Diabetic Complications Consortium

Application Title: Cellular Regulation of Human Proximal Stomach Motor Responses

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1. Project Accomplishments:

All human proximal stomach smooth muscle samples are from the patients of Dr Kent Sasse, undergoing laparoscopic sleeve gastrectomy for obesity. Gender and age data of all patients are on file. We have collected 27 human proximal stomach smooth muscle samples from non-diabetic patients, and 18 human proximal stomach smooth muscle samples from diabetic patients. We have carried out muscle tension studies and western blot studies to establish baseline characteristics of human gastric smooth muscles regarding contractile responses, and the expression levels of contractile and Ca2+ sensitization proteins; since this data is unavailable elsewhere. For Specific Aim 1, we have initially characterized the contractile responses of muscle strips from non-diabetic and diabetic patients to cholinergic stimuli, and the effects of cholinesterase inhibitors on these responses. We are building a database of western blot images for densitometry analysis of the protein expression levels, and the phosphorylation status of contractile and Ca^{2+} sensitization proteins in muscle strips from non-diabetic and diabetic patients. For Specific Aim 2, we have been collecting muscle samples from controls, and from muscles stimulated by cholinergic neurotransmission in the absence and presence of benzbromarone, for western blot analysis of changes in phosphorylation of Ca^{2+} sensitization proteins.

Some of the data we collected was included in my R01DK103675 A1 grant application "Cellular Regulation of Gastric Motility". However, despite the inclusion of innovative, and novel data from *human* proximal stomach smooth muscles, and the clear translational link to the clinically approved pro-kinetic drug Acofide for functional dyspepsia, the application was not discussed.

2. Specific Aims:

Aim 1. Determine the importance of Ca^{2+} sensitization for human gastric fundus smooth muscle contraction by cholinergic neurotransmission. *Hypothesis: The activation of Ca^{2+} sensitization mechanisms by cholinergic neurotransmission is regulated by mechanisms that limit the effective volume of neurotransmitter.*

Aim 2. Evaluate the contribution of loss of ICC function to altered Ca^{2+} sensitization pathways in human gastric fundus smooth muscles. *Hypothesis: The processing of neurotransmitter signals by ICC modulates the activation of Ca^{2+} sensitization mechanisms by cholinergic neurotransmission.*

Aim 1. Determine the importance of Ca^{2+} sensitization for human gastric fundus smooth muscle contraction by cholinergic neurotransmission.

Results: Using proximal stomach smooth muscle strips from resected stomach samples, we carried out myobath tension studies to determine basic contractile properties of human proximal stomach smooth muscles. Despite the gender differences, and wide differences in age, the frequency of spontaneous contractions of the muscle strips was remarkably consistent (3.0 ± 0.5 cycles/min, n=14, P<0.01). We verified that human proximal stomach smooth muscles contract in response to cholinergic neurotransmission as shown in Fig. 1.

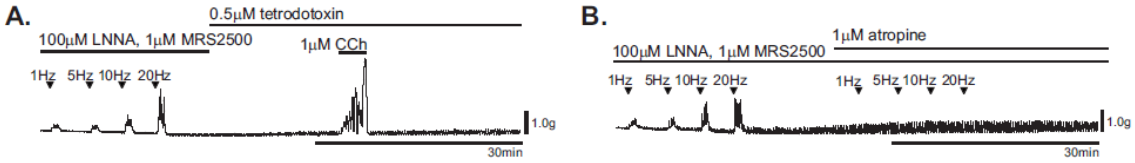

Figure 1. Human proximal stomach smooth muscles contract in response to cholinergic stimuli. A. In the presence of inhibitors of nitrergic and purinergic neurotransmission, different frequencies of electrical field stimulation elicit contractile responses. Exogenously applied carbachol (CCh) elicits contractions. B. The M3 receptor antagonist atropine blocks contractile responses to electrical field stimulation in the presence of inhibitors of nitrergic and purinergic neurotransmission.

We verified that inhibition of acetylcholinesterase activity dramatically increases the force of contractions evoked by electrical field stimulation of cholinergic neurotransmission, as shown in Fig. 2.

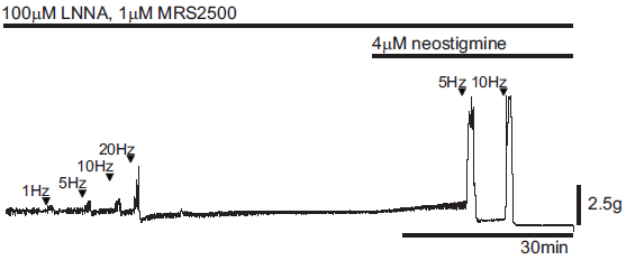

Figure 2. The acetylcholinesterase inhibitor neostigmine increases the contractile response of human proximal stomach smooth muscles to cholinergic neurotransmission. In the presence of inhibitors of nitrergic and purinergic neurotransmission, the contractile responses elicited by different frequencies of electrical field stimulation are increased by neostigmine. n=2.

Also note that the basal level of resting tone, and the amplitudes of spontaneous contractions are increased slightly in the presence of neostigmine. These findings suggest, similar to our findings in murine gastric smooth muscles, that acetylcholine is constitutively released from gastric motor neurons that is degraded by cholinesterase activity.

These experiments are necessary to validate our studies aimed at determining how Ca^{2+} sensitization pathways in human gastric smooth muscles are affected by (i) endogenous cholinergic neurotransmission and exogenously applied cholinergic agonists, and (ii) cholinesterase inhibition. These studies will allow us to compare and analyze the effects of Acofide (acotiamide) on Ca^{2+} sensitization pathways in human gastric smooth muscles.

Acofide relieves the symptoms of functional dyspepsia. Acofide has pro-kinetic actions based on its ability to inhibit cholinesterase, however, the precise cellular and biochemical pathways affected by acotiamide are unknown. Based on our previous findings that neostigmine augments contractile responses and increases CPI-17 and MYPT1 phosphorylation to cholinergic neurotransmission, we hypothesize that acotiamide acts by a similar mechanism. As shown in Fig. 3, similar to neostigmine, acotiamide dramatically increases the force of contractions evoked by electrical field stimulation of cholinergic neurotransmission, of proximal stomach smooth muscle strips from non-diabetic and diabetic patients. Although we have the A1C status of the patients, we have no data as to whether they have been diagnosed with gastroparesis.

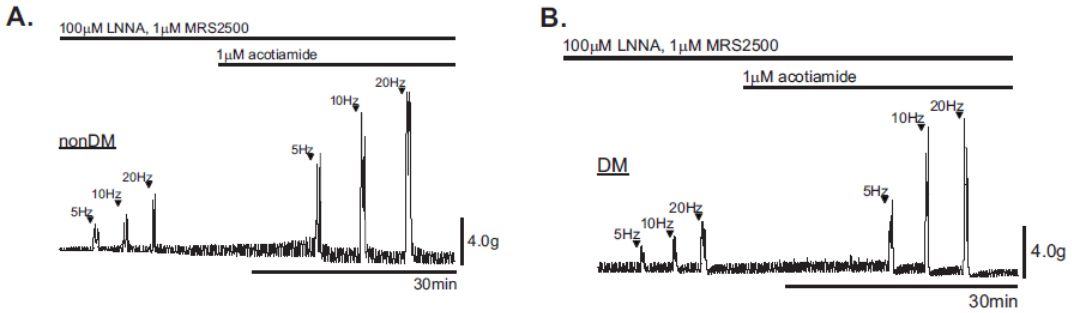

Figure 2. Acotiamide increases the contractile response of human proximal stomach smooth muscles to cholinergic neurotransmission. In the presence of inhibitors of nitrergic and purinergic neurotransmission, the contractile responses elicited by different frequencies of electrical field stimulation are increased by acotimaide. A. Non-diabetic patient. B. Diabetic patient. n=3.

These experiments will allow us to compare the activation of Ca^{2+} sensitization pathways in human proximal stomach smooth muscles in response to cholinergic neurotransmission, in the absence or presence of acotiamide. The goals of these planned experiments are to determine the importance of Ca^{2+} sensitization for human proximal stomach smooth muscle contraction by cholinergic neurotransmission, and to determine whether acotiamide augments the activation of Ca^{2+} sensitization pathways, to provide a possible mechanism for the enhancement of the contractile responses evoked by cholinergic neurotransmission.

CPI-17 and MYPT1 are the major inhibitors of myosin light chain phosphatase in GI smooth muscles. Increases in CPI-17 and MYPT1 phosphorylation by PKC and ROCK, respectively, indicate activation of Ca^{2+} sensitization mechanisms. We are collecting and freezing muscle samples for western blot analysis of CPI-17, MYPT1, and myosin light chain (MLC) phosphorylation. Constitutive phosphorylation levels and phsophorylation levels evoked by cholinergic stimulation from non-diabetic and diabetic patients will be compared. Figure 3 contains representative western blots showing the expression of several key proteins involved in contraction and Ca^{2+} sensitization. Each blot contains proximal stomach smooth muscle lysates from 7 different non-diabetic (nonDM) patients and 7 different diabetic (DM) patients. Figure 3A shows the expression levels of γ-actin and GAPDH; the proteins that we use to normalize the expression of each individual protein. Figure 3B shows the expression of CPI-17, and the constitutive phosphorylation levels of CPI-17 T38. RhoA is a critical activator of ROCK, resulting in MYPT1

phosphorylation. RhoA activity is regulated by (i) guanine nucleotide dissociation inhibitors (GDIα), which sequester RhoA as inactive GDP-RhoA; (ii) guanine nucleotide exchange factors (GEFs), which activate RhoA by ejecting GDP, and allowing GTP to bind to and activate RhoA; and (iii) GTPase activating proteins (GAPs), which inactivate RhoA by stimulating GTP hydrolysis. Figure 3C shows the expression of GDIα, GEFT, and ARHGAP42. GEFT, or p63RhoGEF (ARHGEF25), is a GEF that is a major target of Gαq in smooth muscles, providing a mechanism for Ca^{2+} dependent activation of ROCK. ARHGAP42 is a Rho-specific GAP expressed specifically in smooth muscle cells. Figure 3D shows the expression of myosin light chain (MLC), and the constitutive phosphorylation levels of MLC S19. Finally, Fig. 3E and 3F show the expression levels of RhoA, ROCK2, MYPT1, and the constitutive phosphorylation levels of MYPT1 T853 and T696. These findings provide the foundation for our studies of changes in RhoA activity, and CPI17, and MYPT1 phosphorylation in response to cholinergic stimuli.

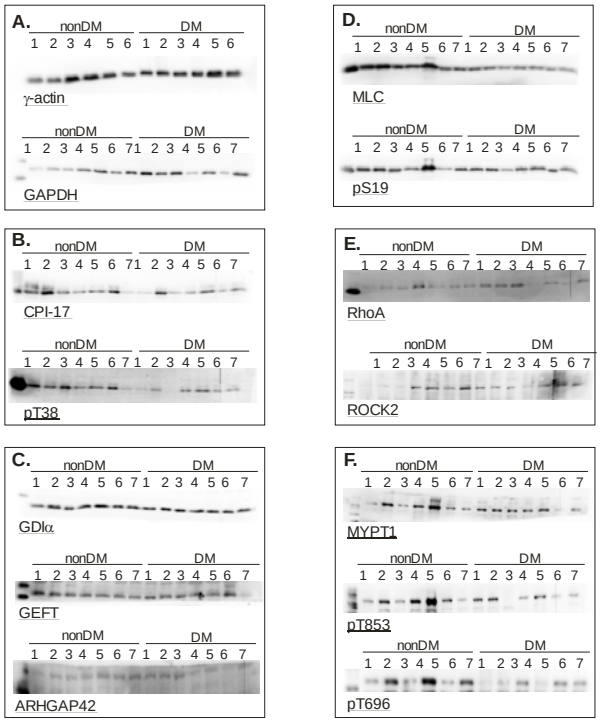

Figure 3. Representative western blots of protein expression and constitutive phosphorylation in proximal stomach smooth muscle lysates from non-diabetic (nonDM) and diabetic (DM) patients.

Specific Aim 2. Evaluate the contribution of loss of ICC function to altered Ca^{2+} sensitization pathways in human gastric fundus smooth muscles.

Results: we have been collecting muscle samples from controls, and from muscles stimulated by cholinergic neurotransmission in the absence and presence of benzbromarone, for western blot analysis of changes in phosphorylation of Ca^{2+} sensitization proteins.

3. Publications:

None