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# HYDROGEN SULFIDE IMPROVES ABERRANT GASTRIC SMOOTH MUSCLE FUNCTION IN DUCHENNE MUSCULAR DYSROPHY MICE

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**HYDROGEN SULFIDE IMPROVES ABERRANT GASTRIC SMOOTH  
MUSCLE FUNCTION IN DUCHENNE MUSCULAR DYSROPHY MICE**

Thesis submitted in partial fulfillment of the requirements for the degree of  
Master of Science at Virginia Commonwealth University.

by

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## **ABSTRACT**

Deficiency of dystrophin, a cytoskeletal protein localized in the inner face of the plasma membrane in skeletal, cardiac and smooth muscle, results in Duchenne Muscular Dystrophy (DMD). Through its interactions with extracellular matrix and plasma membrane proteins, dystrophin plays a role in contraction and signal transduction. In DMD, gastrointestinal disorders such as gastric dilation and intestinal pseudo-obstruction resulting from altered motility have been reported. The role of dystrophin in the regulation of contractile protein expression and smooth muscle function, per se, is not known. Studies have suggested that inflammation contributes to the pathophysiology of DMD. Exogenous H<sub>2</sub>S had been shown to exert beneficial cardiovascular and gastrointestinal functions, probably via exerting anti-inflammatory actions. Aim. To test the hypothesis that a lack of dystrophin causes a decrease in contractile protein expression and smooth muscle function and that treatment with H<sub>2</sub>S restores the effects of dystrophin deficiency. Methods. The role of dystrophin was examined using mice deficient in dystrophin alone (*mdx*) and mice deficient in dystrophin plus telomerase RNA (*mdx/mTR*), which exhibit increased disease severity. The effect of an orally-active, slow releasing H<sub>2</sub>S agent (SG1002) was tested in *mdx/mTR* mice (40 mg/kg body weight in chow/every 3 days starting from 3 weeks to 9 months). Contraction in response to acetylcholine (ACh) was measured in gastric muscle strips isolated from *mdx*, *mdx/mTR* and SG1002-treated *mdx/mTR* mice. Age-matched control mice were used for each group. Contraction was also measured in muscle cells isolated from the stomach of control and *mdx* mice by scanning micrometry and expressed as the percent decrease in muscle cell length. Expression of mRNA and

contractile proteins such as smoothelin, caldesmon, calponin and tropomyosin was measured by qRT-PCR and western blot. Results. Acetylcholine-induced contraction was reduced in muscle strips from *mdx/mTR* mice ( $18 \pm 4$  mN/100 mg tissue) compared to age-matched 9-month old control mice ( $33 \pm 7$  mN/100 mg tissue). Treatment of *mdx/mTR* mice with SG1002 restored contraction to above normal levels ( $56 \pm 8$  mN/100 mg tissue). Contraction was also decreased in gastric muscle strips from *mdx* mice ( $24 \pm 5$  mN/100 mg tissue) compared to age-matched 3-month old control mice ( $45 \pm 4$  mN/100 mg tissue). Both  $\text{Ca}^{2+}$ -dependent initial contraction and  $\text{Ca}^{2+}$ -independent sustained contraction was also decreased in isolated gastric muscle cells from *mdx* mice compared to control mice. Conclusion. The results support our hypothesis that dystrophin deficiency reduces smooth muscle contraction. Furthermore, treatment with  $\text{H}_2\text{S}$  restores gastric smooth muscle function and contractile mRNA expression suggesting therapeutic potential of  $\text{H}_2\text{S}$  in the treatment of motility disorders in DMD.

## CHAPTER 1: INTRODUCTION

### ***Duchenne Muscular Dystrophy***

Duchenne Muscular Dystrophy (DMD) is an X-linked recessive disease that causes rapid skeletal muscle degeneration resulting from mutations in the dystrophin gene. Dystrophin, a trans-sarcolemmal protein, plays an integral role in maintaining membrane integrity by protecting the cell from contraction-induced damage (Manning et al., 2016). The dystrophin gene is the largest identified gene in humans and is comprised of 79 exons located on the short arm of chromosome Xp21 (Rodrigues et al., 2016). The protein product of this gene is known to be around 400 kDa. In DMD, 65–70% mutations are intragenic deletions, which most commonly occur in a region on the DMD gene spanning exons 45–53. Duplications are found in only 7% of patients, and point mutations or small deletions/insertions are observed in a small number of patients (Yiu, 2015). DMD patients suffer from progressive loss of muscle function, leading to paralysis and death in the third decade of life (Sacco et al., 2010)

Studies have shown that the failure of muscle stem cells, also known as satellite cells, to maintain the damage-repair cycle can also contribute to DMD progression. Myocyte regeneration is unable to keep up with constant myocyte damage leading to the replacement of myocytes with fibrotic and adipose tissue. It has been observed that patients with DMD exhibit skeletal muscle weakness by 3 or 4 years of age. The deposition of fibrotic and adipose tissue may lead to pseudohypertrophy of the calves. Children with DMD may have an unusual walk or waddling gait. By the age 13, children with DMD use a wheelchair. Cardiac and respiratory muscle damage eventually leads to serious, life threatening complications by the mid-twenties (Sacco et al., 2010).

## ***The Role of Dystrophin***

Dystrophin is a large sarcolemmal protein located in a membrane-spanning protein complex. Dystrophin links the inner cytoskeleton to the extracellular matrix in skeletal, cardiac and smooth muscle. The majority of dystrophin is produced in skeletal, smooth and cardiac muscle but a small portion can also be found in nerve cells. Dystrophin has an N-terminal domain, a rod-shaped domain, cysteine rich domain and C-terminal domain. The amino terminal domain of dystrophin binds to F-actin, forming a link with internal cytoskeleton. The function of the cysteine rich and carboxyl terminal domains is to bind to the dystrophin-glycoprotein complex (DGC), a protein complex found at the plasma membrane of myofibers. In the presence of dystrophin, the DGC is able to maintain sarcolemmal integrity by serving as a signaling center and a buffer or shock absorber to reduce damage resulting from contraction (Rodrigues et al, 2016).

In DMD the link between the cytoskeleton and the ECM is lost leaving the sarcolemma unable to endure the stress of normal muscle contractions (Lovering et al, 2005). Additionally, the lack of dystrophin triggers membrane deregulation due to the disruption of the dystrophin glycoprotein complex (DGC), a membrane-spanning complex that plays a crucial role in signal transduction, including nitric oxide (NO) signaling. Dystrophin is associated with the DCG and this interaction is thought to play a protective role during muscle contraction. Dystrophin is also needed for binding nitric oxide synthase (NOS), a part of the DCG protein complex, to the inner surface of the sarcolemma. In both DMD patients and in *mdx* mice, an animal model for DMD, it has been established that the sarcolemma is lacking NOS (Vannucchi et al, 2011). Furthermore, DMD has been associated with aberrant cell signaling, Ca<sup>2+</sup> influx due to

increased membrane permeability, mitochondrial dysregulation leading to increased oxidative stress, inflammation, recurrent muscle ischemia and even fibrosis of smooth muscle (Mulè and Serio, 2002).

### ***Anatomy and Function of the Gastrointestinal Tract***

The main functions of the GI tract include absorbing nutrients after digestion, secreting enzymes to aid in digestion and absorption of nutrients. The large surface area of the GI tract allows for optimal nutrient absorption. The GI tract begins with the esophagus. The chief functions of the esophagus are to propel the food bolus from the mouth to the stomach and also the lower esophageal sphincter will prevent reflux of gastric contents into the esophagus. Once the bolus passes into the stomach, it is mixed with HCl for further breakdown. The function of gastric contraction is to grind and mix food to produce chyme, a liquefied state of partially digested food. Chyme is then passed through the small intestine (duodenum) to the jejunum and then the ileum. At this point most of the nutrient absorption has already occurred in the small intestine. The remaining chyme enters the colon ending in the rectum where it can be excreted from the anus as feces (Meerveld et al, 2017)

The GI tract is divided into four layers interconnected by connective tissue, neural and vascular elements. Facing the intestinal lumen is the first and innermost layer known as the Mucosa. This is where the majority of absorption occurs and it is comprised of the innermost single layer epithelium, a middle lamina propria comprised of connective tissue and lymph nodes, and an outer muscularis mucosa. The second layer is the submucosa, which contains lymphatics, inflammatory cells, autonomic nerve

fibers and ganglion cell. The next layer is the muscularis propria, which functions to produce peristaltic waves that propel food through the GI tract. The outer most layer is the known as the serosa and serves as a protective layer by forming a natural barrier from the spread of inflammation (Roa and Wang, 2010).

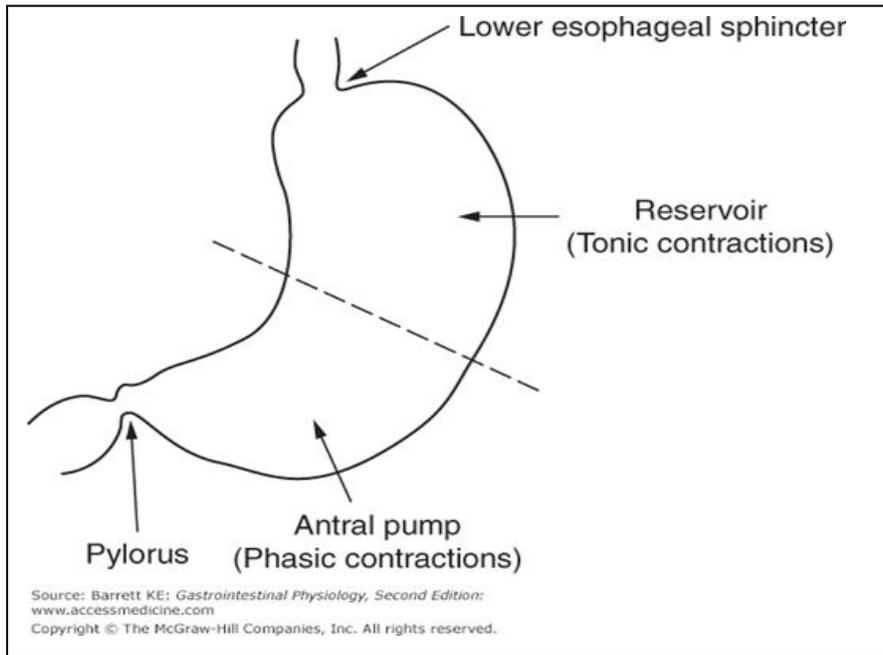
### **Gastric Motility**

The major functions of the stomach include grinding and mixing ingested food with secretions to produce chyme, which can be passed into the small intestine. The stomach can be divided into 3 major regions: the fundus, corpus and antrum (Cooke, 1975). The fundus is the most proximal portion of the stomach and is responsible for the basal tone present in the stomach. Upon ingestion of food, the fundus will relax so that food may enter the GI tract. This drop in gastric pressure immediately after eating is known as receptive relaxation. Receptive relaxation requires vagal input, vago-vagal reflexes, and signals from the enteric nervous system (ENS) (Barrett, 2014). The fundus also displays sustained low frequency, tonic contractions which are responsible to gastric emptying (Cooke, 1975).

The antrum is located in the distal portion of the stomach and displays phasic contractions and strong peristaltic waves, which function largely to grind up the ingested meal by generating a pressure gradient from the stomach to small intestine. In humans, these synchronized waves occur three times every 60 seconds. Interstitial cells of Cajal (ICC) play an important role in the generation of slow waves that determines these 3 cycles per minute synchronized wave activity. Lastly, the pylorus functions as a

sphincter that controls the movement of partially digested food into the small intestine (Barrett, 2014).

ENS and CNS regulation in addition to a complex network of hormones, autocrine/paracrine agents, and other local chemical signals work together to maintain proper gastric motility. The stomach is innervated with both intrinsic and extrinsic neural inputs. The enteric nervous system (ENS) serves as intrinsic control and is required for gastric motility. The parasympathetic and sympathetic control serves as the extrinsic neural input, parasympathetic being more prominent. Sympathetic innervation, which occurs at a much lesser degree, mostly functions to decrease gastric motility. The control of gut motility requires interaction of regulatory systems at all levels including CNS, ENS, interstitial cells of Cajal (ICC) and smooth muscle cells (Manning et al., 2016).



**Figure 1.** This figure provides a schematic of the stomach, specifically displaying the location of phasic contraction and tonic contraction (Barrett, 2014).

### ***GI Motility in mdx Mice***

Along with many skeletal muscle complications, many GI related symptoms have also been observed in DMD. In patients, it has been observed that the absence of dystrophin can lead to GI abnormalities such as nausea, vomiting, delayed gastric emptying, hypo-motility, chronic constipation and even pseudo-obstruction (Manning et al., 2016). When looking at *mdx* mice, diminished GI NO production/function has been reported. NO is produced by neurons and smooth muscle cells in the mammalian stomach and is thought to play a major role gastrointestinal relaxations (Vannucchi et al., 2011). Consequently, significant alterations of the relaxation in the gastric fundus were observed in *mdx* mice when compared to WT. Furthermore, the *mdx* gastric strips develop a major spontaneous tone while the WT mouse's production of sufficient NO in the stomach restricts the level of spontaneous tone (Mulè et al., 2002).

It has also been indicated that while the length of the intestine in both *mdx* and WT mice was very similar, a delay in intestinal transit time and decreased fecal output was observed in the *mdx* mouse (Mulè et al., 2010), suggesting there are significant alterations in *mdx* mice intestines in in vivo conditions. Although, the gastric emptying of a non-nutrient meal consisting carboxymethyl cellulose/phenol red was not significantly different in control and *mdx* mice (Mulè et al., 2010), in DMD patients gastric emptying time was significantly delayed when compared to controls (Borrelli et al., 2005). However, the mechanisms behind the delayed gastric emptying are not known. Specifically, how smooth muscle contractile proteins are affected and what role they are playing in dystrophin deficient smooth muscle is not known. The decrease in electrical field stimulation (EFS)-induced relaxation in carbachol-precontracted fundus

muscle strips from *mdx* mice compared to normal mice was attributed to the impaired nitrergic neurotransmission (Baccari et al., 2010). In addition, the full-length isoform of dystrophin is also expressed in ICC suggesting a dysfunctional ICC and slow wave activity (Vannucchi et al., 2004).

### ***Gastric Smooth Muscle***

Smooth muscle makes up the walls of hollow organs and tracts such as the arteries and veins of the circulatory system, the respiratory, urinary, GI and reproductive systems. Smooth muscle is differentiated from skeletal muscle, in part, due to the lack of striations in smooth muscle. Smooth muscles cells are spindle shaped and mononucleated. Smooth muscle, unlike skeletal muscle, is not organized into sarcomeres. The majority of the smooth muscle cells are comprised of dense bodies, homologous to the Z-lines present in striated muscle that attach to the plasma membrane, and contractile filaments (Gash and Varacallo, 2018).

### ***The Contractile Apparatus in Smooth Muscle***

The principal contractile apparatus in smooth muscle is comprised of thin filaments, thick filaments and intermediate filaments. Intermediate filaments provide a linkage between cytoplasmic dense bodies and dense bands on the plasma membrane. Thin filaments consist of ~42 kDa protein actin, which exists in vivo as filamentous actin (F-actin). Thin filament associated proteins such as smoothelin, tropomyosin, caldesmon and calponin also play a role in the regulation of contraction. Thick filaments are made of myosin molecules, hexamers comprising of a pair of myosin heavy chains

and two pairs of myosin light chains (MLCs). Each of the heavy chains are asymmetrical, containing a globular head at the N-terminus, and form an  $\alpha$ -helical core or tail by coiling around each other. The globular head on the myosin filament is where the ATP binding site, actin binding site and intrinsic ATPase activity is located. The interaction of actin with the myosin head and following hydrolysis of ATP is the fundamental reaction whereby cross bridge cycling and contraction occur generating force as a result of the sliding of the thin and thick filaments (Makhlouf and Murthy, 2015).

### ***Regulation of Smooth Muscle Contraction by Thin-Filament Associated Proteins***

Thin filament associated proteins such as tropomyosin, calponin, caldesmon and smoothelin also play an important role in modulation of smooth muscle contraction by regulating actomyosin interaction (Lovering et al., 2005).

**Caldesmon:** The Caldesmon gene is located on chromosome 7 and is comprised of at least 15 exons (Huber et al., 1997). Caldesmon is a protein that can be found as two isoforms: the smooth muscle specific h-caldesmon (heavy molecular weight) and the non-smooth muscle specific lower molecular weight l-caldesmon. H-caldesmon consists of three main domains. The C-terminus contains two actin-binding sites, which block actin-myosin binding and disrupt actomyosin ATPase activity. It also has a calmodulin (CaM) binding site. The myosin binding N-terminus can also weakly bind to actin, tropomyosin and CaM (Eves, 2006). Lastly, h-Caldesmon contains a spacer region, giving it a larger molecular weight than its isoform, which is not known to strongly bind to actin or myosin. The main function of caldesmon is to tether the actin

thin filaments to myosin thick filaments. Upon smooth muscle stimulation, caldesmon is removed from the actin binding sites and phosphorylated myosin heads are able to bind to stimulate contraction. (Wang, 2001)

**Tropomyosin:** Tropomyosin is a coiled-coil spanning seven-actin monomers. It exists as a heterodimer and is mostly made of  $\alpha$  -helices. Only two isoforms appear to be specific to smooth muscle—one from the  $\beta$  Tm gene and the other from the  $\alpha$  Tm gene. The N-terminus of tropomyosin must be phosphorylated in order for it to have a good binding affinity to actin. As previously discussed, caldesmon inhibits actomyosin ATPase activity and motility. Studies have shown this effect of caldesmon is potentiated in the presence of tropomyosin-actin complex. In the absence of tropomyosin, caldesmon has weaker inhibitory effects on smooth muscle contraction (Marston, 2008). However, the exact mechanism of interaction between tropomyosin and caldesmon is not known (Marston and El-Mezgueldi, 2008).

**Calponin:** Calponin is another smooth muscle specific thin filament associated contractile protein and exists as three isoforms; smooth muscle specific basic h1-calponin, neutral h2-calponin found primarily in cardiac muscle, and an acidic variant which is not tissue-specific. It is able to bind actin,  $Ca^{2+}$ /calmodulin complex and tropomyosin. Like many of the previously discussed proteins, calponin works to inhibit the actomyosin ATPase. Protein kinase C (PKC) or CaM kinase II mediated phosphorylation inhibits these effects of calponin. In response to contractile stimuli such as  $Ca^{2+}$ , calponin gets phosphorylated which allows for cross bridge cycling. Upon dephosphorylation, calponin can reactivate and portray its inhibitory effects on smooth muscle contraction (Winder, 1993).

**Smoothelin:** Smoothelin A and B are both generated from the same gene located on chromosome 22. Smoothelin A is visceral smooth muscle specific and smoothelin B is vascular smooth muscle specific. Smoothelin is an actin binding protein that participates in smooth muscle contraction. The exact mechanism of action is unknown although studies have shown smoothelin knockout mice exhibited decreased contraction in response to contractile agonists. Another study was conducted to examine the effect of a smoothelin A/B knockout on intestinal smooth muscle in mice. The results demonstrated less intestinal flexibility and increased intestinal fragility. Circular and longitudinal muscle in the intestine of smoothelin KO mice showed hypertrophy. Additionally, there was a decrease in intestinal muscle contraction in the absence of smoothelin when compared to the wild type mice (Niessen et al, 2005)

### ***Signaling Mechanisms in Smooth Muscle Contraction***

Additionally, the excitation-contraction coupling mechanism in smooth muscle differs from skeletal muscle as well, including the mechanism that allows  $\text{Ca}^{2+}$  to enter the cell. The 3 mechanisms that increase intracellular concentration are voltage-gated  $\text{Ca}^{2+}$  channels activated by membrane depolarization; the second method is hormones or neurotransmitters binding to and opening ligand-gated channels on the cell membrane allowing  $\text{Ca}^{2+}$  influx and finally, hormones and neurotransmitters can act via the phospholipase-C pathway leading to an increase in intracellular inositol triphosphate (IP<sub>3</sub>) which can signal downstream to cause  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum, intracellular store of  $\text{Ca}^{2+}$  (Webb, 2003).

Once  $\text{Ca}^{2+}$  is released, it binds to a protein called calmodulin. This Ca/calmodulin complex functions to activate the myosin light chain kinase (MLCK), which phosphorylates the myosin light chain. Once phosphorylated, the myosin light chain hydrolyzes ATP due to its myosin- $\text{Mg}^{2+}$ ATPase activity, increasing the myosin light chain's affinity to actin allowing for myosin to bind actin and providing energy for cross-bridge cycling. When looking at the GI tract specifically,  $\text{Ca}^{2+}$ /calmodulin-dependent myosin light-chain kinase (MLCK) phosphorylation of Ser19 on the 20-kDa regulatory light chain of myosin II ( $\text{MLC}_{20}$ ) is vital for initiating smooth muscle contraction (Patel et al, 2006). However this initiation of  $\text{Ca}^{2+}$  dependent contraction and MLCK activity is transient and short-lived. Sustained contraction and  $\text{MLC}_{20}$  phosphorylation only occurs through the inhibition of MLC phosphatase (MLCP) is known as the  $\text{Ca}^{2+}$  independent phase (Murthy, 2006). The small G protein RhoA and its downstream target Rho kinase play an imperative part in the regulation of MLCP activity. MLCP inhibition occurs via two RhoA-dependent pathways. The first one involves by Rho kinase dependent phosphorylation of MYPT1, the regulatory subunit of MLC phosphatase. The second mechanism involves PKC dependent phosphorylation of the MLCP inhibitor, CPI-17 (Patel et al, 2006) (Seko et al, 2003).

### ***Signaling Mechanisms in Smooth Muscle Relaxation***

In order for smooth muscle to relax a reduced intracellular  $\text{Ca}^{2+}$  concentration and up-regulation of MLCP's dephosphorylating activity is required. Relaxation of the GI smooth muscle is induced through inhibitory neurotransmitters through the generation of cAMP and cGMP and activation of cAMP dependent protein kinase (PKA) or cGMP-

dependent protein kinase (PKG). The kinases, PKA and PKG, act downstream to decrease intracellular  $\text{Ca}^{2+}$  levels leading to dephosphorylation of MLC20 and eventually relaxation of smooth muscle. An example of these inhibitory neurotransmitters is nitric oxide (NO), which acts through the cGMP pathway. NO formed in nerve terminals modulates VIP (vasoactive intestinal peptide) release. VIP functions to stimulate eNOS (nitric oxide synthase III) to generate NO in endothelial cells which then diffuses to smooth muscle. NO in smooth muscle cells stimulates the cGMP signaling cascade to cause relaxation of smooth muscle (Murthy, 2006).

### ***Excitation-Transcription Coupling***

Calcium ( $\text{Ca}^{2+}$ ) signals affect not only contraction, but also mediate gene transcription in smooth muscle and many other physiological processes.  $\text{Ca}^{2+}$ -cyclic AMP response element binding protein (CREB) and nuclear factor of activated T-cells (NFAT) are two of the most well studied  $\text{Ca}^{2+}$ -regulated smooth muscle transcription factors. Studies have presented many commonalities and differences in the regulation of CREB and NFAT through  $\text{Ca}^{2+}$  influx leading to expression of smooth muscle cell specific differentiation and proliferation markers.

CREB controls transcription through binding to  $\text{Ca}^{2+}$ -cyclic AMP (cAMP)-response elements (CREs), which are located in the promoter of several genes. In order for activation and recruitment of CREB binding protein (CBP300) to form an active transcriptional complex, phosphorylation of <sup>133</sup>serine must occur. Depolarization-mediated CREB phosphorylation has been associated with increased transcription of *c-fos*, the CRE-containing immediate early gene, and has also been found to be impacted

by inhibitors of voltage gated  $\text{Ca}^{2+}$  channels. However, every  $\text{Ca}^{2+}$  signal does not lead to activation of CREB.  $\text{Ca}^{2+}$  released from ryanodine receptors (RyR) has been shown to have an inhibitory effect on CREB activation. These findings propose the nature of the  $\text{Ca}^{2+}$  signal has great significance on the downstream changes in gene transcription mediated by CREB.

Likewise, NFAT has also recently been indicated to play an essential role in in the regulation of excitation-transcription coupling in smooth muscle. Upon increased intracellular  $\text{Ca}^{2+}$  levels, calcineurin, the  $\text{Ca}^{2+}$ -CaM-dependent phosphatase, dephosphorylates NFAT which translocates the NFAT-calcineurin complex into the nucleus. NFAT translocation and transcriptional activity has been distinctly established in both cultured and native smooth muscle cells.

The serum response factor (SRF) DNA binding site or CArG box (CC[A/T]<sub>6</sub>GG) has also been shown to have an imperative role in regulating smooth muscle specific genes. Voltage gated Ca channels allow for  $\text{Ca}^{2+}$  influx upon depolarization which stimulates expression of SRF-dependent smooth muscle differentiation markers. This mechanism involves both Rho kinase and the SRF coactivator myocardin. The clearly suggests co-activators as well as CREB and NFAT have the capacity to direct expression of SRF-regulated genes in response to  $\text{Ca}^{2+}$  signaling in smooth muscle cells (Barlow et al., 2006.)

## ***Pathogenesis in DMD***

The primary defect of DMD is well established. However, the mechanisms that initiate pathogenesis have not been clearly characterized. Plasma membrane fragility and damage due to mechanical stretch are important factors facilitating diseases, but these mechanisms do not fully explain the onset and progression of the disease. Altered intracellular signaling and augmented inflammation and immune response appear to contribute to the disease onset and progression. Previous studies have shown that calcium plays a significant role in DMD. Without dystrophin, the plasma membrane becomes more susceptible to contraction-induced damage resulting in tears and breaks in the membrane. This leads to an increased membrane permeability and an influx of calcium ( $\text{Ca}^{2+}$ ) through these breaks.  $\text{Ca}^{2+}$  will travel into the myocyte and bind to  $\text{Ca}^{2+}$  dependent proteases.  $\text{Ca}^{2+}$  dependent proteases are thought to accelerate myocyte death when coupled with DMD.  $\text{Ca}^{2+}$  can also lead to mitochondrial dysfunction causing a release of reactive oxygen species (ROS). The exact process underlying this mechanism is not well known however, elevated levels of ROS and oxidative stress have been associated in the pathophysiology of DMD (Choi et al., 2015). Inflammatory gene expression profile in *mdx* muscle is closely similar (~70%) to that observed in DMD patients. Treatment of *mdx* mice with the anti-oxidant N-acetyl cysteine decreased oxidative stress and protected the muscle fiber from stretch-induced damage (Hori et al., 2011). In addition, currently available treatment options for DMD are glucocorticoids and the most the significant effect of glucocorticoids is to inhibit expression of inflammatory genes.

## ***Hydrogen Sulfide (H<sub>2</sub>S)***

Historically, hydrogen sulfide (H<sub>2</sub>S) has been known as a deadly and toxic gas. However, H<sub>2</sub>S has recently been accepted into the family of endogenously produced gasotransmitters which includes nitric oxide (NO) and carbon monoxide (CO) (Hartle and Pluth, 2016). The molecular weight of hydrogen sulfide (H<sub>2</sub>S) is 34.076 g/mol and is known to have a strong odor of rotten eggs. The boiling point of the compound is -60.02 degrees celcius and the melting point is 82.3 degrees Celsius. The vapor density for H<sub>2</sub>S is 1.19 (Wang, 2012). H<sub>2</sub>S can be enzymatically generated from cysteine metabolism by the three enzymes cystathionine  $\gamma$ -lyase (CSE), cystathionine- $\beta$ -synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST) / cysteine aminotransferase (CAT) (Hartle and Pluth, 2016). It has been demonstrated in vascular smooth muscle cells, where hydrogen sulfide is mainly synthesized by CSE, whereas it is predominantly synthesized by CBS in the central nervous system and by 3-MST in cardiac tissues (Kamoun, 2004).

H<sub>2</sub>S levels ranging from 50-160 micromolar have been observed in some mammals and even the human brain (Wang, 2012) implying physiological importance. H<sub>2</sub>S has been shown to have anti-oxidant, anti-inflammatory, vasorelaxant and many other physiological properties (Hu et al., 2010) (Kamoun, 2004). Hydrogen sulfide can also act as a neuromodulator by mediating NMDA receptor responses and also possesses vasorelaxant properties in smooth muscle by enhancing the effects of nitric oxide by up to 13-fold. (Kamoun, 2004). When specifically looking at the GI tract, H<sub>2</sub>S has been shown to increase gastric mucosal blood flow and also support maintenance of gastric mucosal integrity in rats treated with nonsteroidal anti-inflammatory drugs

(NSAIDs), which are known to compromise gastric mucosal blood flow (Fiorucci, et al, 2006).

***H<sub>2</sub>S as an Antioxidant:*** Hydrogen sulfide (H<sub>2</sub>S) exerts a wide range of physiological and cytoprotective functions such as neuroprotection, cardioprotection and antihypertension (Xie et al, 2016). Specifically, the role of H<sub>2</sub>S in oxidative stress has gained a lot of attention. H<sub>2</sub>S is a strong reducing agent that interacts with reactive oxidative species. This effect contributes to the antioxidant role of H<sub>2</sub>S. It is plausible that endogenous H<sub>2</sub>S can scavenge reactive oxygen species to protect against oxidative stress. However, studies demonstrate that instead of functioning directly as an antioxidant, H<sub>2</sub>S increases intracellular reduced glutathione (GSH) levels to protect neurons from oxidative stress. GSH is a very potent antioxidant that serves many protective functions such as shielding neurons from glutamate toxicity. Endogenous levels of GSH are 1–8 mM while those of H<sub>2</sub>S are 50–160 μM. Consequently, due to these low levels of H<sub>2</sub>S, H<sub>2</sub>S itself cannot alleviate cells of oxidative stress and must stimulate production of the more abundant antioxidant, GSH (Kimura and Kimura, 2004).

H<sub>2</sub>S exhibits a cardioprotective role, through its antioxidant properties, on the cardiovascular system specifically when looking at heart failure. S-propargyl-cysteine (SPRC), a modulator of endogenous hydrogen sulfide levels, is designed to act by stimulating the activity of cystathionine-γ-lyase (CSE), which as mentioned previously, is a metabolic enzyme that produces hydrogen sulfide. Studies have shown that administration of SPRC in rats with chronic heart failure conserved the level of antioxidant molecules such as GSH and also preserved cardiomyocyte membrane

integrity, preventing the leakage of metabolic enzymes such as creatine kinase (CK). This study demonstrates that the administration of H<sub>2</sub>S as SPRC in myocardium may exhibit a cardioprotective role through maintaining the balance of oxidative stress associated with the development of heart failure. (Huang et al., 2013).

H<sub>2</sub>S may also exhibit a gasoprotective role through its antioxidative properties. The antioxidant function in the GI tract of H<sub>2</sub>S can be mediated by increasing the expression of enzymes such as superoxide dismutase, glutathione peroxase dismutase, and thioredoxin (Wallace et al, 2012). In previous studies, NaHS, an H<sub>2</sub>S donor was shown to increase the expression of potent antioxidative enzymes such as superoxide dismutase and glutathione peroxase dismutase. Moreover, there was a significant reduction in intestinal ischemia-reperfusion injury and also decreased intestinal MDA activity, which is a marker of radical injury, in rats (Liu et al., 2009). In addition to its role in the intestine, endogenous H<sub>2</sub>S also protects against oxidative stress in gastric ischemia-reperfusion injury in a similar manner (Cui et al., 2012).

***Regulation of smooth muscle function by H<sub>2</sub>S:*** While gastric mucosa expresses both CSE and CBS, CSE appears to play a major role in H<sub>2</sub>S generation. The mechanism through which H<sub>2</sub>S exerts its relaxant properties is not fully understood, although it is likely mediated by the opening of K<sup>+</sup><sub>ATP</sub> channels. It is thought that in vascular smooth muscle cells, the opening of K<sup>+</sup><sub>ATP</sub> channels hyperpolarizes the cell membrane. This results in the inactivation of voltage-dependent L-type Ca<sup>2+</sup> channels, which results in relaxation. H<sub>2</sub>S -induced vasorelaxation has been established in the aorta, portal vein, mesenteric artery, cerebral arteries and vas deferens from many different species including humans (Wang, 2012). Additionally, H<sub>2</sub>S relaxes smooth

muscle in the colon through the same mechanism via  $K^{+}_{ATP}$  channels.  $H_2S$  has also been known to enhance the smooth muscle relaxant effect of Nitric Oxide (NO). While the primary observed outcome of  $H_2S$  in smooth muscle is relaxation, it is noteworthy that  $H_2S$  can exert potent contractile responses in certain tissues and species (Fiorucci, et al, 2006). It has been demonstrated that  $H_2S$  produces excitatory motor responses in the rat urinary bladder by activating capsaicin-sensitive primary afferent neurons (Patacchini et al., 2004)

### ***Mouse models: mdx mice versus mdx/mTR mice***

As previously mentioned, DMD is due to a mutation in the dystrophin gene, which has been shown to lead to fatal skeletal muscle degeneration. Several animal models of DMD have been developed over the last 35 years and *mdx* mice are the most commonly employed animal models in studies to understand the pathophysiology of DMD. Dystrophin deficiency, however, does not display the same severity of DMD in mice (*mdx*), which only exhibit mild skeletal muscle defects and this could be due to species-specific up-regulation of compensatory mechanisms. A number of mouse models with more severe dystrophic phenotypes have been created and these include double KO mice lacking dystrophin and utrophin, MyoD,  $\alpha 7$  integrin,  $\alpha$ -dystrobrevin or glycan processing. Later studies showed that species-specific differences in the telomere length account for the differences in the disease severity. Telomerase consisting protein telomerase reverse transcriptase and telomerase RNA component (TERC or mTR) maintains the length of the telomeres. In order to achieve an animal model with severe muscular dystrophy that more closely resembles DMD that occurs in

humans, a telomerase mutation is added to the dystrophin mutation (*mdx/mTR*) resulting in double knockout mice. A lack in the RNA for telomerase causes shortened telomeres, which contribute to the progression of DMD severity with age and this more closely represents the symptoms we see in human DMD (Rodrigues et al., 2016). Compared to single KO mice, the double KO mouse models exhibit exacerbated disease phenotypes based on various criteria such as increased serum creatine kinase levels, skeletal muscle fibrosis, exhaustion of mice on treadmill, kyphosis, skeletal and cardiac muscle myopathies and shortened life span (Yucel et al., 2018).

### ***Rationale and Hypothesis***

Gastrointestinal complications are common in DMD. The mechanisms underlying the gastrointestinal motility dysfunction in DMD are unknown. It is our hypothesis that dystrophin deficiency in DMD leads to a decrease in the level of contractile protein expression and gastric smooth muscle function. The aim of this study was to determine the levels of thin-filament associated proteins such as caldesmon, calponin, tropomyosin, and smoothelin while also assessing gastric smooth muscle function in using 2 mouse model of DMD, *mdx* and *mdx/mTR*.

Altered Ca<sup>2+</sup> levels in DMD may lead to mitochondrial dysfunction and ROS production. ROS have been associated in the pathophysiology of DMD. Oxidative stress has been shown to cause changes in the levels of thin filament-associated proteins in visceral smooth muscle. We also hypothesized that due to its anti-oxidant properties, treatment with H<sub>2</sub>S will restore the level of contractile protein expression and smooth muscle function.

## Chapter 2- MATERIALS AND METHODS

### Reagents:

RNAqueous™ kit, TRIzol Reagent, High-capacity cDNA Reverse Transcription Kit, PCR Primers for Calponin (Cnn1 Mm00487032-m1 59 bp), Caldesmon (Cald1Mm00513995-m1 102 bp), Tropomyosin (Tpm2 Mm00437172-g1 97 bp), Smoothelin (Smtn Mm00449973 m1 66 bp), GAPDH (NM-008084.2 107 bp),  $\beta$ -actin (Actb Mm02619580-g1), and 18-S (4332641) were obtained from Thermo Fisher (Waltham, MA); Antibodies for Calponin, Caldesmon, and Smoothelin were obtained from Abcam (Cambridge, MA). Antibodies for Tropomyosin were obtained from Santa Cruz Biotechnologies, Inc. (Dallas, TX); GAPDH was obtained from Cell Signaling Technology (Danvers, MA). Western blotting materials, 2x Laemmli Sample Buffer, Clarity Max™ Western ECL Substrate and DC™ Protein Assay Reagents were obtained from Bio-Rad Laboratories (Hercules, CA); SuperSignal® Western Blotting materials, 2x Laemmli Sample Buffer, DC™ Protein Assay Reagents, Clarity Max Western ECL Substrate, Clarity Western ECL were obtained from Bio-Rad Laboratories (Hercules, CA). T-PER® Tissue Protein Extraction Reagent was obtained from Thermo Scientific (Rockford, IL). All other supplies from Sigma (St.Louis, MO); and Fisher Scientific, (Asheville, NC).

### Animals

9-month-old male wild type, *mdx/mTR* and *mdx/mTR* treated with SG1002 were received from Dr. Solloum. *mdx/mTR* mice in Dr. Salloum's lab were treated with

SG1002, an orally active slow-releasing H<sub>2</sub>S pro-drug. SG1002 was administered at 21 days old to 9 months (40 mg/kg of body weight in chow/every 3 days in *mdx/mTR* mice).

3-month old wild-type and *mdx* female mice (C57BL/6) were purchased from Jackson Laboratories (Sacramento, California). The mice were housed in an animal facility directed by the Division of Animal Resources at Virginia Commonwealth University. They were euthanized by asphyxiation by carbon dioxide (CO<sub>2</sub>) under approval by the Institutional Animal Care and Use committee of Virginia Commonwealth University.

#### ***Preparation of dispersed smooth muscle cells.***

Smooth muscle cells were isolated from mouse stomach by sequential enzymatic digestion, filtration, and centrifugation. The mucosa of the gastric tissue was carefully removed and the tissue was chopped via surgical shears. It was then incubated at 31°C for 10-15 minutes in SMB [NaCl 120 mM, KCl 4 mM, KH<sub>2</sub>PO<sub>4</sub> 2.6 mM, CaCl<sub>2</sub> 2.0 mM, MgCl<sub>2</sub> 0.6 mM, HEPES (N-2-hydroxyethylpiperazine-N' 2-ethanesulfonic acid) 25 mM, glucose 14 mM, and Basic Eagle Medium (essential amino mixture) 2.1% (pH 7.4)] containing 0.1 % collagenase and 0.01% of soybean trypsin inhibitor. The partially degraded tissue was washed once with collagenase-free SMB and permitted to disperse spontaneously for 20 minutes. Cells were then collected utilizing a 500 um Nitex every 10 minutes.

### ***Measurement of contraction and relaxation in freshly isolated muscle cells.***

Contraction in freshly dispersed gastric smooth muscle cells was determined by scanning micrometry. Freshly isolated muscle cells aliquots ( $10^4$  cells/ml) were treated with 0.5 ml of medium containing  $10 \mu\text{M}$  acetylcholine for 30 s or 5 minutes and the reaction was terminated with 1% acrolein at a final concentration of 0.1%. The resting cell length was determined in control experiments in which muscle cells were incubated with 0.5 ml of 0.1% bovine serum albumin in the absence of the ACh. The mean lengths of 50 muscle cells treated with  $10 \mu\text{M}$  acetylcholine was measured by scanning micrometry and compared with the mean lengths of untreated cells. ACh induced contraction was expressed as the percent decrease in mean cell length from control cell length.

Relaxation was also examined in intact muscle cells contracted with  $1 \mu\text{M}$  ACh. Muscle cells were treated for 5 minutes with  $10 \mu\text{M}$  SNP or  $10 \mu\text{M}$  isoproterenol followed by ACh for 30 s or 5 min. The reaction was terminated with 1% acrolein. The length of 50 cells treated with acetylcholine was measured in sequential microscopic fields by scanning micrometry. Relaxation was expressed as percent increase in the length of cells contracted with ACh.

### **Preparation of gastric smooth muscle strips**

1 L of Krebs buffer [ $118 \text{ mM NaCl}$ ,  $4.8 \text{ mM KCl}$ ,  $1 \text{ mM MgSO}_4$ ,  $1.15 \text{ mM NaH}_2\text{PO}_4$ ,  $15 \text{ mM NaHCO}_3$ ,  $10.5 \text{ mM glucose}$  and  $2.5 \text{ mM CaCl}_2$  ( $95\% \text{ O}_2/5\% \text{ CO}_2$ , pH 7.4,  $37^\circ\text{C}$ ) was prepared. Mice were euthanized with  $\text{CO}_2$  inhalation, vertical abdominal incisions were made, and the stomach was removed and placed into a beaker

containing Krebs buffer. Gastric contents were removed with a syringe filled with Krebs buffer. The mucosa was removed and strips were cut from the fundus of the stomach. Each end of the muscle strip was tied using a silk thread and mounted vertically on organ baths that were kept oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) at 37 degrees Celsius. Each organ bath was filled with either 7ml or 3.5 ml of Krebs's (depending on the size of the organ bath). One side of the strip was hooked onto the transducer and the other side was hooked onto a glass rod.

Every 15 min, Krebs buffer was flushed and refilled and the preparations were allowed to equilibrate for 1 hour at a resting tension of 0.7-0.8 grams. Contraction was induced via muscarinic receptor activation (acetylcholine) at a concentration of 10  $\mu$ M of acetylcholine (ACh). Relaxation was measured in response to 10  $\mu$ M of sodium-nitroprusside (SNP), a NO donor. The contractile response of the gastric segments to muscarinic receptor activation was measured and expressed as an increase in tension in grams. SNP induced relaxation was calculated as percent decrease in maximum contraction. After completion of the experiment, the strips were taken down, blotted dry with a kimwipe and the tissue wet weight (7mg  $\pm$  0.8) was obtained.

### **Western blot analysis of thin filament-associated proteins**

Gastric smooth muscle tissue was taken from animal models. The tissue was cleaned and the mucosa was removed. This tissue was then transferred into new tubes with appropriate amounts of 1.44mm beads and TPER (lysis buffer) in the presence of 5  $\mu$ L of protease inhibitor. Bead number was calculated as 5 times the tissue weight. TPER was then added and was calculated as two times the tissue weight. Tissues were

homogenized using a bullet blender. The supernatant was collected and centrifuged at 13.3 rpm at 4°C for 10 minutes. The supernatant was transferred to new tubes and the protein concentration was then measured using a DC protein assay kit from Bio-Rad. Equal amounts of proteins were fractionated by SDS/PAGE, and transferred on to Polyvinylidene difluoride (PVDF) membrane. Blots were blocked with 1X TBS with 1% casein blocker for 1 hour at room temperature and then incubated overnight at 4 °C with primary antibodies in 1X TBS with 1% casein (Calponin 1:5000, Tropomyosin 1:1000, Caldesmon 1:10,000, Smoothelin 1:5000). The following morning the membranes were washed with TBS-T and incubated for 1 hour with horseradish-peroxidase-conjugated corresponding secondary antibody (1:5000) in 1X TBS with 1% casein. Immunoreactive proteins were visualized using Clarity Max Western ECL Substrate, Clarity Western ECL Substrate, or SuperSignal West Pico Chemiluminescent Substrate kit. Enhanced chemiluminescence reagent identified the protein bands.

### ***Qualitative Real Time PCR***

25-50 mg of gastric tissue was isolated from mice stomach and transferred to a tube with 1 ml of TRIzol. The mixture was homogenized via Fisher PowerGen 125 homogenizer. 0.2 ml of chloroform was added. The tubes were vortexed, incubated at room temperature for 5 min and then centrifuged at 13.3rpm for approximately 5 min. At the end of centrifugation the mixture separates into two distinct layers, a lower red, phenol-chloroform phase and an upper clear aqueous phase. The RNA containing upper aqueous phase was carefully transferred into a new autoclaved 1.5ml tube. RNA was precipitated from the aqueous phase by adding 0.5 ml of isopropyl alcohol. The

mixture was then centrifuged at 13.3rpm for 20 min at 4°C and the precipitated RNA appeared as a white pellet. The pellet was washed with 1 ml of 70% ethanol, mixed by vortexing and centrifuged at 13.3 for 10 min at 4°C and completely vacuum dried. RNase and DNase free ultrapure distilled water (25 µL) is added once the pellet is completely dry and the samples are then left to incubate at 65°C for 10 minutes. Immediately following incubation the samples are placed on ice. The concentration was measured with a NanoDrop 8000.

cDNA was prepared by reversely transcribing RNA from each preparation using the High Capacity cDNA reverse transcription kits to prepare the 2x RT Master Mix [10xRT Buffer, 25x dNTP Mix, 10x RT Random primers, Multiscribe Reverse Transcriptase, Nuclease Free H<sub>2</sub>O]. An equal volume of RNA was added to 2xRT Master Mix. Quantitative RT-PCR was then performed on cDNA samples using the primers specific to tropomyosin, caldesmon, calponin, and smoothelin, based on known sequences in mouse and Taqman gene expression master mix. 18-S and β-actin were used to normalize expression of the target genes. Each real-time PCR reactions were performed in triplicate. With 18-S/β-actin acting as controls, a quantitative comparison between *mdx*, *mdx/mTR*, and *mdx/mTR* treated with SG1002 and their age-matched control was calculated using the  $2^{-\Delta\Delta CT}$  method. Final calculations were expressed as a fold difference in the expression of *mdx*, *mdx/mTR*, and *mdx/mTR* treated with SG1002 mice cohorts relative to their age-matched control.

### **Statistical Analysis**

Results were expressed as means ± standard error of the mean of n experiments. Each experiment was performed on tissues obtained from different

animals. Experiments were designed to compare treatment to control conditions, with each individual strip serving as its own control. Paired t-tests were conducted in GraphPad Software Prism 6 (La Jolla, CA) with significance set at  $P < 0.05$ .

## Chapter 3: RESULTS

### **1. Effect of dystrophin deficiency on gastric smooth muscle function.**

Dystrophin deficiency in DMD patients and mouse models such as *mdx* is associated with delayed gastric emptying suggesting altered gastric motility. However, the role of dystrophin in gastric smooth muscle function is not known. To investigate the role of dystrophin in normal smooth muscle function, muscle contraction and relaxation was measured in both muscle strips and isolated muscle cells.

**Contraction in gastric muscle strips.** Contraction in response to the main excitatory transmitter acetylcholine (ACh) was measured. Experiments were carried out in gastric muscle strips on 3-month old control and *mdx* mice. The strips were mounted in organ bath and after equilibration of 1 g of resting tension, 10  $\mu$ M of ACh was added. Addition of ACh to the bath medium caused a rapid contraction that reached a plateau of sustained contraction. Contraction was recorded as increase in tension in grams above basal tension. In the gastric muscle strips from control mice ACh caused  $1.0 \pm 0.2$  g of contraction and in the gastric muscle the strips from *mdx* mice ACh caused  $0.5 \pm 0.2$  g of contraction (Figure 2). Contraction in grams was normalized to tissue weight and presented as mN/100 mg tissue. Gastric muscle contraction was significantly inhibited in *mdx* mice. ACh-induced contractions in gastric muscle strips controls mice was  $45 \pm 4$  mN/100 mg tissue, whereas in gastric muscle strips from *mdx* mice contraction in response to ACh was  $24 \pm 5$  mN/100 mg tissue (Figure 2) ( $p < 0.05$ ,  $n = 4-5$ ).

Although *mdx* mice were extensively used, this model exhibits only mild phenotype compared to DMD patients. Recent studies have developed a more humanized mouse model, *mdx/mTR*, that lacks both dystrophin and telomerase RNA

component. This model exhibits exacerbated disease phenotype and closely approximated human DMD. Dr. Salloum's group at Virginia Commonwealth University has shown that the abnormalities in cardiac function were more robust in this model and treatment of *mdx/mTR* mice with treated with SG1002, an orally active slow-releasing compound that provides stable, non-toxic serum and tissue levels of H<sub>2</sub>S, provided cardioprotection. Gastric tissues from 9-month old control and *mdx/mTR* mice, and *mdx/mTR* mice treated with SG1002 obtained Dr. Salloum laboratory were used to examine gastric muscle function

Contraction in response to ACh was also significantly decreased in gastric muscle strips from *mdx/mTR* mice compared to their age-matched control ( $32.3 \pm 6.8$  mN/100 mg tissue in control and  $17.7 \pm 3.8$  mN/100 mg tissue in *mdx/mTR* mice;  $p < 0.05$ ,  $n = 4$ ) (Figure 3). These results suggest that H<sub>2</sub>S completely reversed the decrease in smooth muscle contraction in *mdx/mTR* mice.

***Relaxation in gastric muscle strips.*** Changes in smooth muscle relaxation also affects gastric motility. Nitric oxide (NO) is the inhibitory transmitter in the gastrointestinal tract. Relaxation in response to NO donor, sodium nitroprusside (SNP) was measured as inhibition of ACh-induced contraction in gastric muscle strips. To examine the effect of SNP on ACh-induced contraction, gastric muscle strips were treated with ACh (10  $\mu$ M) to increase the tension and after the tension plateaued, SNP (10  $\mu$ M) was added to induce inhibition of sustained tone and the inhibition of contraction was calculated as percent relaxation. SNP induced relaxation in muscle strips from control and *mdx* mice. The effect of SNP to induce relaxation was not different between control and *mdx* mice (control:  $86 \pm 3\%$  decrease in contraction; *mdx*:

88±4% decrease in contraction) (Figure 4). Similarly SNP-induced relaxation was not different in muscle strips from control and *mdx/mTR* mice and *mdx/mTR* mice treated with SG1002 (control: 85±3%; *mdx/mTR*: 91±2%; *mdx/mTR* treated with SG1002: 95±7% decrease in contraction) (Figure 5). These results suggest that, unlike contraction in response to ACh, relaxation in response to NO donor was not affected in gastric muscle of *mdx* and *mdx/mTR* mice.

**Contraction in isolated muscle cells.** The results in *mdx* and *mdx/mTR* mice suggest that lack of dystrophin impairs gastric muscle contraction. One of the limitations of the studies in muscle strips is involvement of multiple cell types. Although decreased response to ACh in gastric muscle strips from *mdx* and *mdx/mTR* mice suggest changes in response in post-synaptic cells, the involvement of enteric neurons and interstitial cells of Cajal (ICC) cannot be excluded. To preclude the effects of enteric neurons and ICC and see the direct effect of dystrophin deficiency on smooth muscle function, gastric muscle cell devoid of enteric neurons and ICC were prepared and contraction in response to ACh was measured by scanning micrometry in dispersed muscle cells. Experiments were carried out in gastric muscle cells isolated from 3-month old control and *mdx* mice. Previous studies have shown that contraction in gastric muscle cells, as in muscle strips, in response to several contractile agonists is biphasic. A rapid initial peak contraction reflecting activation of Ca<sup>2+</sup>/calmodulin-dependent myosin light chain (MLC) kinase activity and a sustained Ca<sup>2+</sup>-independent phase reflecting inhibition of MLC phosphatase activity has been seen. Activation of MLC kinase initiates phosphorylation of MLC<sub>20</sub>, a prerequisite for muscle contraction, whereas inhibition MLC phosphatase sustains MLC<sub>20</sub> phosphorylation and muscle

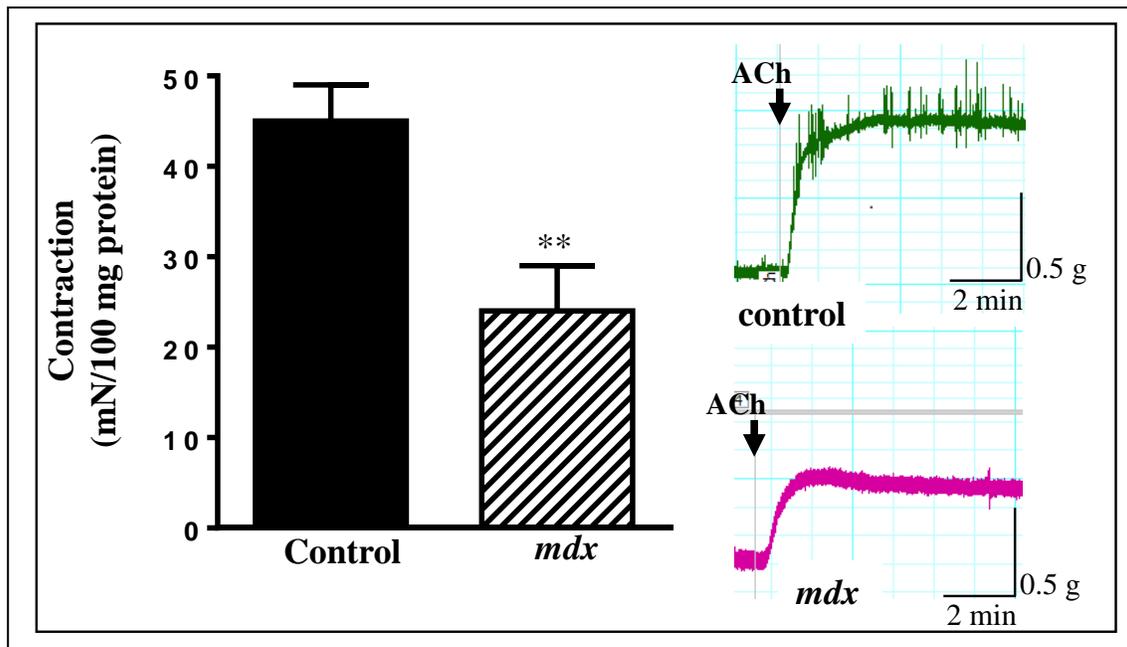
contraction. Dispersed gastric muscle cells were treated with ACh (1  $\mu$ M) for 0.5 min to measure initial contraction and for 5 min to measure sustained contraction. Contraction was measured as decrease in cells length compared to basal cell length.

The basal cell lengths of colonic smooth muscle cells were not significantly different in control mice and *mdx* mice (63 $\pm$ 3  $\mu$ m in control mice and 67 $\pm$ 5  $\mu$ m in *mdx* mice). Treatment with ACh for 0.5 min caused a significant decrease in cell length in muscle cells isolated from the stomach of control and *mdx* mice. However, the contraction in response to ACh was significantly inhibited in muscle cells from the stomach of *mdx* mice compared to control mice (40 $\pm$ 4% decrease in cell length in control mice and 24 $\pm$ 3% decrease in muscle cell length in *mdx* mice;  $p < 0.05$ ,  $n = 4-5$ ) (Figure 6). Treatment with ACh for 5 min also caused a significant decrease in cell length in muscle cells isolated from the stomach of control and *mdx* mice. However, the contraction in response to ACh was significantly inhibited in muscle cells from the stomach of *mdx* mice compared to control mice (41 $\pm$ 4% in control and 25 $\pm$ 2 in *mdx* mice;  $p < 0.05$ ,  $n = 4-5$ ) (Figure 7). The results in isolated muscle cells are consistent with the results in muscle strips and provide evidence that lack of dystrophin affects smooth muscle contraction.

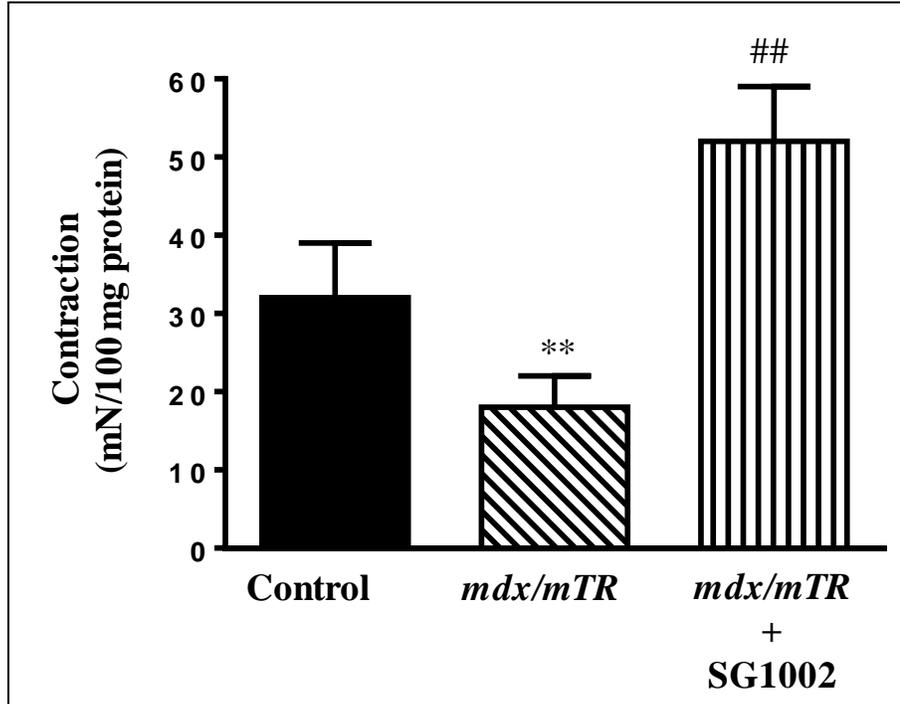
***Relaxation in isolated muscle cells.*** In contrast to an increase in MLC<sub>20</sub> phosphorylation, which mediates acto-myosin interaction and muscle contraction, decrease in MLC<sub>20</sub> phosphorylation mediates muscle relaxation. The main inhibitory transmitters are nitric oxide, which causes relaxation via activation of soluble guanylyl cyclase, generation of cGMP and activation of cGMP-dependent protein kinase (PKG), and vasoactive intestinal peptide, which causes relaxation via activation of Gs-coupled

adenylyl cyclase, generation of cAMP and activation of cAMP-dependent protein kinase (PKA). We next examined whether lack of dystrophin affects the pathways that mediate muscle relaxation, Sodium nitroprusside, a NO-donor that activates soluble guanylyl cyclase and cGMP/PKG pathway, and isoproterenol, an agonist that activates  $\beta$ -adrenergic receptor coupled to Gs and activation of adenylyl cyclase, cAMP/PKA pathway were used. Experiments were carried out in gastric muscle cells isolated from 3-month old control and *mdx* mice.

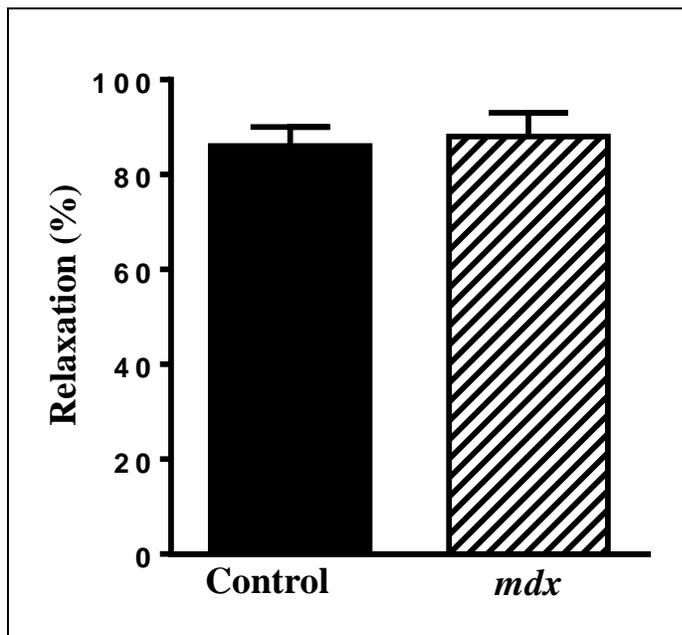
To measure relaxation, muscle cells were pretreated with SNP (10  $\mu$ M) or isoproterenol (10  $\mu$ M) for 5 min and initial contraction in response to ACh (1  $\mu$ M) was measured. Inhibition of contraction in response to ACh in the presence of SNP or isoproterenol was considered as relaxation. Relaxation in response to SNP was not significantly different in muscle cells isolated from the stomach of control and *mdx* mice. SNP caused  $82\pm 10\%$  relaxation in muscle cells from control mice and  $76\pm 7\%$  relaxation in muscle cells from *mdx* mice (Figure 8). Relaxation in response to isoproterenol also was not significantly different in muscle cells isolated from the stomach of control and *mdx* mice. Isoproterenol caused  $86\pm 9\%$  relaxation in muscle cells from control mice and  $74\pm 8\%$  relaxation in muscle cells from *mdx* mice (Figure 9). These results suggest that either NO-dependent relaxation mediated via cGMP/PKG pathway or Gs-coupled receptor relaxation mediated via cAMP/PKA pathways mediated was not affected in gastric muscle cells from *mdx* mice.



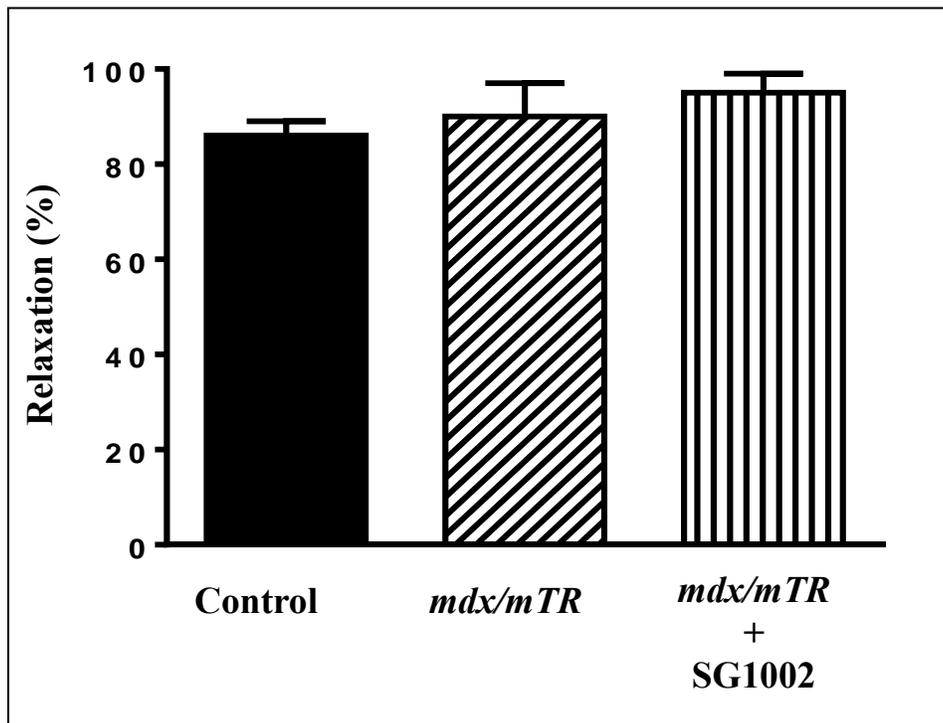
**Figure 2. Inhibition of gastric muscle contraction in mdx mice.** Gastric muscle strips were isolated from 3-month old control and mdx mice. Contraction in response to acetylcholine (ACh, 10  $\mu$ M) was measured in organ bath experiments. Contraction was recorded as increase in grams and calculates as mN/100g mg tissue. **Left panel:** Bar graphs of calculated values representing mean  $\pm$  SEM of 4-5 separate experiments. **Right panel:** Representative tracing of acetylcholine-induced contraction in gastric muscle strips from control and mdx mice. Contraction was significantly decreased in mdx mice (\*\*  $p < 0.05$ )



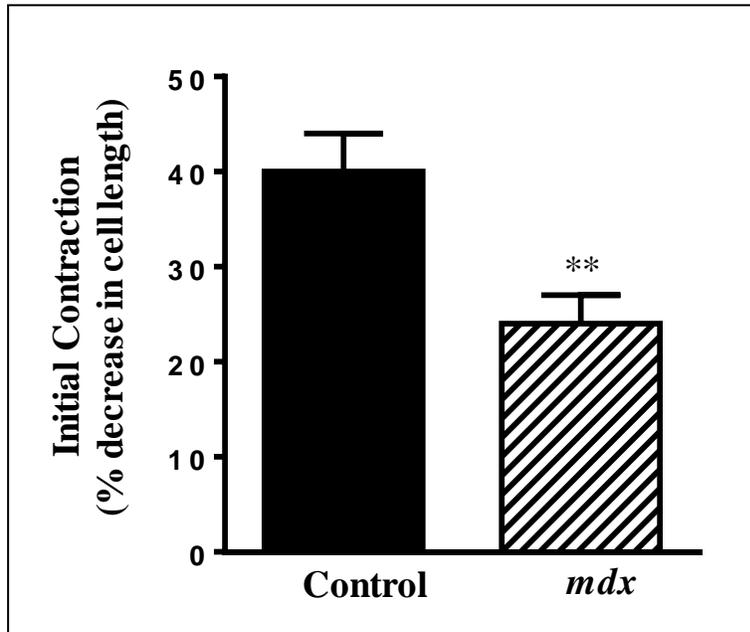
**Figure 3. Inhibition of gastric muscle contraction in *mdx/mTR* mice and reversal of inhibition by H<sub>2</sub>S.** Gastric muscle strips were isolated from 9-month old control and *mdx/mTR* mice and *mdx/mTR* mice treated with SG1002, an orally active H<sub>2</sub>S donor. Contraction in response to acetylcholine (10 μM) was measured in organ bath experiments. Contraction was recorded as increase in grams and calculated as mN/100g mg tissue. Values are mean ± SEM of 4 separate experiments. Contraction was significantly decreased in *mdx/mTR* (\*\* *p* < 0.05). Treatment with SG1002 completely reversed the inhibition in contraction (## *p* < 0.05).



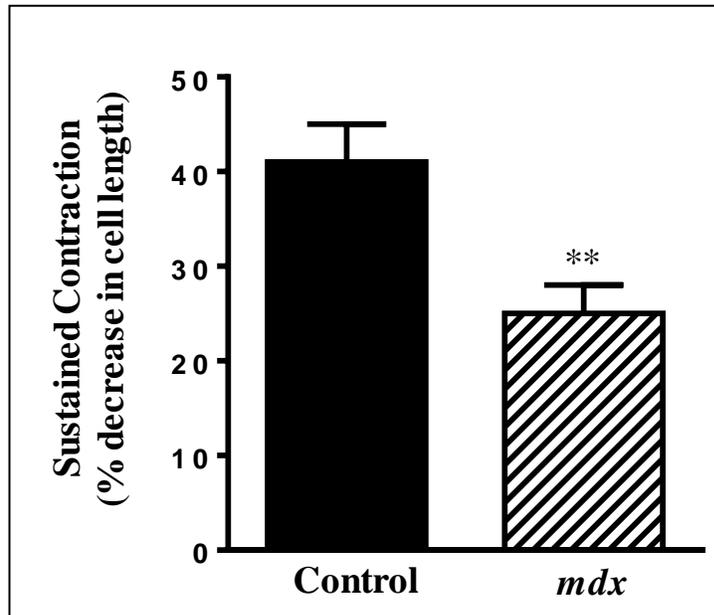
**Figure 4.** *SNP-induced relaxation in gastric muscle cells from control and mdx mice.* Gastric muscle strips were isolated from 3-month old control and mdx mice. Relaxation in response to nitric oxide donor, sodium nitroprusside (SNP, 10  $\mu$ M) was measured as inhibition of acetylcholine (10  $\mu$ M)-induced contraction and calculated as percent inhibition of contraction. Values are mean  $\pm$  SEM of 4 separate experiments. SNP-induced relaxation was not different between gastric muscle strips from control and mdx mice.



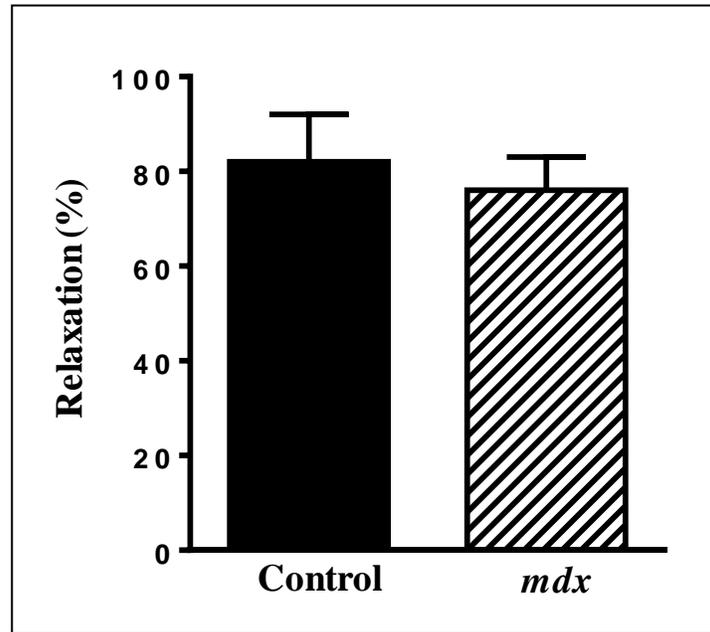
**Figure 5.** *SNP-induced relaxation in gastric muscle cells from control and mdx mice and mdx/mTR mice treated with SG1002.* Gastric muscle strips were isolated from 3-month old control and mdx mice. Relaxation in response to nitric oxide donor, sodium nitroprusside (SNP, 10  $\mu$ M) was measured as inhibition of acetylcholine (10  $\mu$ M)-induced contraction and calculated as percent inhibition of contraction. Values are mean  $\pm$  SEM of 4 separate experiments. SNP-induced relaxation was not different between gastric muscle strips from control and mdx/mTR mice and mdx/mTR mice treated with SG1002.



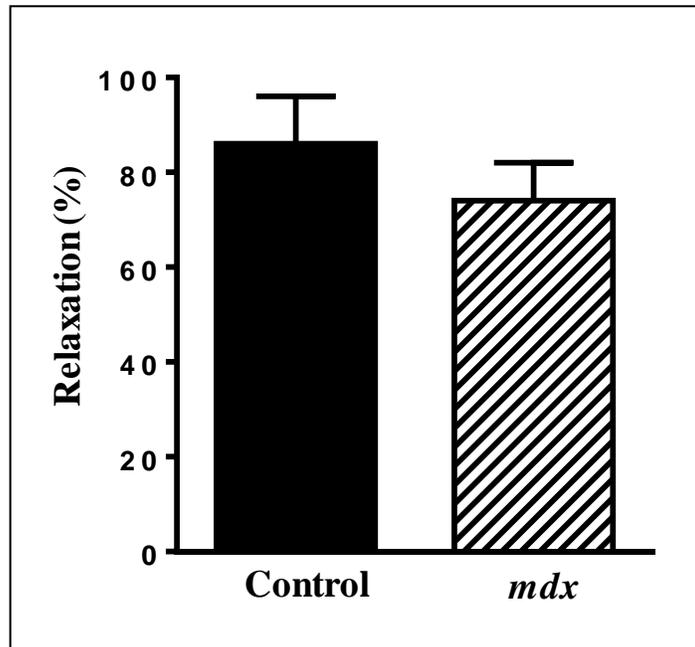
**Figure 6. Inhibition of initial muscle contraction in gastric muscle cells from mdx mice.** Smooth muscle cells were isolated from the stomach of 3-month old control and mdx mice. Cells were treated with acetylcholine ( $1 \mu\text{M}$ ) for 0.5 min and contraction was measured as decrease in cell length from the basal length (control:  $63 \pm 3 \mu\text{m}$  and mdx:  $67 \pm 5 \mu\text{m}$ ) and expressed as percent decrease in cell length. Values are mean  $\pm$  SEM of 4-5 separate experiments. Initial contraction was significantly decreased in mdx mice (\*\*  $p < 0.05$ ).



**Figure 7. Inhibition of sustained muscle contraction in gastric muscle cells from mdx mice.** Smooth muscle cells were isolated from the stomach of 3-month old control and mdx mice. Cells were treated with acetylcholine (1  $\mu$ M) for 5 min and contraction was measured as decrease in cell length from the basal length (control:  $63\pm 3$   $\mu$ m and mdx:  $67\pm 5$   $\mu$ m) and expressed as percent decrease in cell length. Values are mean  $\pm$  SEM of 4-5 separate experiments. Sustained contraction was significantly decreased in mdx mice (\*\*  $p < 0.05$ ).



**Figure 8.** *SNP-induced relaxation in gastric muscle cells from control and mdx mice.* Smooth muscle cells were isolated from the colon of 3-month old control and mdx mice were pretreated with the nitric oxide donor, sodium nitroprusside (SNP, 10  $\mu$ M) for 5 min and contraction in repose to acetylcholine (1  $\mu$ M) was measured. Percent inhibition of ACh-induced contraction in the presence of SNP was presented as relaxation. Values are mean  $\pm$  SEM of 4-5 separate experiments. SNP-induced relaxation was not different in colonic smooth muscle cells from both control versus mdx mice.



**Figure 9.** *Isoproterenol-induced relaxation in gastric muscle cells from control and mdx mice.* Smooth muscle cells were isolated from the colon of 3-month old control and mdx mice were pretreated with the Gs-coupled  $\beta$ -adrenergic receptor agonist isoproterenol ( $10 \mu\text{M}$ ) for 5 min and contraction in repose to acetylcholine ( $1 \mu\text{M}$ ) was measured. Percent inhibition of ACh-induced contraction in the presence of isoproterenol was presented as relaxation. Values are mean  $\pm$  SEM of 4-5 separate experiments. Isoproterenol-induced relaxation was not different in colonic smooth muscle cells from both control versus mdx mice.

## **2. Effect of dystrophin deficiency on the expression of thin filament-associated proteins**

The decrease in contraction could be due to changes in the intrinsic signaling pathways that regulate MLC<sub>20</sub> phosphorylation or changes in the expression of proteins that regulate acto-myosin interaction. Previous studies have that thin filament-associated proteins regulate acto-myosin interaction. Hence, studies were carried out to examine whether the expression of thin filament-associated proteins are altered in muscle cells in *mdx* mice. Experiments were carried out in gastric muscle cells isolated from 3-month old control and *mdx* mice. Expression of caldesmon, calponin, tropomyosin and smoothelin were measured by quantitative RT-PCR and western blot analysis.

**Caldesmon.** Caldesmon is a thin-filament associated protein and shown to inhibit acto-myosin interaction and muscle contraction. Expression of smooth muscle specific h-caldesmon was analyzed in gastric smooth muscle from control and *mdx* mice by qRT-PCR using specific primers. The results showed that mRNA expression of caldesmon was significantly decreased in gastric smooth muscle from *mdx* mice compared to gastric smooth muscle control mice ( $p < 0.05$ ,  $n=4-5$ ) (Figure 10). Western blot analysis using antibody that detect both muscle specific h-caldesmon or non-muscle caldesmon demonstrated expression of h-caldesmon (~90 kDa) in gastric muscle and showed no difference in the expression between gastric muscle from control and *mdx* mice (Figure 10).

Expression of caldesmon mRNA was also significantly decreased in gastric smooth muscle *mdx/mTR* mice compared to age-matched control mice ( $p < 0.05$ ,  $n=4$ ).

Treatment of *mdx/mTR* mice with SG1002 significantly reversed the decrease in caldesmon expression ( $p < 0.05$ ,  $n = 4$ ) (Figure 11).

**Calponin.** Calponin is also filament-associated protein expressed in smooth muscle and several types of non-muscle cells. Three isoforms of calponin (calponin 1, 2, and 3) are present and all three isoforms bind actin and inhibit its interaction with myosin. Calponin isoform is smooth muscle specific and plays a role in fine-tuning smooth muscle contractility. Expression of smooth muscle specific calponin 1 was analyzed in gastric smooth muscle from control and *mdx* mice by qRT-PCR using specific primers. The results showed that mRNA expression of calponin was significantly decreased in gastric smooth muscle from *mdx* mice compared to gastric smooth muscle control mice ( $p < 0.05$ ,  $n = 4-5$ ) (Figure 12). Western blot analysis using antibody that detects both muscle specific and non-muscle calponin demonstrated expression of calponin 1 (~35 kDa) in gastric muscle and showed no difference in the expression between gastric muscle from control and *mdx* mice (Figure 12).

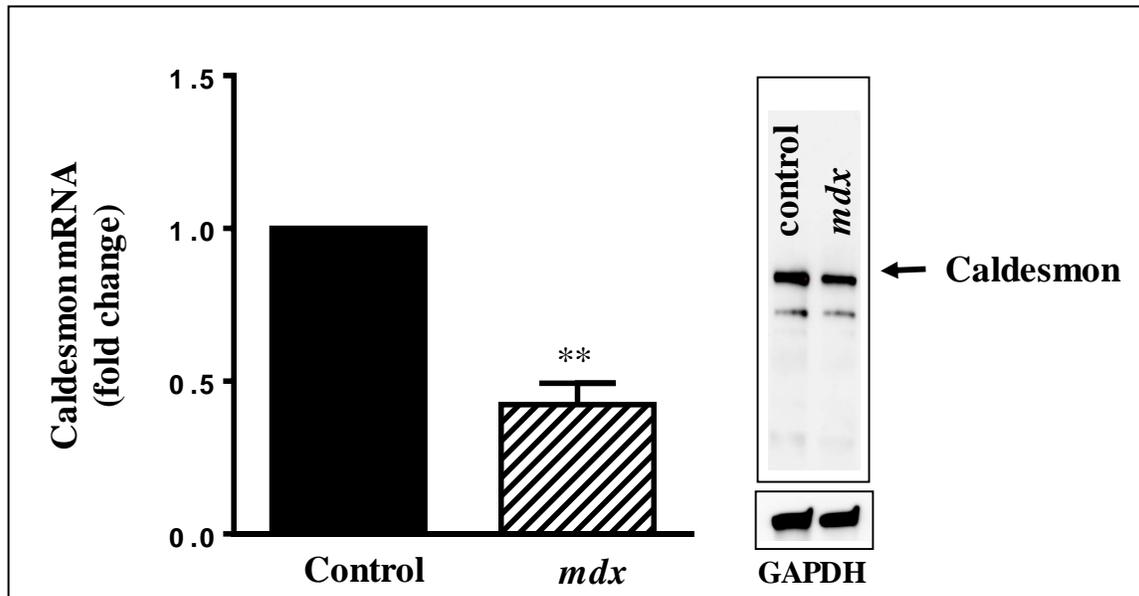
Expression of calponin 1 mRNA was also significantly decreased in gastric smooth muscle *mdx/mTR* mice compared to age-matched control mice ( $p < 0.05$ ,  $n = 4$ ). Treatment of *mdx/mTR* mice with SG1002 significantly reversed the decrease in calponin 1 expression ( $p < 0.05$ ,  $n = 4$ ) (Figure 13).

**Tropomyosin:** Smooth muscle tropomyosin exists in heterodimer of  $\alpha/\beta$  isoforms, and unlike skeletal muscle, smooth muscle tropomyosin by itself augments actomyosin ATPase activity. This action is in an opposite manner to that of caldesmon and calponin. Expression of tropomyosin was analyzed in gastric smooth muscle from control and *mdx* mice by qRT-PCR using primers that are not isoform-specific. The

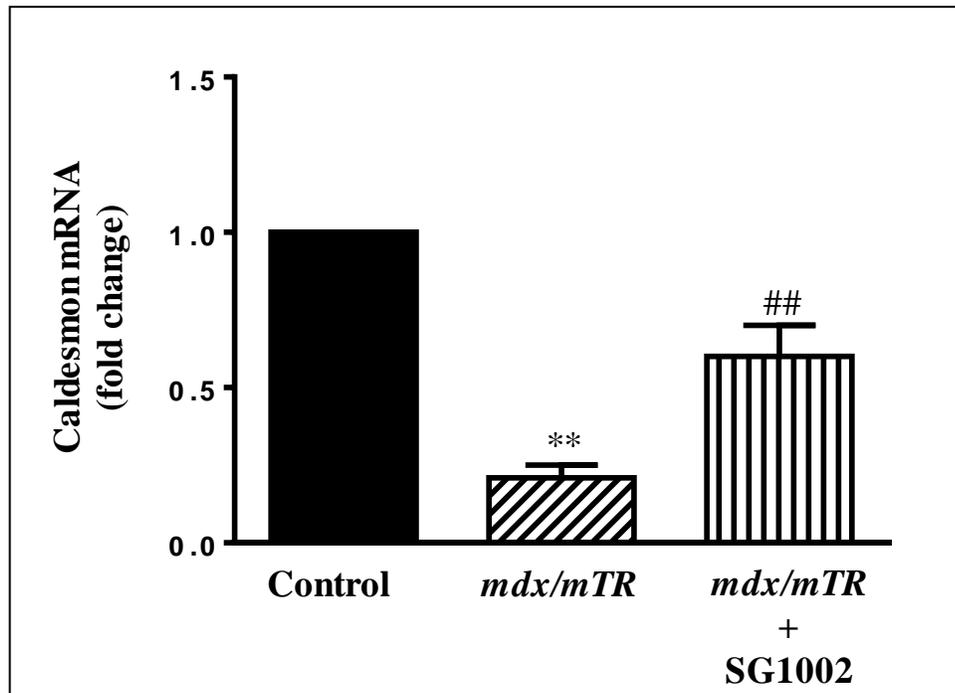
results showed that mRNA expression of tropomyosin was significantly decreased in gastric smooth muscle from *mdx* mice compared to gastric smooth muscle control mice ( $p < 0.05$ ,  $n=4-5$ ) (Figure 14). Western blot analysis using antibody demonstrated expression of tropomyosin (~70 kDa) in gastric muscle and showed no difference in the expression between gastric muscle from control and *mdx* mice (Figure 14).

Expression of tropomyosin mRNA was also significantly decreased in gastric smooth muscle *mdx/mTR* mice compared to age-matched control mice ( $p < 0.05$ ,  $n=4$ ). Treatment of *mdx/mTR* mice with SG1002 significantly reversed the decrease in tropomyosin expression ( $p < 0.05$ ,  $n=4$ ) (Figure 15).

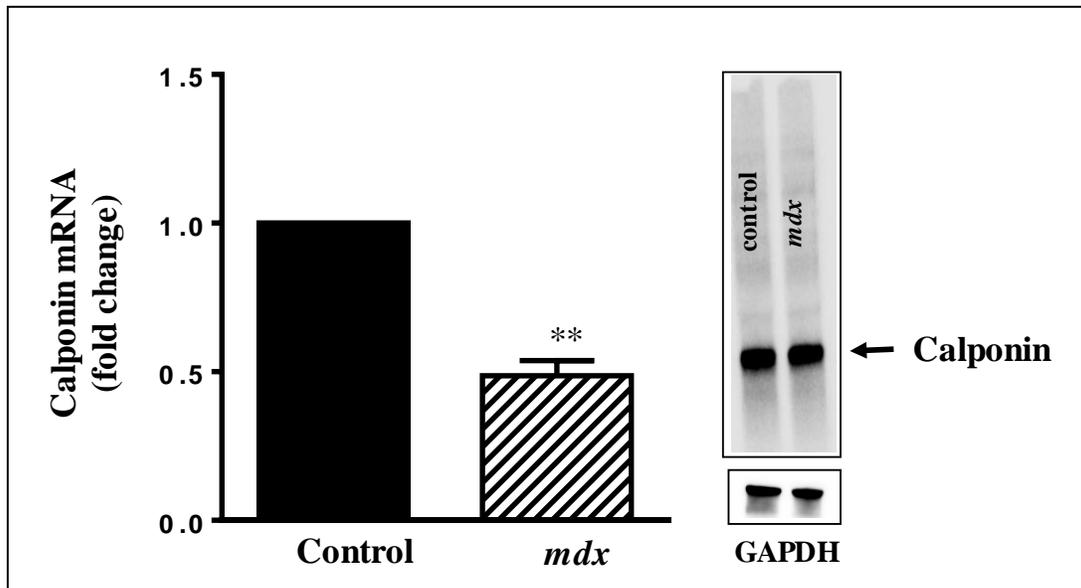
**Smoothelin.** Smoothelin is an important constituent smooth muscle cytoskeleton and a marker of fully differentiated contractile smooth muscle. There are two isoforms of smoothelins, and their expression is tissue specific. Smoothelin-A (~ 59 kDa) is expressed in visceral smooth muscle and smoothelin-B (~110 kDa) is expressed in vascular smooth muscle. Gene knockout studies demonstrated that smoothelin-A is essential for smooth muscle contraction in the gastrointestinal tract (Niessen et al., 2005). Expression of smoothelin-A was analyzed in gastric smooth muscle from control and *mdx* mice by qRT-PCR using specific primers. The results showed that mRNA expression of smoothelin-A was significantly decreased in gastric smooth muscle from *mdx* mice compared to gastric smooth muscle control mice ( $p < 0.05$ ,  $n=4-5$ ) (Figure 16). Western blot analysis using antibody that detects both smoothelin-A and smoothelin-B demonstrated expression of both isoforms in gastric muscle and showed no difference in the expression between gastric muscle from control and *mdx* mice (Figure 16).



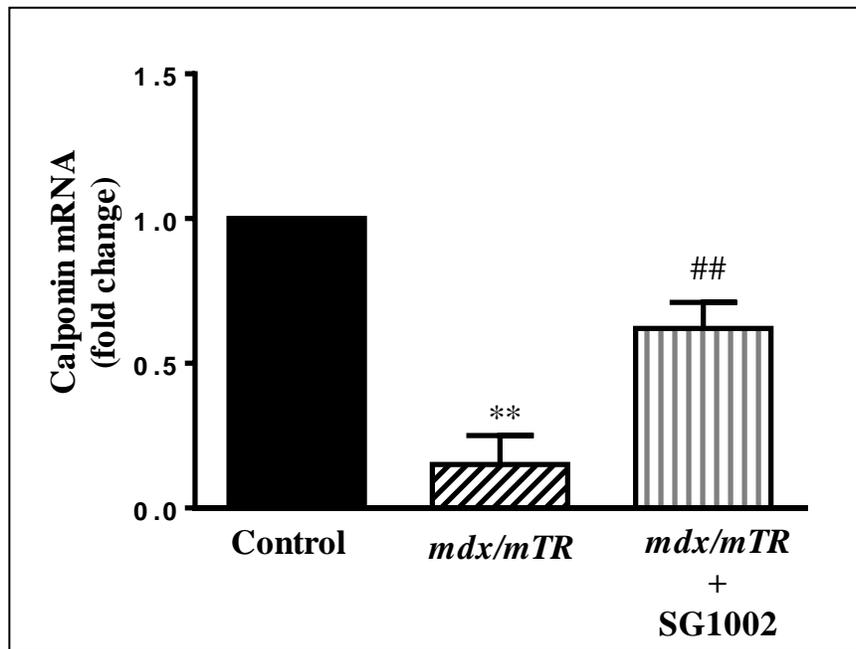
**Figure 10. Expression of caldesmon in gastric smooth muscle from control and mdx mice.** *Left Panel: RNA was isolated from gastric smooth muscle of 3-month old control and mdx mice. mRNA expression of caldesmon was measured using specific primers by quantitative RT-PCR and expressed as fold change over control. Values are mean  $\pm$  SEM of 4-5 separate experiments. mRNA expression of caldesmon was significantly decreased in gastric smooth muscle from mdx mice compared to control (\*\*  $p < 0.05$ ). Right panel: Lysates were prepared from gastric smooth muscle of control and mdx mice and expression of caldesmon was measured by western blot using specific antibody. Representative western blot image of 4 separate experiments was shown in the figure. Densitometry analysis of all the images showed no difference in the expression of caldesmon in gastric smooth muscle between control and mdx mice.*



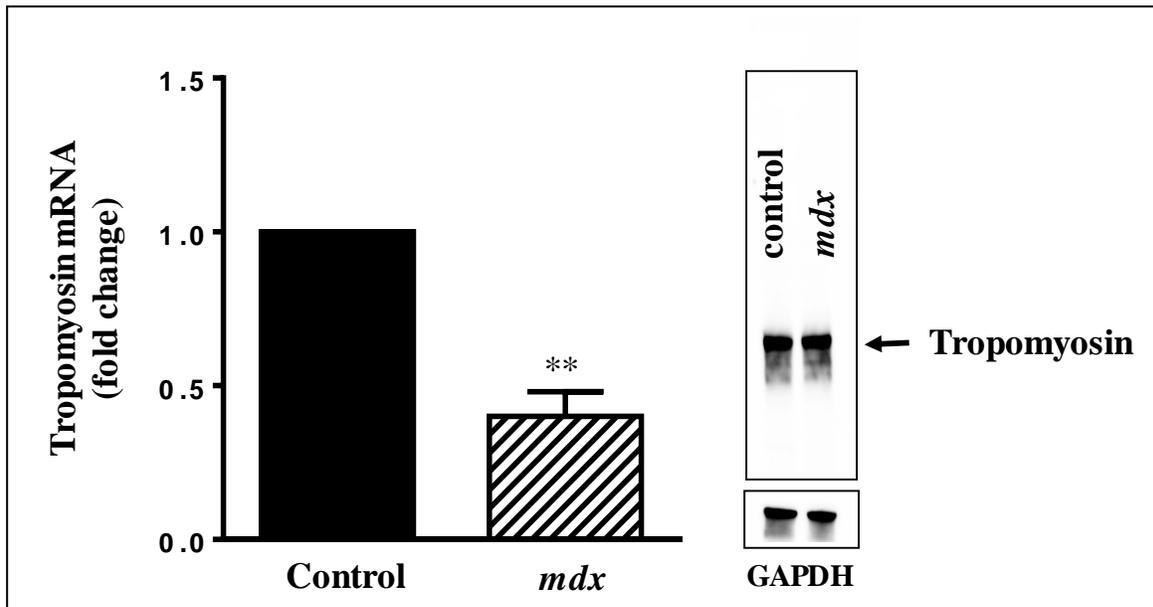
**Figure 11.** *Effect of H<sub>2</sub>S on caldesmon expression in gastric smooth muscle of mdx/mTR mice. RNA was isolated from gastric smooth muscle of 9-month old control and mdx/mTR mice and mdx/mTR mice treated with SG1002, an orally active H<sub>2</sub>S donor. mRNA expression of caldesmon was measured using specific primers by quantitative RT-PCR and expressed as fold change over control. Values are mean ± SEM of 4 separate experiments. mRNA expression of caldesmon was significantly decreased in colonic smooth muscle from mdx/mTR mice compared to control (\*\* p<0.05). Treatment with SG1002 significantly reversed the inhibition in caldesmon mRNA expression (## p<0.05).*



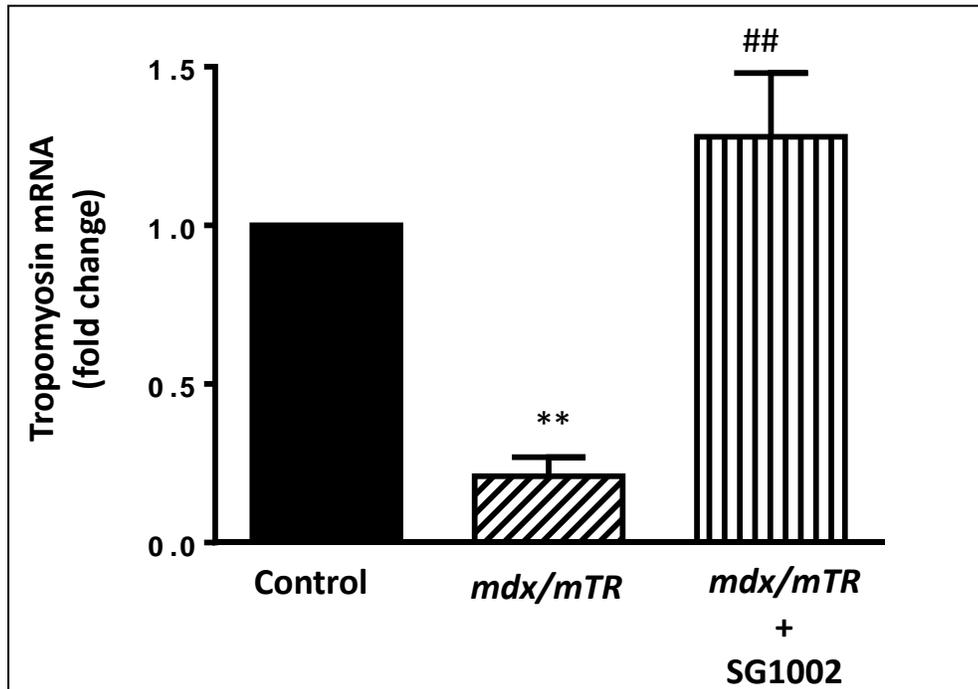
**Figure 12.** *Expression of calponin in gastric smooth muscle from control and mdx mice.* Left Panel: RNA was isolated from gastric smooth muscle of 3-month old control and mdx mice. mRNA expression of calponin was measured using specific primers by quantitative RT-PCR and expressed as fold change over control. Values are mean  $\pm$  SEM of 4-5 separate experiments. mRNA expression of calponin was significantly decreased in gastric smooth muscle from mdx mice compared to control (\*\*  $p < 0.05$ ). Right panel: Lysates were prepared from gastric smooth muscle of control and mdx mice and expression of calponin was measured by western blot using specific antibody. Representative western blot image of 4 separate experiments was shown in the figure. Densitometry analysis of all the images showed no difference in the expression of calponin in gastric smooth muscle between control and mdx mice.



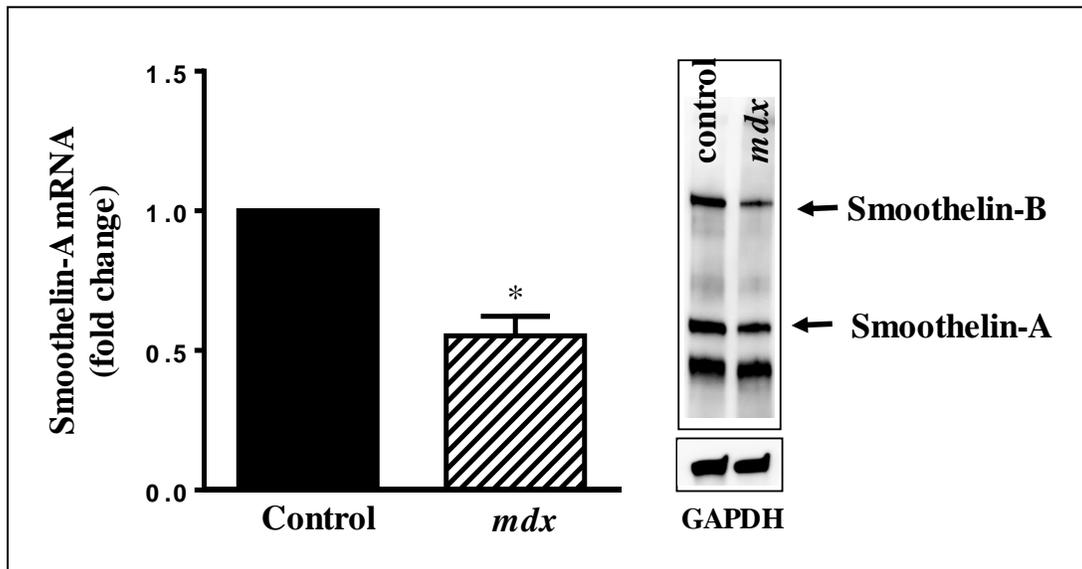
**Figure 13.** *Effect of H<sub>2</sub>S on calponin expression in gastric smooth muscle of mdx/mTR mice. RNA was isolated from gastric smooth muscle of 9-month old control and mdx/mTR mice and mdx/mTR mice treated with SG1002, an orally active H<sub>2</sub>S donor. mRNA expression of calponin was measured using specific primers by quantitative RT-PCR and expressed as fold change over control. Values are mean ± SEM of 4 separate experiments. mRNA expression of calponin was significantly decreased in colonic smooth muscle from mdx/mTR mice compared to control (\*\* p<0.05). Treatment with SG1002 significantly reversed the inhibition in calponin mRNA expression (## p<0.05).*



**Figure 14. Expression of tropomyosin in gastric smooth muscle from control and mdx mice.** *Left Panel: RNA was isolated from gastric smooth muscle of 3-month old control and mdx mice. mRNA expression of tropomyosin was measured using specific primers by quantitative RT-PCR and expressed as fold change over control. Values are mean  $\pm$  SEM of 4-5 separate experiments. mRNA expression of tropomyosin was significantly decreased in gastric smooth muscle from mdx mice compared to control (\*\*  $p < 0.05$ ). Right panel: Lysates were prepared from gastric smooth muscle of control and mdx mice and expression of tropomyosin was measured by western blot using specific antibody. Representative western blot image of 4 separate experiments was shown in the figure. Densitometry analysis of all the images showed no difference in the expression of tropomyosin in gastric smooth muscle between control and mdx mice.*



**Figure 15.** *Effect of H<sub>2</sub>S on tropomyosin expression in gastric smooth muscle of mdx/mTR mice. RNA was isolated from gastric smooth muscle of 9-month old control and mdx/mTR mice and mdx/mTR mice treated with SG1002, an orally active H<sub>2</sub>S donor. mRNA expression of tropomyosin was measured using specific primers by quantitative RT-PCR and expressed as fold change over control. Values are mean ± SEM of 4 separate experiments. mRNA expression of tropomyosin was significantly decreased in colonic smooth muscle from mdx/mTR mice compared to control (\*\* p<0.05). Treatment with SG1002 significantly reversed the inhibition in tropomyosin expression (## p<0.05).*



**Figure 16. Expression of smoothelin in gastric smooth muscle from control and mdx mice.** Left Panel: RNA was isolated from gastric smooth muscle of 3-month old control and mdx mice. mRNA expression of smoothelin-A was measured using specific primers by quantitative RT-PCR and expressed as fold change over control. Values are mean  $\pm$  SEM of 4-5 separate experiments. mRNA expression of smoothelin-A was significantly decreased in gastric smooth muscle from mdx mice compared to control (\*\*  $p < 0.05$ ). Right panel: Lysates were prepared from gastric smooth muscle of control and mdx mice and expression of smoothelin was measured by western blot using specific antibody. Representative western blot image of 4 separate experiments was shown in the figure. Densitometry analysis of all the images showed no difference in the expression of smoothelin in gastric smooth muscle between control and mdx mice.

### 3. Summary of Results.

- Contraction in response to acetylcholine was decreased in *gastric muscle strips* from *mdx* mice compared to age-matched controls.
- Relaxation in response to SNP was not different between gastric muscle strips from control and *mdx* mice
- Contraction in response to acetylcholine was also decreased in *isolated gastric smooth muscle cells* from *mdx* mice compared to age-matched controls.
- Relaxation in response to SNP or isoproterenol was not different between gastric muscle cells from control and *mdx* mice
- mRNA expression of caldesmon, calponin, tropomyosin and smoothelin was decreased in gastric smooth muscle from *mdx* compared to control mice.
- Protein expression of caldesmon, calponin, tropomyosin and smoothelin mRNA was not different between gastric smooth muscle from control and *mdx* mice.
- Contraction in response to acetylcholine was decreased in *gastric muscle strips* from *mdx/mTR* mice compared to age-matched controls.
- mRNA expression of caldesmon, calponin, tropomyosin and smoothelin was decreased in gastric smooth muscle from *mdx/mTR* mice compared to age-matched control mice.
- Treatment of *mdx/mTR* mice with H<sub>2</sub>S releasing agent SG1002 reversed the decrease in the mRNA expression of caldesmon, calponin and tropomyosin and also muscle contraction to normal levels.

## Chapter 4: DISCUSSION

Dystrophin is an important structural protein present in skeletal, cardiac and smooth muscle and mutations in the dystrophin gene lead to complex diseases affecting several aspects of skeletal, cardiac and smooth muscle physiology. Deficiency of dystrophin due to a loss-of-function mutation in the dystrophin gene is the main genetic defect in Duchenne's muscular dystrophy (DMD). Clinical and experimental studies, in addition to the established skeletal and cardiac muscle disorders, have revealed motility disorders in the gut of DMD patients and in animal models of DMD. The clinical manifestations of motility disorders in DMD patients include bloating, early satiety; feeling of fullness and constipation suggesting delayed gastric emptying and decreased intestinal motility. The treatment recommended to relieve gastrointestinal symptoms are prokinetics that are mainly aimed at correcting motility disorders. Despite the established role in skeletal and cardiac muscle, and the emerging role in vascular and airway smooth muscle biology, very little is known about the functional role of dystrophin in the gastric smooth muscle that is responsible for generating force for gastric motility and gastric emptying.

In this study we hypothesized that dystrophin is a determinant of gastric smooth muscle function, and this underpins a role in determining gastric motility *in vivo*. We used *mdx* and *mdx/mTR* mice lacking dystrophin to perform biochemical and functional studies. We measured isometric contraction studies *ex vivo* using gastric muscle strips. We also measured smooth muscle function using isolated muscle cells. Acetylcholine was used as a contractile agonist as it is the main excitatory neurotransmitter in the gut. We examined the changes in the expression of calponin, caldesmon, tropomyosin and

smoothelin by qualitative RT-PCR and western blot comparing tissue from *mdx* and wild-type mice. We demonstrated that dystrophin is a determinant of gastric smooth muscle contraction, as there is a decrease in contraction in *mdx* mice compared to controls. This effect was associated with decrease in the expression of all thin-filament associated proteins in gastric smooth muscle cells.

Our studies in muscle strips showed that contraction in response to acetylcholine was decreased compared to response in muscle strips from normal mice. Because both enteric neurons and ICC express dystrophin, a decrease in contraction in response to EFS in muscle strip might be due to decrease in transmitter release and or altered function of ICC. Studies by Mule and Serio (2002) showed that the amplitude of spontaneous contractions in gastric muscle strips from *mdx* are similar to that in normal mice. The spontaneous tone developed by gastric muscle was higher in *mdx* mice than that of normal mice and blockade of nNOS with L-NAME augmented contraction in normal mice without affecting contractions in *mdx* mice (Mule et al, 2006). These studies suggests that impairment of nitrenergic neurotransmission but not ICC or post-junctional effector cells. Our studies provide evidence that dystrophin in smooth muscle plays an important role in the contractile properties of gastric smooth muscle. Contraction in response to the main contractile transmitter, acetylcholine, showed a decrease in muscle strips as well in isolated muscle cells. Studies in isolated muscle cells conclusively demonstrate that lack of dystrophin leads to decreased muscle contraction. A limitation of our study is that we did not go beyond acetylcholine to assess the smooth muscle function. Although smooth muscle expresses both muscarinic m2 and m3 receptors, contraction in response to acetylcholine is mainly

mediated by activation of m3 receptors. A decrease in contraction in response to acetylcholine may reflect a decrease in the expression of m3 receptors and/or changes in the signaling pathways activated by m3 receptors such as  $G\alpha_q$ , phospholipase- $\beta$ 1 (PLC- $\beta$ 1) and  $IP_3$ -dependent  $Ca^{2+}$  release. One approach to address this issues is to examine the response to other contractile agonist such as substance P which acts on distinct  $G\alpha_q$ -coupled receptor or examine the response to depolarizing concentrations of KCl that causes  $Ca^{2+}$  influx to mediate contraction bypassing the GPCR activation and  $IP_3$ -dependent  $Ca^{2+}$  release mechanism. Future studies are planned to understand the intracellular mechanisms responsible for altered contraction to examine the role of dystrophin in the regulation of receptors for excitatory transmitters and the intracellular signaling pathways that mediate muscle contraction. Although the intracellular mechanisms responsible for altered muscle contraction in *mdx* mice have not been established in our study, some studies provide clues in this area. A decrease in the expression of reduced type-2-ryanodine receptor expression resulting in the compromised sarcoplasmic reticulum  $Ca^{2+}$  release was attributed to a decrease in contraction of duodenal smooth muscle strips in *mdx* mice (Morel et al. 2004). Our findings from studies with gastric smooth muscle of *mdx* mice demonstrate that the dystrophin plays a similar role in gut smooth muscle, as it does in vascular and airway smooth muscle.

Lack of effective animal models mimicking human DMD limited our understanding of the pathophysiology and the development of effective therapeutic strategies. The most widely used *mdx* model exhibits only mild phenotype compared to DMD patients. A number of mouse models with more severe dystrophic phenotypes

have been created and these include double KO mice lacking dystrophin and utrophin, MyoD,  $\alpha$ 7integrin,  $\alpha$ -dystrobrevin or glycan processing. Except dystrophin all other proteins are not absent in the muscle of DMD patients and thus do not faithfully mimic DMD. Later studies showed that species-specific differences in the telomere length account for the differences in the disease severity. The severity of DMD patients is postulated to be due to loss of functional muscle stem cells and the mild phenotype of *mdx* is postulated to be due to greater reserve of muscle stem cells, which is facilitated by longer telomeres in mice (>40 kilobases) compared to humans (~5-15 kilobases). Length of the telomeres determines the replicative life of cells and a reduction in telomere length in the muscle stem cells limits the stem cell pool and muscle repair. Telomerase consisting protein telomerase reverse transcriptase and telomerase RNA component (TERC or mTR) maintains the length of the telomeres. Ablation of RNA component of telomerase results in shortened telomeres. Mice lacking both dystrophin and the RNA component of telomerase (*mdx/mTR*) closely approximates human DMD. Compared to single KO mice, the double KO mouse models exhibit exacerbated disease phenotypes based on various criteria such as increased serum creatine kinase levels, skeletal muscle fibrosis, exhaustion of mice on treadmill, kyphosis, skeletal and cardiac muscle myopathies and shortened life span. The cardiomyocytes of DMD patients have 45% shorter telomeres compared to cardiomyocytes of normal individuals highlighting the importance of telomere shortening (Mourkioti et al., 2013). There is a 14-fold greater shortening of telomeres in skeletal muscle of DMD patients compared to normal (Decary et al., 2000). Similarly, tibialis anterior muscle from ~24 month old *mdx* mice have 30% shorter telomeres compared to age-matched control. Shortening to

telomeres results from unlimited proliferation of degenerating muscle in DMD. These findings also suggests that DMD is initiated by lack of dystrophin due to genetic defect and progresses into premature cardiovascular and muscle aging. In our study we used both *mdx* and *mdx/mTR* mice to demonstrate the importance of dystrophin in gastric smooth muscle function. Our studies also demonstrate that the changes in the mRNA expression of thin filament associated proteins are greater in *mdx/mTR* mice compared *mdx* mice.

Several pharmacological approaches to treat DMD have been investigated. These include exon skipping, augmentation of cGMP pathway via inhibition of phosphodiesterase 5 and corticosteroids; the latter is more established and currently used. Increased oxidative stress in skeletal muscle has been implicated in the pathogenesis of DMD. In DMD muscle biopsies, there is 2 fold increase in protein carbonyl groups and isoprostanes reflecting protein damage and lipid peroxidation, respectively, due to reactive oxygen species. Similar increase in protein oxidation and lipid peroxidation was also observed in muscle from *mdx* mice. Genes involved in ECM remodeling and fibrosis (different types of collagen, matrix metalloproteinases, transforming growth factor  $\beta$ 1) are altered both during the onset and disease progression. Similarly, DNA microarray studies in *mdx* muscle indicate that nearly 30% of genes that are differentially expressed are associated with inflammation and these include TNF $\alpha$ , IL-1 $\beta$ , inflammatory chemokines and their receptors. Inflammatory gene expression profile in *mdx* muscle is closely similar (~70%) to that observed in DMD patients (Evans et al., 2009). Administration of anti-oxidants in *mdx* mice was shown to reduce muscle damage and improve muscle function. Treatment of *mdx* mice with the

anti-oxidant N-acetyl cysteine decreased oxidative stress and protected the muscle fiber from stretch-induced damage. Studies also showed that long-term treatment of *mdx* mice with resveratrol (a naturally occurring polyphenol compound) that reduces oxidative stress decreased reactive oxygen species and attenuated fibrosis in skeletal muscle (Hori et al., 2011) (Kumo and Horio, 2016) (Terrill et al., 2013).

Hydrogen sulfide (H<sub>2</sub>S) has been recognized as an important signaling molecule to mitigate oxidative stress. The anti-oxidant effect of H<sub>2</sub>S include quenching reactive oxygen species (ROS) and reactive nitrogen species (RNS), modulating cellular levels of glutathione and thioredoxin, and activating nuclear factor (erythroid-derived 2)-like 2 (*NRF2*), a key transcription factor in the expression of antioxidant enzymes. This suggests that exogenous supplementation of H<sub>2</sub>S could be employed as a beneficial strategy to improve the injury induced by oxidative stress. In this study we showed that long-term treatment of *mdx/mTR* mice with H<sub>2</sub>S donor SG1002 reversed the decrease in contractile protein expression and restored the contractile function to near normal levels. SG1002 has been shown to prevent cardiac dysfunction in *mdx/mTR* mice (Dr. Salloum's unpublished observation). Thus, these results demonstrate the significant therapeutic potential of H<sub>2</sub>S donor in dystrophin-deficient *mdx/mTR* mice.

In conclusion, our functional data using *mdx* and *mdx/ mTR* mice indicate that the dystrophin plays an important role in contractile properties of gastric smooth muscle and suggests that it is also important gastric motility in vivo. Our studies also suggest that with better understanding of the precise mechanism behind the role of H<sub>2</sub>S in reducing oxidative stress, treatment with H<sub>2</sub>S donor could be a promising therapeutic approach for DMD patients.

## REFERENCES

- Alves, G., Silva, L., Rosa, E., Aboulaflia, J., Freymüller-Haapalainen, E., Souccar, C., & Nouailhetas, V. (2014). Intestine of dystrophic mice presents enhanced contractile resistance to stretching despite morphological impairment. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, 306(3), G191-9.
- Barlow, C., Rose, P., Pulver-Kaste, R., & Lounsbury, K. (2006). Excitation–transcription coupling in smooth muscle. *Journal of Physiology*, 570(1), 59-64.
- Barrett, K. (2014). *Gastrointestinal physiology* (2nd ed., Lange physiology series).
- Borrelli, O., Salvia, G., Mancini, V., Santoro, L., Tagliente, F., Romeo, E., & Cucchiara, S. (2005). Evolution of gastric electrical features and gastric emptying in children with Duchenne and Becker muscular dystrophy. *American Journal Of Gastroenterology*, 100(3), 695-702.
- Berridge M. J. (2008). Smooth muscle cell calcium activation mechanisms. *The Journal of physiology*, 586(21), 5047-61.
- Choi, M. H., Ow, J. R., Yang, N. D., & Taneja, R. (2015). Oxidative Stress-Mediated Skeletal Muscle Degeneration: Molecules, Mechanisms, and Therapies. *Oxidative medicine and cellular longevity*, 2016, 6842568.
- Cooke, Allan R., (1975) Control of Gastric Emptying and Motility *Gastroenterology*, Volume 68 , Issue 4 , 804 - 816
- Cui, J., Liu, L., Zou, J., Qiao, W., Liu, H., Qi, Y., & Yan, C. (2012). Protective effect of endogenous hydrogen sulfide against oxidative stress in gastric ischemia-reperfusion injury. *Experimental and therapeutic medicine*, 5(3), 689-694.
- Decary, Ben Hamida, Mouly, Barbet, Hentati, & Butler-Browne. (2000). Shorter telomeres in dystrophic muscle consistent with extensive regeneration in young children. *Neuromuscular Disorders*, 10(2), 113-120.
- Evans, N. P., Misyak, S. A., Robertson, J. L., Bassaganya-Riera, J. W., & Grange, R. (2009). Dysregulated Intracellular Signaling and Inflammatory Gene Expression During Initial Disease Onset in Duchenne Muscular Dystrophy. *American Journal of Physical Medicine & Rehabilitation*, 88(6), 502-522.

- Eves, R., Webb, B., Zhou, S., & Mak, A. (2006). Caldesmon is an integral component of podosomes in smooth muscle cells. *Journal of Cell Science*, 119(Pt 9), 1691-702.
- Fiorucci S., Distrutti E., Cirino G., & Wallace J.,(2006).The Emerging Roles of Hydrogen Sulfide in the Gastrointestinal Tract and Liver. *Gastroenterology*, 131(1), 259-271.
- Garella, & Baccari. (2010). Contribution of endogenous nitregeric and peptidergic influences to the altered neurally-induced gastric contractile responses in strips from dystrophic (mdx) mice. *Regulatory Peptides*, 160(1), 57-63.
- Gash M., Varacallo M., (2018) Physiology, Muscle Contraction. Treasure Island (FL): StatPearls.
- Hammar, O. (2013). *Gastrointestinal motility and the role of gonadotropin-releasing hormone (GnRH)* Internal Medicine Research Unit
- Hartle, M. D., & Pluth, M. D. (2016). A practical guide to working with H<sub>2</sub>S at the interface of chemistry and biology. *Chemical Society reviews*, 45(22), 6108-6117.
- Hori, Y., Kuno, A., Hosoda, R., Tanno, M., Miura, T., Shimamoto, K., & Horio, Y. (2011). Resveratrol ameliorates muscular pathology in the dystrophic mdx mouse, a model for Duchenne muscular dystrophy. *The Journal of Pharmacology and Experimental Therapeutics*, 338(3), 784-94.
- Hu, L. , Lu, M. , Tiong, C. X., Dawe, G. S., Hu, G. and Bian, J. (2010), Neuroprotective effects of hydrogen sulfide on Parkinson's disease rat models. *Aging Cell*, 9: 135-146.
- Huber, P. (1997). Caldesmon. *International Journal of Biochemistry and Cell Biology*,29(8), 1047-1051.
- Huang, Chengrong, Huang, Yu, Kan, Juntao, Liu, Xu, Ma, Fenfen, Tran, Ba Hieu, . . . Zhu, Yi Zhun. (2013). Cardioprotective Effects of a Novel Hydrogen Sulfide Agent–Controlled Release Formulation of S-Propargyl-Cysteine on Heart Failure Rats and Molecular Mechanisms.
- Kabil, O., Motl, N., & Banerjee, R. (2014). H<sub>2</sub>S and its role in redox signaling. *Biochimica Et Biophysica Acta (BBA) - Proteins and Proteomics*, 1844(8), 1355-1366.
- Kamoun, P. (2004). Endogenous production of hydrogen sulfide in mammals. *Amino Acids*, 26(3), 243-254.

- Kimura, Y, & Kimura, H. (2004). Hydrogen sulfide protects neurons from oxidative stress. *Faseb Journal*, 18(7), 1165-1167.
- Lapidos, K. A., Kakkar, R. M., McNally, E., & Kass, D. (2004). The Dystrophin Glycoprotein Complex: Signaling Strength and Integrity for the Sarcolemma. *Circulation Research: Journal of the American Heart Association*, 94(8), 1023-1031.
- Lovering, R. M., Porter, N. C., & Bloch, R. J. (2005). The muscular dystrophies: from genes to therapies. *Physical therapy*, 85(12), 1372-88.
- Liu, H., Bai, X., Shi, S., & Cao, Y. (2009). Hydrogen sulfide protects from intestinal ischaemia–reperfusion injury in rats. *Journal of Pharmacy and Pharmacology*, 61(2), 207-212.
- Makhlouf, G. M. and Murthy, K. S. (2015). Smooth muscle of the gut. In Yamada' s Textbook of Gastroenterology (eds D. K. Podolsky, M. Camilleri, J. G. Fitz, A. N. Kalloo, F. Shanahan and T. C. Wang).
- Manning, J., Buckley, M. M., O'Halloran, K. D. and O'Malley, D. (2016), *In vivo* neutralization of IL-6 receptors ameliorates gastrointestinal dysfunction in dystrophin-deficient *mdx* mice. *Neurogastroenterol. Motil.*, 28: 1016-1026.
- Marston S., El-Mezgueldi M. (2008) Role of Tropomyosin in the Regulation of Contraction in Smooth Muscle. In: Gunning P. (eds) Tropomyosin. *Advances in Experimental Medicine and Biology*, vol 644. Springer, New York, NY
- Meerveld B., Johnson A.C., Grundy D. (2017) Gastrointestinal Physiology and Function. In: Greenwood-Van Meerveld B. (eds) Gastrointestinal Pharmacology. *Handbook of Experimental Pharmacology*, vol 239. Springer, Cham
- Morel, JI, Rakotoarisoa, L, Jeyakumar, LH, Fleischer, S, Mironneau, C, & Mironneau, J. (2004). Decreased expression of ryanodine receptors alters calcium-induced calcium release mechanism in *mdx* duodenal myocytes. *Journal Of Biological Chemistry*, 279(20), 21287-21293.
- Mourkioti, F., Kustan, J., Kraft, P., Day, J., Zhao, M., Kost-Alimova, M., . . . Blau, H. (2013). Role of telomere dysfunction in cardiac failure in Duchenne muscular dystrophy. *Nature Cell Biology*, 15(8), 895-U300.
- Mulè, F., Amato, A., & Serio, R. (2010). Gastric emptying, small intestinal transit and fecal output in dystrophic (*mdx*) mice. *The Journal of Physiological Sciences*, 60(1), 75-79.

- Mulè, F., & Serio, R. (2002). Spontaneous mechanical activity and evoked responses in isolated gastric preparations from normal and dystrophic (*mdx*) mice. *Neurogastroenterology & Motility*, 14(6), 667-675.
- Murthy, K. (2006). Signaling for contraction and relaxation in smooth muscle of the gut. *Annual Review of Physiology*, 68, 345-74.
- Nowak, K., & Davies, K. (2004). Duchenne muscular dystrophy and dystrophin: Pathogenesis and opportunities for treatment. *EMBO Reports*, 5(9), 872-876.
- Niessen, Rensen, Van Deursen, De Man, De Laet, Vanderwinden, Van Eys. (2005). Smoothelin-A Is Essential for Functional Intestinal Smooth Muscle Contractility in Mice. *Gastroenterology*, 129(5), 1592-1601.
- Patacchini, R., Santicoli, P., Giuliani, S., & Maggi, C. A. (2004). Hydrogen sulfide (H<sub>2</sub>S) stimulates capsaicin-sensitive primary afferent neurons in the rat urinary bladder. *British journal of pharmacology*, 142(1), 31-4.
- Patel, C., & Rattan, S., (2006). Spontaneously tonic smooth muscle has characteristically higher levels of RhoA/ROK compared with the phasic smooth muscle. *American journal of physiology. Gastrointestinal and liver physiology*, 291 5, G830-7.
- Punnoose, A., Burke, A., & Golub, Robert. (2011). Muscular Dystrophy. *JAMA*, 306(22), 2526.
- Rao, J., & Wang, J.-Y. (2011). *Regulation of gastrointestinal mucosal growth* (Vol. 3, Colloquium digital library of life sciences). San Rafael, Calif.: Morgan & Claypool.
- Rodrigues, M., Echigoya, Y., Fukada, S. I., & Yokota, T. (2016). Current Translational Research and Murine Models For Duchenne Muscular Dystrophy. *Journal of neuromuscular diseases*, 3(1), 29-48.
- Sacco, A., Mourkioti, F., Tran, R., Choi, J., Llewellyn, M., Kraft, P., Shkreli, M., Delp, S., Pomerantz, J. H., Artandi, S. E., ... Blau, H. M. (2010). Short telomeres and stem cell exhaustion model Duchenne muscular dystrophy in *mdx/mTR* mice. *Cell*, 143(7), 1059-71.
- Seko, Ito, Kureishi, Okamoto, Moriki, Onishi, Nakano. (2003). Activation of RhoA and inhibition of myosin phosphatase as important components in hypertension in vascular smooth muscle. *Circulation Research*, 92(4), 411-418.
- Sinha, R., Sarkar, S., Khaitan, T., & Dutta, S. (2017). Duchenne muscular dystrophy: Case report and review. *Journal of family medicine and primary care*, 6(3), 654-656.

- Terrill, J., Radley-Crabb, H., Iwasaki, T., Lemckert, F., Arthur, P., & Grounds, M. (2013). Oxidative stress and pathology in muscular dystrophies: Focus on protein thiol oxidation and dysferlinopathies. *FEBS Journal*, 280(17), 4149-4164.
- Unno, Matsuyama, Okamoto, Sakamoto, Yamamoto, Tanahashi Komori. (2006). Muscarinic cationic current in gastrointestinal smooth muscles: Signal transduction and role in contraction. *Autonomic and Autacoid Pharmacology*, 26(3), 203-217.
- Vannucchi, M., Garella, R., Cipriani, G., & Baccari, M. (2011). Relaxin counteracts the altered gastric motility of dystrophic (mdx) mice: Functional and immunohistochemical evidence for the involvement of nitric oxide. *American Journal of Physiology. Endocrinology and Metabolism*, 300(2), E380-91.
- Vannucchi, M., Zizzo, M., Zardo, C., Pieri, L., Serio, R., Mulè, F., & Fausone-Pellegrini, M. (2004). Ultrastructural changes in the interstitial cells of Cajal and gastric dysrhythmias in mice lacking full-length dystrophin mdx mice. *Journal of Cellular Physiology*, 199(2), 293-309.
- Wang, R., (2012). Physiological Implication of Hydrogen Sulfide: A Whiff Exploration that Blossomed. *Physiological Reviews*, 92(2), 791-896.
- Wallace, J., Ferraz, J., & Muscara, M. (2012). Hydrogen Sulfide: An Endogenous Mediator of Resolution of Inflammation and Injury. *Antioxidants & Redox Signaling*, 17(1), 58-67.
- Wang, C-L. (2001). Caldesmon and Smooth-Muscle Regulation. *Cell biochemistry and biophysics*. 35. 275-88.
- Webb, R. (2003). Smooth muscle contraction and relaxation. *Advances in Physiology Education*, 27(1-4), 201-6.
- Winder, & Walsh. (1993). Calponin: Thin filament-linked regulation of smooth muscle contraction. *Cellular Signalling*, 5(6), 677-686.
- Xie, Z. Z., Liu, Y., & Bian, J. S. (2016). Hydrogen Sulfide and Cellular Redox Homeostasis. *Oxidative medicine and cellular longevity*, 2016, 6043038.
- Yiu, E., & Kornberg, A. (2015). Duchenne muscular dystrophy. *Journal of Paediatrics and Child Health*, 51(8), 759-764.