissues rather than impaired relaxation of the tissue to NO itself. As has been reported (Ogulener et al., 2001), L-NNA did not alter SNP-induced immediate relaxation of ilea from control and diabetic animals (Fig. 7D) suggesting a direct effect of NO released from SNP to stimulate soluble guanylyl cyclase resulting in an increase in intracellular cGMP promoting smooth muscle relaxation (Moncada et al., 1991). Hence, the ability of exogenous NO to relax diabetic ileum was not compromised.

Generation of nitric oxide from L-arginine

The depressed inhibitory tone of diabetic ileum may result from inability of enteric inhibitory neurons to generate NO from NOS after nicotinic neural receptor activation. Relaxation in response to exogenous L-arginine, the substrate used by NOS to generate NO, was less pronounced in diabetic as compared to control ileum (Fig. 7A). Hence, reduced relaxation of diabetic ileum after L-arginine treatment was due either to impaired transport of exogenous L-arginine into enteric neurons or to decreased amount or activity of NOS enzyme. Treatment of diabetic rats with exogenous L-arginine normalized intestinal L-arginine content suggesting that transport of the amino acid into neurons was not impaired in diabetic intestine (Umathe et al., 2009). In diabetic animals, decreased nNOS mRNA and protein expression (Cellek et al., 2003; Shotton and Lincoln, 2006; Surendran and

Kondapaka, 2005; Takahashi et al., 1997; Watkins et al., 2000; Zandecki et al., 2008), as well as impaired nerve-stimulated NANC relaxations (Jenkinson and Reid, 1995; Kaputlu et al., 1999; Martinez-Cuesta et al., 1995; Takahashi et al., 1997; Watkins et al., 2000; Zandecki et al., 2008) and low tissue nitrite levels (Umathe et al., 2009) have been reported. In STZ- guinea pigs, there were similar numbers of myenteric neurons containing nNOS in control and diabetic ilea, yet weaker fluorescent intensities of nNOS-ir nerve processes in diabetic ileum (LePard, 2005), as well as reduced generation of NO by NOS as inferred from weaker L-arginine-induced smooth muscle relaxation of diabetic ileum (Fig. 7A), confirming that early degenerative changes in nNOS-ir nerve fibers (Surendran and Kondapaka, 2005), but not cell bodies, had occurred. In addition, L-NNA alone increased force production in control, but not diabetic, ileum (Fig. 6) suggesting that in the absence of exogenous L-arginine substrate, nNOS in control, but not diabetic, ileum tonically generated sufficient NO to measurably suppress contractility. Taken together, data suggest that the enteric inhibitory pathway was significantly depressed in diabetic as compared to control ileum. Therefore, reduced generation of NO by NOS after activation of the inhibitory pathway contributed to stronger neostigmine-induced contractions of diabetic as compared to control ileum (Fig. 9).

Role for inducible nitric oxide synthase

In guinea pig ileum, some myenteric neurons expressed inducible (i) NOS (LePard, 2005) suggesting this isoform may participate in production of NO after L-arginine treatment. In the absence of any NOS inhibitor, weak relaxations of diabetic ileum in response to 10 mM L-arginine reflected reduced nNOS enzymatic activity (Martinez-Cuesta et al., 1995) and nerve fiber density (LePard, 2005). At this low L-arginine substrate concentration, more NO would be generated using nNOS than iNOS as indicated by the lower nNOS K_m (nNOS, 1.2–3 μM ; iNOS, 6–19 μM) (Boer et al., 2000; Furfine et al., 1993; Griffith and Stuehr, 1995). To confirm the role of nNOS in L-arginine-induced relaxation in these studies, tissues were pretreated with L-NNA, a rapid-acting, competitive inhibitor of NOS with a 50- to 300-fold greater selectivity for nNOS than iNOS, a lower K_m for nNOS than iNOS, and a reported IC_{50} of 3 μM (Alderton et al., 2001; Boer et al., 2000; Furfine et al., 1993; Griffith and Stuehr, 1995; Klatt et al., 1994). Surprisingly, pretreatment with L-NNA significantly augmented, rather than weakened, L-arginine-induced relaxation of diabetic ileum (Fig. 7B). Taken together, generation of NO by iNOS may contribute to stronger L-arginine-induced relaxation of diabetic ileum in the presence of L-NNA. Since prolonged suppression of nNOS by exogenous administration of 7-nitroindazole resulted in upregulation of intestinal iNOS mRNA, protein, and activity (QU et al., 2001), one could speculate that diabetes-induced decrease in nNOS mRNA, protein, and enzyme activity (Cellek et al., 2003; Martinez-Cuesta et al., 1995; Shotton and Lincoln, 2006; Surendran and Kondapaka, 2005; Takahashi et al., 1997; Watkins et al., 2000; Zandecki et al., 2008) may result in upregulation of iNOS mRNA, protein, and activity. A physiologic role for iNOS in peristalsis of normal mouse colon has been proposed (Mancinelli et al., 2001); however, additional experiments using many NOS inhibitors with different selectivities for nNOS and iNOS are required to fully explore a physiologic role for iNOS in smooth muscle relaxation in diabetic animals.

Recovery from nitrergic relaxation

In general, ilea recovered better from L-arginine (3–30 mM) (Fig. 8A) than SNP (0.3–300 μM) (Fig. 8C) induced relaxations even though the magnitudes of smooth muscle relaxations were similar (Figs. 6A, C). The amount of NO generated by the highest dose of SNP was likely lower than that generated by 100 mM L-arginine since those strong relaxations were maintained at 3 min. Despite lower NO levels, recovery from SNP was

more sluggish perhaps due to more sustained generation of NO throughout the 3 min recovery period (Badovinac et al., 2000).

Surprisingly, minimizing generation of NO from L-arginine by nNOS using L-NNA increased relaxations of diabetic as compared to control ileum (Fig. 7B), yet also significantly shortened diabetic ileum recovery time (Fig. 8B). As suggested above, in the presence of L-NNA, more NO may be generated by iNOS from L-arginine which has faster kinetics than nNOS (Anggard, 1994) permitting more rapid NO generation yet quicker recovery of diabetic ileum. The ability of L-NNA to improve recovery of control ileum more than diabetic ileum from SNP-induced relaxations (Fig. 8D) may reflect the ability of L-NNA alone to increase force production in control but not diabetic ileum (Fig. 6).

Conclusion

In control and diabetic ilea, the cholinergic neuro-neuronal and the neuromuscular pathways were not mutually dependent in generating contractions in response to neostigmine. If nicotinic receptors were blocked, strong neostigmine-induced contractions persisted, but if muscarinic receptors were blocked, neostigmine-induced contractions were weak. In diabetic ileum, stronger neostigmine-induced contractions were attributed to stronger activation of postjunctional muscarinic receptors by a greater amount of accumulated Ach, due to higher enteric neuronal content and poor feedback control by presynaptic muscarinic autoreceptors on enteric cholinergic motor neurons. Noncholinergic, excitatory neurotransmitter(s) released by activation of nicotinic receptors on enteric motor neurons contributed to stronger neostigmine-induced contractions of diabetic ileum. In contrast, the inhibitory nitrergic pathway played a weaker role in diabetic ileum indirectly contributing to stronger neostigmine-induced contractions. Relaxation of smooth muscle to NO was not compromised in diabetic ileum, but less NO was generated by nNOS after activation of nicotinic receptors on enteric inhibitory motor neurons. Despite enteric neuropathy and myopathy, neostigmine produced strong contractions of isolated ileum suggesting that intestinal transit in patients with DM might be normalized by suppressing noncholinergic excitatory input to intestinal smooth muscle, activating presynaptic muscarinic receptors to moderate endogenous Ach release, and activating iNOS in enteric neurons to generate normal levels of NO.

Acknowledgments

Grants

Supported by R21 NS039768 and R15 NS047106 to KJL and by ORSP at Midwestern University.

Reference List

- Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. *Biochem J.* 2001; 357:593–615. [PubMed: 11463332]
- Anggard E. Nitric oxide: mediator, murderer, and medicine. *Lancet.* 1994; 343:1199–1206. [PubMed: 7909873]
- Anjaneyulu M, Ramarao P. Studies on gastrointestinal tract functional changes in diabetic animals. *Methods Find Exp Clin Pharmacol.* 2002; 24:71–75. [PubMed: 12040885]
- Badovinac V, Trajkovic V, Mostarica-Stojkovic M. Nitric oxide promotes growth and major histocompatibility complex-unrestricted cytotoxicity of interleukin-2-activated rat lymphocytes. *Scand J Immunol.* 2000; 52:62–70. [PubMed: 10886785]
- Battle WM, Cohen JD, Snape WJ Jr. Disorders of colonic motility in patients with diabetes mellitus. *Yale J Biol Med.* 1983; 56:277–283. [PubMed: 6670291]

- Battle WM, Snape WJ Jr, Alavi A, Cohen S, Braunstein S. Colonic dysfunction in diabetes mellitus. *Gastroenterol.* 1980; 79:1217–1221.
- Bennett WA, Berge KG, Sprague RG. The intestinal tract in diabetic diarrhea; a pathologic study. *Diabetes.* 1956; 5:289–294. [PubMed: 13356718]
- Berthoud HR, Carlson NR, Powley TL. Topography of efferent vagal innervation of the rat gastrointestinal tract. *Am J Physiol.* 1991; 260:R200–R207. [PubMed: 1992820]
- Berthoud HR, Jedrzejewska A, Powley TL. Simultaneous labeling of vagal innervation of the gut and afferent projections from the visceral forebrain with dil injected into the dorsal vagal complex in the rat. *J Comp Neurol.* 1990; 301:65–79. [PubMed: 1706359]
- Boer R, Ulrich WR, Klein T, Mirau B, Haas S, Baur I. The inhibitory potency and selectivity of arginine substrate site nitric-oxide synthase inhibitors is solely determined by their affinity toward the different isoenzymes. *Mol Pharmacol.* 2000; 58:1026–1034. [PubMed: 11040050]
- Bornstein JC, Costa M, Grider JR. Enteric motor and interneuronal circuits controlling motility. *Neurogastroenterol Motil.* 2004; 16(Suppl 1):34–38. [PubMed: 15066002]
- Brookes SJ. Classes of enteric nerve cells in the guinea-pig small intestine. *Anat Rec.* 2001; 262:58–70. [PubMed: 11146429]
- Brown NJ, Rumsey RD, Bogentoft C, Read NW. The effect of adrenoceptor antagonists on the ileal brake mechanism in the rat. *Br J Pharmacol.* 1992; 105:751–755. [PubMed: 1352722]
- Carrier GO, Aronstam RS. Increased muscarinic responsiveness and decreased muscarinic receptor content in ileal smooth muscle in diabetes. *J Pharmacol Exp Ther.* 1990; 254:445–459. [PubMed: 2384882]
- Cellek S, Foxwell NA, Moncada S. Two phases of nitrgic neuropathy in streptozotocin-induced diabetic rats. *Diabetes.* 2003; 52:2353–2362. [PubMed: 12941776]
- Cellini J, DiNovo K, Harlow J, LePard KJ. Regional differences in neostigmine-induced contraction and relaxation of stomach from diabetic guinea pig. *Auton Neurosci Basic Clin.* 2010; 160:69–81.
- Chandrasekharan B, Srinivasan S. Diabetes and the enteric nervous system. *Neurogastroenterol Motil.* 2007; 19:951–960. [PubMed: 17971027]
- Costa M, Brookes SJH, Steele PA, Gibbins IL, Burcher E, Kandiah CJ. Neurochemical classification of myenteric neurons in the guinea-pig ileum. *Neurosci.* 1996; 75:949–967.
- Coulson FR, Jacoby DB, Fryer AD. Increased function of inhibitory neuronal M2 muscarinic receptors in trachea and ileum of diabetic rats. *Br J Pharmacol.* 2002; 135:1355–1362. [PubMed: 11906948]
- Curro D, Ipavec V, Preziosi P. Neurotransmitters of the non-adrenergic non-cholinergic relaxation of proximal stomach. *Eur Rev Med Pharmacol Sci.* 2008; 12(Suppl 1):53–62. [PubMed: 18924444]
- Dave KR, Katyare SS. Effect of alloxan-induced diabetes on serum and cardiac butyrylcholinesterases in the rat. *J Endocrinol.* 2002; 175:241–250. [PubMed: 12379509]
- Dzieniszewski P, Kilbinger H. Muscarinic modulation of acetylcholine release evoked by dimethylphenylpiperazinium and high potassium from guinea-pig myenteric plexus. *Eur J Pharmacol.* 1978; 50:385–391. [PubMed: 699963]
- Ellenberg M. Diabetic neuropathy, with special reference to visceral neuropathy. *Adv Intern Med.* 1964; 12:11–32. [PubMed: 14268277]
- Elliott KR, Pogson CI. The effects of starvation and experimental diabetes on phosphoenol-pyruvate carboxykinase in the guinea pig. *Biochem J.* 1977; 164:357–361. [PubMed: 141929]
- Feldman M, Corbett DB, Ramsey EJ, Walsh JH, Richardson CT. Abnormal gastric function in longstanding, insulin-dependent diabetic patients. *Gastroenterol.* 1979; 77:12–17.
- Feldman M, Schiller LR. Disorders of gastrointestinal motility associated with diabetes mellitus. *Ann Intern Med.* 1983; 98:378–384. [PubMed: 6402969]
- Forrest A, Molleman A, Parsons M. The responses to manipulation of extracellular and intracellular calcium are altered in the streptozotocin-diabetic rat colon and ileum. *Eur J Pharmacol.* 2005; 509:77–83. [PubMed: 15713432]
- Furfin ES, Harmon MF, Paith JE, Garvey EP. Selective inhibition of constitutive nitric oxide synthase by L-NG-nitroarginine. *Biochemistry.* 1993; 32:8512–8517. [PubMed: 7689333]
- Furness, JB.; Costa, M. *The Enteric Nervous System.* Churchill Livingstone; Edinburgh: 1987.

- Gershon MD. Review article: serotonin receptors and transporters -- roles in normal and abnormal gastrointestinal motility. *Aliment Pharmacol Ther.* 2004; 20(Suppl 7):3–14. [PubMed: 15521849]
- Gorray KC, Baskin D, Brodsky J, Fujimoto WY. Responses of pancreatic β cells to alloxan and streptozotocin in the guinea pig. *Pancreas.* 1986; 1:130–138. [PubMed: 2953022]
- Griffith OW, Stuehr DJ. Nitric oxide synthases: properties and catalytic mechanism. *Annu Rev Physiol.* 1995; 57:707–736. [PubMed: 7539994]
- Grover M, Farrugia G, Lurken MS, Bernard CE, Faussone-Pellegrini MS, Smyrk TC, Parkman HP, Abell TL, Snape WJ, Hasler WL, Unalp-Arida A, Nguyen L, Koch KL, Calles J, Lee L, Tonascia J, Hamilton FA, Pasricha PJ. Cellular changes in diabetic and idiopathic gastroparesis. *Gastroenterol.* 2011; 140:1575–1585.
- Harberson J, Thomas RM, Harbison SP, Parkman HP. Gastric neuromuscular pathology in gastroparesis: analysis of full-thickness antral biopsies. *Dig Dis Sci.* 2010; 55:359–370. [PubMed: 19997975]
- Harrison DG, Bates JN. The nitrovasodilators. New ideas about old drugs. *Circulation.* 1993; 87:1461–1467. [PubMed: 8491000]
- Hasler WL. Gastroparesis: symptoms, evaluation, and treatment. *Gastroenterol Clin North Am.* 2007; 36:619–47. ix. [PubMed: 17950441]
- Hawkins RA, Mans AM, Davis DW. Regional ketone body utilization by rat brain in starvation and diabetes. *Am J Physiol.* 1986; 250:E169–E178. [PubMed: 2937307]
- He CL, Soffer EE, Ferris CD, Walsh RM, Szurszewski JH, Farrugia G. Loss of interstitial cells of cajal and inhibitory innervation in insulin-dependent diabetes. *Gastroenterol.* 2001; 121:427–434.
- Hodges FJ, Rundles RW, Hanelin J. Roentgenologic study of the small intestine; dysfunction associated with neurologic diseases. *Radiology.* 1947; 49:659–674. [PubMed: 18921561]
- Holgate AM, Read NW. Effect of ileal infusion of intralipid on gastrointestinal transit, ileal flow rate, and carbohydrate absorption in humans after ingestion of a liquid meal. *Gastroenterol.* 1985; 88:1005–1011.
- Hootman SR, Jones JE, Kapoor R, Nguyen KL, de Ondarza J. Sodium, potassium-activated adenosine triphosphatase activity is impaired in the guinea pig pancreatic duct system in streptozotocin-induced diabetes. *Biochem Biophys Res Commun.* 1998; 243:869–873. [PubMed: 9501017]
- Howell SL, Edwards JC, Whitfield M. Preparation of B cell deficient guinea pig islets of Langerhans. *Horm Metab Res.* 1971; 3:37–43. [PubMed: 4942751]
- Iber FL, Parveen S, Vandrunen M, Sood KB, Reza F, Serlovsky R, Reddy S. Relation of symptoms to impaired stomach, small bowel, and colon motility in long-standing diabetes. *Dig Dis Sci.* 1993; 38:45–50. [PubMed: 8420759]
- Jenkinson KM, Reid JJ. Effect of diabetes on relaxations to non-adrenergic, non-cholinergic nerve stimulation in longitudinal muscle of the rat gastric fundus. *Br J Pharmacol.* 1995; 116:1551–1556. [PubMed: 8564218]
- Johnson FM Jr. The importance of adrenocortical steroids in the pathogenesis of ketosis in diabetic guinea pigs (*Caviae porcellus*). *Cornell Vet.* 1978; 68:150–160. [PubMed: 205388]
- Jorgensen CS, Ahrensberg JM, Gregersen H, Flyvberg A. Tension-strain relations and morphometry of rat small intestine in experimental diabetes. *Dig Dis Sci.* 2001; 46:960–967. [PubMed: 11341665]
- Junod A, Lambert AE, Stauffacher W, Renold AE. Diabetogenic action of streptozotocin: relationship of dose to metabolic response. *J Clin Invest.* 1969; 48:2129–2139. [PubMed: 4241908]
- Kaputlu I, Ozdem S, Sadan G, Gokalp O. Effects of diabetes on non-adrenergic, non-cholinergic relaxation induced by GABA and electrical stimulation in the rat isolated duodenum. *Clin Exp Pharmacol Physiol.* 1999; 26:724–728. [PubMed: 10499162]
- Kerlin P, Phillips S. Differential transit of liquids and solid residue through the human ileum. *Am J Physiol.* 1983; 245:G38–G43. [PubMed: 6869546]
- Kilbinger H. Modulation by oxotremorine and atropine of acetylcholine release evoked by electrical stimulation of the myenteric plexus of the guinea-pig ileum. *Naunyn Schmiedeberg's Arch Pharmacol.* 1977; 300:145–151. [PubMed: 593436]
- Kilbinger H, Wessler I. Inhibition by acetylcholine of the stimulation-evoked release of [3H]acetylcholine from the guinea-pig myenteric plexus. *Neurosci.* 1980a; 5:1331–1340.

- Kilbinger H, Wessler I. Pre- and postsynaptic effects of muscarinic agonists in the guinea-pig ileum. *Naunyn Schmiedebergs Arch Pharmacol.* 1980b; 314:259–266. [PubMed: 7231570]
- Klatt P, Schmidt K, Brunner F, Mayer B. Inhibitors of brain nitric oxide synthase. Binding kinetics, metabolism, and enzyme inactivation. *J Biol Chem.* 1994; 269:1674–1680. [PubMed: 7507482]
- Kniel PC, Junker U, Perrin IV, Bestetti GE, Rossi GL. Varied effects of experimental diabetes on the autonomic nervous system of the rat. *Lab Invest.* 1986; 54:523–530. [PubMed: 3702343]
- Kortezova NI, Shikova LI, Milusheva EA, Itzev DE, Bagaev VA, Mizhorkova ZN. Muscarinic modulation of nitrergic neurotransmission in guinea-pig gastric fundus. *Neurogastroenterol Motil.* 2004; 16:155–165. [PubMed: 15086869]
- Kumar A, Attaluri A, Hashmi S, Schulze KS, Rao SS. Visceral hypersensitivity and impaired accommodation in refractory diabetic gastroparesis. *Neurogastroenterol Motil.* 2008; 20:635–642. [PubMed: 18282170]
- LePard KJ. Choline acetyltransferase and inducible nitric oxide synthase are increased in myenteric plexus of diabetic guinea pig. *Auton Neurosci.* 2005; 118:12–24. [PubMed: 15795174]
- Lincoln J, Bokor JT, Crowe R, Griffith SG, Haven AJ, Burnstock G. Myenteric plexus in streptozotocin-treated rats. Neurochemical and histochemical evidence for diabetic neuropathy in the gut. *Gastroenterol.* 1984; 86:654–661.
- Lucas PD, Sardar AM. Effects of diabetes on cholinergic transmission in two rat gut preparations. *Gastroenterol.* 1991; 100:123–128.
- Lundquist I, Sundler F, Hakanson R, Larsson LI, Heding LG. Differential changes in the 5-hydroxytryptamine and insulin content of guinea-pig B-cells. *Endocrinology.* 1975; 97:937–947. [PubMed: 127702]
- Maggs D, MacDonald I, Nauck MA. Glucose homeostasis and the gastrointestinal tract: insights into the treatment of diabetes. *Diabetes Obes Metab.* 2008; 10:18–33. [PubMed: 17490426]
- Maljaars PW, Peters HP, Mela DJ, Masclee AA. Ileal brake: a sensible food target for appetite control. A review. *Physiol Behav.* 2008; 95:271–281. [PubMed: 18692080]
- Mancinelli R, Fabrizi A, Vargiu R, Morrone L, Bagetta G, Azzena GB. Functional role of inducible nitric oxide synthase on mouse colonic motility. *Neurosci Lett.* 2001; 311:101–104. [PubMed: 11567788]
- Martin MT, Azpiroz F, Malagelada JR. Ileal brake failure in streptozotocin-induced diabetic rat. *Scand J Gastroenterol.* 2004; 39:423–427. [PubMed: 15180178]
- Martinez-Cuesta MA, Massuda H, Whittle BJR, Moncada S. Impairment of nitrergic-mediated relaxation of rat isolated duodenum by experimental diabetes. *Br J Pharmacol.* 1995; 114:919–924. [PubMed: 7540094]
- Mathison R, Davison JS. Modified smooth muscle responses of jejunum in streptozotocin-diabetic rats. *J Pharmacol Exp Ther.* 1988; 244:1045–1050. [PubMed: 2908044]
- McNally EF, Reinhard AE, Schwartz PE. Small bowel motility in diabetics. *Am J Dig Dis.* 1969; 14:163–169. [PubMed: 5773913]
- Miller MR, Megson IL. Recent developments in nitric oxide donor drugs. *Br J Pharmacol.* 2007; 151:305–321. [PubMed: 17401442]
- Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev.* 1991; 43:109–142. [PubMed: 1852778]
- Muri JW. Nocturnal diarrhea in diabetes mellitus. *Acta Med Scand.* 1953; 146:143–145. [PubMed: 13091724]
- Murthy KS. Signaling for contraction and relaxation in smooth muscle of the gut. *Annu Rev Physiol.* 2006; 68:345–374. [PubMed: 16460276]
- Nowak TV, Harrington B, Kalbfleisch J. Adaptation of cholinergic enteric neuromuscular transmission in diabetic rat small intestine. *Diabetes.* 1990; 39:891–897. [PubMed: 1973670]
- Ogulener N, Ergun Y, Dondas N, Dikmen A. The influence of nitric oxide donors on the responses to nitrergic nerve stimulation in the mouse duodenum. *Eur J Pharmacol.* 2001; 421:121–131. [PubMed: 11399268]
- Olgart C, Iversen HH. Nitric oxide-dependent relaxation induced by M1 muscarinic receptor activation in the rat small intestine. *Br J Pharmacol.* 1999; 127:309–313. [PubMed: 10369487]

- Ordog T. Interstitial cells of Cajal in diabetic gastroenteropathy. *Neurogastroenterol Motil.* 2008; 20:8–18. [PubMed: 18173559]
- Park MI, Camilleri M. Gastroparesis: clinical update. *Am J Gastroenterol.* 2006; 101:1129–1139. [PubMed: 16696789]
- Pasricha PJ, Pehlivanov ND, Gomez G, Vittal H, Lurken MS, Farrugia G. Changes in the gastric enteric nervous system and muscle: a case report on two patients with diabetic gastroparesis. *BMC Gastroenterol.* 2008; 8:21. [PubMed: 18513423]
- Patel BA, Galligan JJ, Swain GM, Bian X. Electrochemical monitoring of nitric oxide released by myenteric neurons of the guinea pig ileum. *Neurogastroenterol Motil.* 2008; 20:1243–1250. [PubMed: 18694441]
- Patrick A, Epstein O. Review article: gastroparesis. *Aliment Pharmacol Ther.* 2008; 27:724–740. [PubMed: 18248660]
- Peterssen B, Hellerström C, Gunnarsson R. Structure and metabolism of the pancreatic islets in streptozotocin treated guinea pigs. *Horm Metab Res.* 1970; 2:313–317. [PubMed: 5521644]
- Qu XW, Wang HAO, De Plaen IG, Rozenfeld RA, Hsueh WEI. Neuronal nitric oxide synthase (NOS) regulates the expression of inducible NOS in rat small intestine via modulation of nuclear factor kappa B. *FASEB J.* 2001; 15:439–446. [PubMed: 11156959]
- Rayner CK, Horowitz M. New management approaches for gastroparesis. *Nat Clin Pract Gastroenterol Hepatol.* 2005; 2:454–462. [PubMed: 16224477]
- Regalia J, Cai F, Helke C. Streptozotocin-induced diabetes and the neurochemistry of vagal afferent neurons. *Brain Res.* 2002; 938:7–14. [PubMed: 12031529]
- Reiche D, Michel K, Pfannkuche H, Schemann M. Projections and neurochemistry of interneurons in the myenteric plexus of the guinea-pig gastric corpus. *Neurosci Lett.* 2000; 295:109–112. [PubMed: 11090986]
- Robertson DM, Sima AA. Diabetic neuropathy in the mutant mouse [C57BL/ks(db/db)]: a morphometric study. *Diabetes.* 1980; 29:60–67. [PubMed: 6991317]
- Rosztoczy A, Roka R, Varkonyi TT, Lengyel C, Izbeki F, Lonovics J, Wittmann T. Regional differences in the manifestation of gastrointestinal motor disorders in type 1 diabetic patients with autonomic neuropathy. *Z Gastroenterol.* 2004; 42:1295–1300. [PubMed: 15558439]
- Samsom M, Jebbink RJ, Akkermans LM, vanBerge-Henegouwen GP, Smout AJ. Abnormalities of antroduodenal motility in type I diabetes. *Diabetes Care.* 1996; 19:21–27. [PubMed: 8720528]
- Samsom M, Roelofs JM, Akkermans LM, van Berge-Henegouwen GP, Smout AJ. Proximal gastric motor activity in response to a liquid meal in type I diabetes mellitus with autonomic neuropathy. *Dig Dis Sci.* 1998; 43:491–496. [PubMed: 9539642]
- Samsom M, Salet GA, Roelofs JM, Akkermans LM, van Berge-Henegouwen GP, Smout AJ. Compliance of the proximal stomach and dyspeptic symptoms in patients with type I diabetes mellitus. *Dig Dis Sci.* 1995; 40:2037–2042. [PubMed: 7555462]
- Sanger GJ, Alpers DH. Development of drugs for gastrointestinal motor disorders: translating science to clinical need. *Neurogastroenterol Motil.* 2008; 20:177–184. [PubMed: 18257767]
- Sarosiek I, Selover KH, Katz LA, Semler JR, Wilding GE, Lackner JM, Sitrin MD, Kuo B, Chey WD, Hasler WL, Koch KL, Parkman HP, Sarosiek J, McCallum RW. The assessment of regional gut transit times in healthy controls and patients with gastroparesis using wireless motility technology. *Aliment Pharmacol Ther.* 2010; 31:313–322. [PubMed: 19814743]
- Sawyer GW, Ehlert FJ. Contractile roles of the M2 and M3 muscarinic receptors in the guinea pig colon. *J Pharmacol Exp Ther.* 1998; 284:269–277. [PubMed: 9435187]
- Scarpello JH, Greaves M, Sladen GE. Small intestinal transit in diabetics. *Br Med J.* 1976; 2:1225–1226. [PubMed: 990859]
- Scarpello JH, Sladen GE. Diabetes and the gut. *Gut.* 1978; 19:1153–1162. [PubMed: 217803]
- Schlosser MJ, Kapeghian JC, Verlangieri AJ. Effects of streptozotocin in the male guinea pig: a potential animal model for studying diabetes. *Life Sci.* 1984; 35:649–655. [PubMed: 6235425]
- Schlosser MJ, Kapeghian JC, Verlangieri AJ. Selected physical and biochemical parameters in the streptozotocin-treated guinea pig: insights into the diabetic guinea pig model. *Life Sci.* 1987; 41:1345–1353. [PubMed: 2957557]

- Shotton HR, Lincoln J. Diabetes only affects nitric oxide synthase-containing myenteric neurons that do not contain heme oxygenase 2. *Brain Res.* 2006; 1068:248–256. [PubMed: 16375869]
- Spiller RC, Trotman IF, Higgins BE, Ghatei MA, Grimble GK, Lee YC, Bloom SR, Misiewicz JJ, Silk DB. The ileal brake--inhibition of jejunal motility after ileal fat perfusion in man. *Gut.* 1984; 25:365–374. [PubMed: 6706215]
- Surendran S, Kondapaka SB. Altered expression of neuronal nitric oxide synthase in the duodenum longitudinal muscle-myenteric plexus of obesity induced diabetes mouse: implications on enteric neurodegeneration. *Biochem Biophys Res Commun.* 2005; 338:919–922. [PubMed: 16256069]
- Takahashi T. Pathophysiological significance of neuronal nitric oxide synthase in the gastrointestinal tract. *J Gastroenterol.* 2003; 38:421–430. [PubMed: 12768383]
- Takahashi T, Nakamura K, Itoh H, Sima AAF, Owyang C. Impaired expression of nitric oxide synthase in the gastric myenteric plexus of spontaneously diabetic rats. *Gastroenterol.* 1997; 113:1535–1544.
- Talubmook C, Forrest A, Parsons M. Streptozotocin-induced diabetes modulates presynaptic and postsynaptic function in the rat ileum. *Eur J Pharmacol.* 2003; 469:153–158. [PubMed: 12782197]
- Troncon LE, Rosa-e-Silva Oliveira RB, Iazigi N, Gallo L Jr, Foss MC. Abnormal intragastric distribution of a liquid nutrient meal in patients with diabetes mellitus. *Dig Dis Sci.* 1998; 43:1421–1429. [PubMed: 9690375]
- Umathe SN, Kochar NI, Jain NS, Dixit PV. Gastrointestinal dysfunction in diabetic rats relates with a decline in tissue L-arginine content and consequent low levels of nitric oxide. *Nitric Oxide.* 2009; 20:129–133. [PubMed: 19041728]
- Vinik AI, Maser RE, Mitchell BD, Freeman R. Diabetic autonomic neuropathy. *Diabetes Care.* 2003; 26:1553–1579. [PubMed: 12716821]
- Vinnik IE, Kern F Jr, Struthers JE Jr. Malabsorption and the diarrhea of diabetes mellitus. *Gastroenterol.* 1962; 43:507–520.
- Watkins CC, Sawa A, Jaffrey S, Blackshaw S, Barrow RK, Snyder SH, Ferris CD. Insulin restores neuronal nitric oxide synthase expression and function that is lost in diabetic gastropathy. *J Clin Invest.* 2000; 106:373–384. [PubMed: 10930440]
- Whalen GE, Soergel KH, Geenen JE. Diabetic diarrhea. A clinical and pathophysiological study. *Gastroenterol.* 1969; 56:1021–1032.
- Yang R, Arem R, Chan L. Gastrointestinal tract complications of diabetes mellitus. Pathophysiology and management. *Arch Intern Med.* 1984; 144:1251–1256. [PubMed: 6375614]
- Yoshida MM, Schuffler MD, Sumi SM. There are no morphologic abnormalities of the gastric wall or abdominal vagus in patients with diabetic gastroparesis. *Gastroenterol.* 1988; 94:907–914.
- Zandecki M, Vanden Berghe P, Depoortere I, Geboes K, Peeters T, Janssens J, Tack J. Characterization of myenteric neuropathy in the jejunum of spontaneously diabetic BB-rats. *Neurogastroenterol Motil.* 2008; 20:818–828. [PubMed: 18312542]

Figure 1.

Effect of scopolamine on bethanechol-induced contractions. Scopolamine at high doses (300 and 3000 μM) greatly reduced ileal rapid, initial peak contractions and latter, sustained contractions to bethanechol (100 μM) ($^{\dagger}p < 0.05$). In contrast, low doses of scopolamine (3 or 30 μM) increased initial peak contractions of both control and diabetic ilea ($^{\dagger}p < 0.05$) [Main effect: dose; $F(4,206) = 97.169$; $p < 0.05$], but only sustained contractions of control ileum ($^{\dagger}p < 0.05$) [Interaction: control/diabetic*dose; $F(4,206) = 2.667$; $p < 0.05$]. At 30 μM scopolamine, sustained contractions of diabetic ileum were weaker than control ileum ($*p < 0.05$). Control: $n = 8-32$ tissue preparations, Diabetic: $n = 16-48$ tissue preparations. $*p < 0.05$ as compared to control, $^{\dagger}p < 0.05$ as compared to 0 μM scopolamine.

Figure 2.

Representative traces of motility of control and diabetic ilea. Tissues were pretreated with either vehicle, 4-DAMP (1 μ M), hexamethonium (300 μ M), or TTX (3 μ M), then either not treated with any drug (no drug, first column) or treated with neostigmine at the arrow (1 μ M, remaining columns). The arrow indicates the time from which area under the curve was calculated. The scale bars indicate 1 g contraction and 1 min of time with the total trace being 6 min.

Figure 3.

Neostigmine-induced contractions of ilea from control and diabetic animals in the absence and presence of TTX. A. In the absence of TTX, neostigmine-induced contractions of diabetic ileum were significantly stronger than control ileum at 0.3, 0.5, and 1 μ M neostigmine ($*p < 0.05$) [Interaction: control/diabetic*dose; $F(5,543)=3.176$, $p < 0.05$]. Within control ileum, weaker contractions were observed at 30 μ M and 50 μ M as compared to 10 μ M neostigmine ($^{\dagger}p < 0.05$) [Interaction: control/diabetic*dose; $F(5,543)=3.176$, $p < 0.05$]. Control $n=15-40$ tissue preparations, Diabetic $n=23-39$ tissue preparations. B. In the presence of TTX (3 μ M), neostigmine-induced contractions of control and diabetic ilea were similar [Interaction: control/diabetic*dose; $F(3,322)=2.210$; $p=0.087$]. Control $n=15-18$ tissue preparations, Diabetic $n=15-16$ tissue preparations. C. Data from Fig. 3A and 2B were replotted for control ileum. Weaker and stronger neostigmine-induced contractions of control ileum were observed at 0.1 and 1 μ M neostigmine, respectively, in the presence as compared to the absence of TTX ($^{\dagger}p < 0.05$) [Interaction: dose*presence/absence of TTX; $F(3,322)=17.099$; $p < 0.05$]. D. Data from Fig. 3A and 2B were replotted for diabetic ileum. Weaker and stronger neostigmine-induced contractions of diabetic ileum were observed at 0.1 and 1 μ M neostigmine, respectively, in the presence as compared to the absence of TTX ($^{\dagger}p < 0.05$) [Interaction: dose*presence/absence of TTX; $F(3,322)=17.099$; $p < 0.05$].

Figure 4.

Role of muscarinic and nicotinic receptors in neostigmine-induced contractions of ilea from control and diabetic animals. Tissues were pretreated with either hexamethonium (C6, 300 μM) or 4-DAMP (1 μM) then treated with neostigmine (1 μM). Force productions at baseline (Vehicle/-Neostigmine) and after neostigmine treatment (Vehicle/+Neostigmine) are shown in the first and last bars, respectively. Neostigmine-induced contractions in the presence of 4-DAMP or hexamethonium were stronger in diabetic as compared to control ileum (* $p < 0.05$) [Interaction: control/diabetic*drug; $F(2,140)=7.763$, $p < 0.05$]. For both control and diabetic ilea, stronger neostigmine-induced contractions were observed in the presence of hexamethonium, as compared to baseline († $p < 0.05$) and as compared to 4-DAMP/neostigmine (‡ $p < 0.05$) [Interaction: control/diabetic*drug; $F(2,140)=7.763$, $p < 0.05$]. The force produced by neostigmine after 4-DAMP pretreatment was similar to baseline force for both control and diabetic ilea [Interaction: control/diabetic*drug; $F(2,140)=7.763$, $p < 0.05$]. Number in bar indicates number of tissue preparations.

Figure 5.

Histamine-induced initial contractions of ilea from control and diabetic animals. Histamine-induced initial contractions were weaker in ileum from diabetic as compared to control animals [Main effect: control/diabetic; $F(1,141)=5.085$; $p<0.05$]. Control $n=16$ tissue preparations, Diabetic $n=28$ tissue preparations.

Figure 6.

Effect of L-NNA on ileum from control and diabetic animals. L-NNA alone at 3 or 30 μ M contracted ileum from control, but not diabetic, animals [Interaction: control/diabetic*dose; $F(3,247)=5.457$; $p<0.05$]. Number in bar indicates number of tissue preparations. * $p<0.05$ control vs. diabetic, † $p<0.05$ as compared to 0 and 0.3 μ M L-NNA.

Figure 7.

Effect of vehicle or L-NNA on L-arginine or SNP induced immediate relaxations of histamine (30 μ M) contracted ilea from control and diabetic animals. A. Tissues were pretreated with vehicle then treated with increasing concentrations of L-arginine. At 10, 30, and 100 mM, L-arginine was less effective at relaxing ileum from diabetic as compared to control animals ($*p < 0.05$) with minimum effective doses of 3 and 10 mM for diabetic and control ileum, respectively [Interaction: control/diabetic*dose; $F(7,352)=8.073$; $p < 0.05$]. Control $n=6-36$ tissue preparations, Diabetic $n=6-39$ tissue preparations. B. Pretreatment with L-NNA was more effective at increasing L-arginine (10 mM)-induced relaxation of ileum from diabetic as compared to control animals [Main effect: control/diabetic; $F(1,150)=25.290$, $*p < 0.05$]. C. Tissues were pretreated with vehicle, then treated with increasing concentrations of SNP. SNP was 8% more effective (10 μ M) and 10% less effective (300 μ M) at promoting relaxation of ileum from diabetic as compared to control animals ($*p < 0.05$) [Interaction: control/diabetic*dose; $F(7,302)=3.693$; $p < 0.05$]. Control $n=6-24$ tissue preparations, Diabetic $n=16-24$ tissue preparations. D. Pretreatment with L-NNA did not alter SNP (100 μ M)-induced relaxation of control or diabetic ilea [Main effect: dose; $F(2,76)=0.033$, $p=0.967$]. Number in bar indicates number of tissue preparations.

Figure 8.

Effect of vehicle or L-NNA on recovery of control and diabetic ilea from L-arginine or SNP induced relaxations. A. Tissues were pretreated with vehicle, then exposed to increasing concentrations of L-arginine. Control ileum recovered from L-arginine-induced relaxation more completely than diabetic ileum [Main effect: control/diabetic; $F(1,192)=14.138$; $p<0.05$]. At 100 mM L-arginine, relaxation of both control and diabetic ilea was sustained with no recovery observed after 3 min [Main effect: dose; $F(3,192)=34.748$; $p<0.05$]. Control: $n=13$ –32 tissue preparations, Diabetic: $n=21$ –36 tissue preparations. B. Pretreatment with L-NNA improved recovery of both control and diabetic ilea from L-arginine (10 mM)-induced relaxation [Main effect: dose; $F(3,202)=42.060$; $p<0.05$] with control ileum producing greater force than diabetic ileum [Main effect: control/diabetic; $F(1,202)=21.872$; $p<0.05$]. Maximal recovery of control and diabetic ilea was observed at 3 μ M L-NNA [Main effect: dose; $F(3,202)=42.060$; $p<0.05$]. $^{\dagger}p<0.05$ as compared to 0 μ M L-NNA, $^{\ddagger}p<0.05$ as compared to 0.3 μ M L-NNA. Number in bar indicates number of tissue preparations. C. Recovery of control and diabetic ilea from SNP-induced relaxation was similar [Main effect: control/diabetic; $F(1,216)=0.828$; $p=0.364$]. Maximal recovery was observed at 30 μ M SNP [Main effect: dose; $F(6,216)=18.802$; $p<0.05$]. Control: $n=6$ –23 tissue preparations, Diabetic: $n=14$ –23 tissue preparations. D. In the presence of L-NNA, recovery from SNP (100 μ M)-induced relaxation was more complete in control than diabetic ileum [Main effect: control/diabetic; $F(1,78)=17.897$; $p<0.05$] with 30 μ M L-NNA improving recovery of both control and diabetic ilea [Main effect: dose; $F(2,78)=4.737$; $p<0.05$]. $^{\dagger}p<0.05$ as compared to 0 μ M L-NNA. Number in bar indicates number of tissue preparations.

Figure 9.

Cartoon depicting excitatory and inhibitory pathways innervating ileal smooth muscle from control and diabetic animals. **Control.** In the presence of neostigmine, endogenous Ach activated postjunctional muscarinic (M) receptors on smooth muscle, M autoreceptors on cholinergic neurons, and nicotinic receptors on excitatory and inhibitory enteric motor neurons. **Diabetic.** Cholinergic agonists were more effective at activating postjunctional M receptors resulting in stronger contractions. Choline tissue content was increased in ileum reflecting alterations in autonomic innervation by cholinergic intrinsic neurons. Presynaptic M autoreceptors were less effective at blunting endogenous Ach release. Activation of nicotinic receptors on noncholinergic excitatory motor neurons contributed more strongly to neostigmine-induced contraction. Nitric relaxation by NO generated by NOS after activation of nicotinic receptors on inhibitory enteric motor neurons was reduced indirectly contributing to stronger neostigmine-induced contractions. Densities of ChAT-ir and nNOS-ir nerve processes were increased and decreased, respectively, and numbers of total myenteric neurons and nNOS-ir myenteric neurons were decreased and unchanged, respectively, in ileum from diabetic guinea pigs (LePard, 2005). **Legend:** Circle (cell body): grey, vagal preganglionic neuron; black, enteric neuron; white, diabetes-induced decrease in total myenteric cell number. Line (axon with nerve terminal): solid (medium, thin), density or strength of neural innervation; thick, diabetes-induced increase in density; dashed, diabetes-induced decrease in density. Box (receptor): small rectangle, nicotinic; large M square, muscarinic; large white square, ENT. Degree of receptor or enzyme activation: black or dark grey, normal activation; light grey, diabetes-induced decreased activation; upward diagonal, diabetes-induced increased activation. Smooth muscle: grey, myopathy. Oval: enzyme. Abbreviations: AchE: acetylcholinesterase, cGMP: cyclic guanosine

monophosphate, ENT: excitatory neurotransmitter, INT: inhibitory neurotransmitter, NOS: nitric oxide synthase, ↑: increase, ↓: decrease

Table 1

Force, but not frequency, of basal contractions was decreased by 4-DAMP but increased by hexamethonium as compared to vehicle. Data are mean $\pm$ SEM for percent change with negative and positive values representing a decrease and an increase, respectively.

Pretreatment	Frequency		Force	
	control (n=8)	diabetic (n=10-11)	control (n=8)	diabetic (n=11)
Vehicle	2 $\pm$ 6	2 $\pm$ 4	-2 $\pm$ 2	-1 $\pm$ 1
4-DAMP (1 μ M)	-38 $\pm$ 19	-10 $\pm$ 19	-15 $\pm$ 4 [†]	-13 $\pm$ 2 [†]
Hexamethonium (100 μ M)	12 $\pm$ 6	24 $\pm$ 11	9 $\pm$ 3 [†]	8 $\pm$ 4 [†]

[†] p<0.05 within control or diabetic as compared to vehicle.

Table 2

Contraction of control and diabetic ilea after cholinergic receptor activation. Data are mean±SEM for area (g^{*} s)/g tissue wt.

Pretreatment	Treatment	Control	Diabetic
Vehicle	Bethanechol (10 μM)	14.1±2.1 (n=10)	19.9±1.8 [*] (n=17)
4-DAMP (1 μM)	Bethanechol (10 μM)	1.5±0.4 [†] (n=10)	0.6±0.2 [†] (n=17)
Vehicle	Neostigmine (1 μM)	15.8±2.4 (n=10)	19.8±2.2 [*] (n=17)
Neostigmine (0.05–0.5 μM)	Neostigmine (1 μM)	12.5±1.0 (n=40)	17.6±1.5 [*] (n=23)

^{*} p<0.05 control vs. diabetic,

[†] p<0.05 as compared to vehicle/bethanechol.

Table 3

Increasing the dose of hexamethonium did not result in greater inhibition of neostigmine-induced contractions of control or diabetic ileum. Data are mean $\pm$ SEM for area (g^{*} s)/tissue wt (g).

Pretreatment	Treatment	Control	Diabetic
Vehicle	Neostigmine (1 μ M)	15.4 $\pm$ 0.9 (n=34)	21.3 $\pm$ 2.2 [*] (n=17)
Hexamethonium (100 μ M)	Neostigmine (1 μ M)	12.3 $\pm$ 1.7 [†] (n=10)	14.7 $\pm$ 1.8 ^{*†} (n=16)
Hexamethonium (300 μ M)	Neostigmine (1 μ M)	10.1 $\pm$ 1.6 [†] (n=10)	15.5 $\pm$ 1.6 ^{*†} (n=17)

^{*}
p<0.05 control vs. diabetic,

[†]
p<0.05 within control or diabetic as compared to vehicle/neostigmine.

Table 4

Histamine (0.03–30 μ M)-induced initial peak contractions and latter sustained contractions were weaker in diabetic as compared to control animals. Data are mean $\pm$ SEM for contraction (g)/tissue wt (g).

	Control	Diabetic
Initial	53.8 $\pm$ 4.6 (n=73)	48.8 $\pm$ 4.0 * (n=78)
Sustained	59.8 $\pm$ 2.6 (n=29)	42.9 $\pm$ 2.8 * (n=24)

*
p<0.05 control vs diabetic.