Changes in the Expression of Thin Filament-Associated Proteins in Colonic Smooth Muscle from Mice During Inflammation

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Changes in the Expression of Thin Filament-Associated Proteins in Colonic Smooth Muscle from Mice During Inflammation

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at Virginia Commonwealth University.

By

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Bachelor of Science in Clinical Laboratory, King Saud University, 2004.

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgments</td>
<td>ii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>Abstract</td>
<td>viii</td>
</tr>
<tr>
<td>Chapter 1: INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1. Smooth Muscle</td>
<td>1</td>
</tr>
<tr>
<td>1.1. Mechanisms involved in the regulation of MLC20 phosphorylation</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1 MLCK</td>
<td>3</td>
</tr>
<tr>
<td>1.1.2 MLCP</td>
<td>4</td>
</tr>
<tr>
<td>1.2. Mechanisms involved in the regulation of acto-myosin interaction by thin-filament associated proteins</td>
<td>6</td>
</tr>
<tr>
<td>1.2.1 Tropomyosin</td>
<td>6</td>
</tr>
<tr>
<td>1.2.2 Caldesmon</td>
<td>6</td>
</tr>
<tr>
<td>1.2.3 Calponin</td>
<td>7</td>
</tr>
<tr>
<td>1.2.4 Smoothelin</td>
<td>7</td>
</tr>
</tbody>
</table>
1.3. Mechanisms involved in the regulation of cytoskeleton remodeling.........................8

2. Inflammation..............................................................................................................8

2.1. Animal models......................................................................................................8

2.2. Colonic dysmotility.............................................................................................9

2.2.1. Enteric nervous system.....................................................................................10

2.2.2. Interstitial cells of Cajal..................................................................................12

2.2.3. Smooth muscle...............................................................................................12

Chapter 2: Materials and Methods..............................................................................16

1. Materials..................................................................................................................16

1.1. Chemical and Antibodies....................................................................................16

1.2. Animals................................................................................................................16

2. Methods..................................................................................................................17

2.1. Induction of Colitis..............................................................................................17

2.2. Collection of Tissue..............................................................................................17

2.3. Preparation of dispersed muscle cells.................................................................18

2.4. Real-time RT-PCR..............................................................................................18
2.5. Western blot analysis.................................................................20
2.6. Immunohistochemistry.............................................................21
2.7. Measurement of contraction in dispersed smooth muscle cells......................22
3. Statistical Analysis........................................................................22
Chapter 3: RESULTS........................................................................29
3.1. Evaluation of colonic inflammation................................................30
3.2. Changes in the expression of thin-filament associated proteins in TNBS-colitis mice........36
  3.2.1. Actins..................................................................................36
  3.2.2. Caldesmon............................................................................37
  3.2.3 Calponin...............................................................................37
  3.2.4. Tropomyosin.....................................................................38
  3.2.5. Smoothelin.........................................................................39
3.3. Changes in the expression of thin-filament associated proteins in DSS-induced colitis......52
3.4. Effect of inflammation on muscle contraction........................................61

Chapter 4: DISCUSSION..................................................................64
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1:</td>
<td>Expression of IL-1β in the colonic muscle after TNBS or DSS treatment</td>
<td>32</td>
</tr>
<tr>
<td>Figure 2:</td>
<td>Expression of TNF-α in the colonic muscle after TNBS or DSS treatment</td>
<td>34</td>
</tr>
<tr>
<td>Figure 3:</td>
<td>Expression of α-actin in the colonic muscle after TNBS treatment</td>
<td>40</td>
</tr>
<tr>
<td>Figure 4:</td>
<td>Expression of γ-actin in the colonic muscle after TNBS treatment</td>
<td>42</td>
</tr>
<tr>
<td>Figure 5:</td>
<td>Expression of h-caldesmon in the colonic muscle after TNBS treatment</td>
<td>44</td>
</tr>
<tr>
<td>Figure 6:</td>
<td>Expression of h1-calponin in the colonic muscle after TNBS treatment</td>
<td>46</td>
</tr>
<tr>
<td>Figure 7:</td>
<td>Expression of α-tropomyosin in the colonic muscle after TNBS treatment</td>
<td>48</td>
</tr>
<tr>
<td>Figure 8:</td>
<td>Expression of smoothelin-A in the colonic muscle after TNBS treatment</td>
<td>50</td>
</tr>
<tr>
<td>Figure 9:</td>
<td>Expression of h-caldesmon in the colonic muscle after DSS treatment</td>
<td>53</td>
</tr>
<tr>
<td>Figure 10:</td>
<td>Expression of h1-calponin in the colonic muscle after DSS treatment</td>
<td>55</td>
</tr>
<tr>
<td>Figure 11:</td>
<td>Expression of α-tropomyosin in colonic muscle after DSS treatment</td>
<td>57</td>
</tr>
<tr>
<td>Figure 12:</td>
<td>Expression of smoothelin-A in the colonic muscle after TNBS or DSS treatment</td>
<td>59</td>
</tr>
<tr>
<td>Figure 13:</td>
<td>Effect of TNBS treatment on acetylcholine-induced muscle contraction</td>
<td>63</td>
</tr>
</tbody>
</table>
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>RT-PCR TaqMan primers</td>
<td>23</td>
</tr>
<tr>
<td>Table 2</td>
<td>RT-PCR SYBRgreen primers</td>
<td>25</td>
</tr>
<tr>
<td>Table 3</td>
<td>Western blot antibodies</td>
<td>27</td>
</tr>
</tbody>
</table>
Abstract

Changes in the Expression of Thin Filament-Associated Proteins in Colonic Smooth Muscle from Mice during Inflammation

By
Reem Alkahtani

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2011

Director: S. Murthy Karnam, Ph.D.
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The contractility of smooth muscle in inflammatory bowel disease and experimental colitis is reduced due to inhibition of neurotransmitter release and a decrease in the response of smooth muscle to contractile agonists. We and others have shown that inflammation induced by TNBS treatment alters the expression and/or activity of signaling molecules involved in the regulation of Ca$^{2+}$ mobilization, MLC$_{20}$ phosphorylation and contraction in colonic smooth muscle. Although, thin filament-associated proteins such as calponin, caldesmon, tropomyosin and smoothelin do not directly participate in contraction, they regulate acto-myosin interaction and thus, muscle contraction. Calponin, caldesmon and tropomyosin inhibit actomyosin interaction
and the inhibition is relieved upon phosphorylation of these proteins. Recent studies have shown that visceral smooth muscle from smoothelin knockout mice exhibited decreased contraction. However, the effect of inflammation on the expression of thin filament-associated proteins is not known. The aim of the present study is to determine the changes in the expression of calponin, caldesmon, tropomyosin, and smoothelin in colonic circular smooth muscle from TNBS- and DSS-induced colitis in mice. The animals were euthanized on day 3 and a segment of inflamed distal colon was removed. Colonic muscle strips from colitis mice and control mice were dissected for western blot and real-time RT-PCR analysis; contraction was measured by scanning micrometry in cells isolated from the muscle strips. Contraction in response to acetylcholine in muscle cells isolated from colonic muscle strips derived from mice with TNBS colitis was significantly inhibited compared with the response of cells derived from untreated colon or colon treated with ethanol. Expression of α-actin, γ-actin calponin, caldesmon, smoothelin-A and tropomyosin mRNA in muscle strips from TNBS or DSS colitis was significantly increased compared to control animals. Similarly, expression of α-actin, calponin, caldesmon, smoothelin-A and tropomyosin protein as determined by western blot was significantly increased compared to control animals. We conclude that the expression of α-actin, γ-actin calponin, caldesmon, smoothelin-A and tropomyosin is upregulated in colonic circular smooth muscle from TNBS or DSS colitis. Increase in the expression of calponin, caldesmon and tropomyosin, which act to inhibit acto-myosin interaction, could contribute to decrease in smooth muscle contraction.
CHAPTER 1: INTRODUCTION

1. SMOOTH MUSCLE

Smooth muscle has wide distribution in the body. The contractile activity of smooth muscle is critical for normal physiology of vascular system, respiratory system, gastrointestinal system and the genitourinary systems. Malfunction of smooth muscle in these systems leads to a variety of clinical disorders. To mediate the contractile function smooth muscle expresses selective catalog of genes that define the smooth muscle cells from other cell types. The main contractile apparatus in the smooth muscle consists of two types of filaments: thin filaments and thick filaments (28, 29, 38, 67, 69, 90, 91). Thin filaments consist of ~42 kDa protein actin, which exists in vivo as filamentous actin (F-actin), and associated proteins such as caldesmon, calponin, tropomyosin and smoothelin. Smooth muscle contains similar amounts of actin as skeletal muscle. Thick filaments are aggregates of myosin molecule, the molecular motor of all muscles. Each myosin molecule is a hexamer consisting of a pair of myosin heavy chain (MHC), a pair of 17-kDa essential light chains, and a pair of 20-kDa myosin light chains (MLC20). Each MHC is asymmetric and contains a globular head at the N-terminus and a coiled-tail at the C-terminus. The globular head contains ATP binding site, actin binding site and intrinsic ATPase activity (28, 67). The interaction of actin with myosin and subsequent hydrolysis of ATP is the fundamental reaction whereby chemical energy is converted into mechanical energy. This interaction generates force and shortens muscle as a result of the
sliding of overlapping, interdigitating thin and thick filaments. Smooth muscle contains less myosin than skeletal muscle, but generates as much force as striated muscle. Alternative splicing of smooth muscle MHC produces 2 C-terminal isoforms (SM1 and SM2) and 2 N-terminal isoforms (SM-A and SM-B). The SM1 isoform has been demonstrated to form better contractile filaments compared to SM2 isoform, whereas the SM-B isoform is associated with increased myosin ATPase activity and increased shortening velocity, a characteristic of phasic phenotype of smooth muscle, compared with the SM-A isoforms (28, 67).

“Excitation-contraction coupling” in all muscle is initiated by increase in intracellular Ca$^{2+}$ leading to changes in the activity of, or interactions of, actin and myosin. However, the contraction of smooth muscle is far more than a simple calcium switch and involves complex, and often redundant, signaling pathways that lead to actomyosin interaction. The fine-tuning of smooth muscle function is made possible mainly because of the dependent acto-myosin interaction on 20-kDa myosin light chain (MLC20) phosphorylation at Ser19, which is both necessary and sufficient for acto-myosin interaction (28, 29, 38, 67, 69, 81, 89-91). Phosphorylation and dephosphorylation of MLC$_{20}$ are directly correlated to smooth muscle contraction and relaxation, respectively, and the levels of MLC$_{20}$ phosphorylation are regulated by MLC kinase (MLCK) and MLC phosphatase (MLCP) activity. The complex mechanisms that regulate MLC$_{20}$ phosphorylation and acto-myosin interaction include: 1) mechanisms that regulate acto-myosin interaction through changes in MLC$_{20}$ phosphorylation levels; 2) mechanisms that regulate directly acto-myosin interaction via a set of proteins associated with actin; and 3) mechanisms that regulate cytoskeleton remodeling and transmission and maintenance of force developed by acto-myosin interaction (67).
1.1. Mechanisms involved in the regulation of MLC20 phosphorylation.

1.1.1. MLCK: An essential step in smooth muscle contraction is phosphorylation of the MLC20 at Ser19, which increases significantly the actin-activated myosin ATPase activity (67, 69, 90, 91). To date, the best-characterized and physiologically important pathway in MLC20 phosphorylation is Ca2+/calmodulin(CaM)-dependent activation of MLCK in response to increase in intracellular Ca$^{2+}$ by contractile agonists (38). In vitro studies suggest that MLC$_{20}$ phosphorylation at Ser19 is also regulated by Ca$^{2+}$-independent kinases such as integrin-linked kinase (ILK), zipper interacting protein kinase (ZIPK) and Rho kinase (69, 90, 91). The physiological significance of these kinases in terms of MLC$_{20}$ phosphorylation, however, remains unclear. MLCK is strictly dependent on Ca2+/CaM for its activation. Stimulation of smooth muscle by contractile agonists elevates the intracellular concentration of Ca$^{2+}$ ([Ca$^{2+}$]$_i$) via activation of voltage-gated Ca$^{2+}$ channels, and/or IP3 receptors/Ca2+ channels and causes Ca$^{2+}$ to bind to calmodulin (CaM). The affinity of MLCK for CaM is strongest among the CaM-dependent protein kinases (38, 98). There are three isoforms of MLCK: ubiquitous smooth muscle MLCK (smMLCK), restricted cardiac muscle MLCK (cMLCK) and skeletal muscle MLCK (skMLCK). MLCK of smooth muscle consists of an N-terminal actin-binding domain, C-terminal myosin-binding domain and a central catalytic domain that phosphorylates MLC$_{20}$. The region between the catalytic domain and C-terminal domain has been characterized as the regulatory domain for kinase activity. Due to the interaction of MLCK with actin and myosin, it is proposed that a mechanism, referred as non-kinase activity of MLCK regulated acto-myosin interaction, however, the physiological significance of non-kinase activity of MLCK is unknown.
Targeted deletion of MLCK in smooth muscle leads to decreased gut motility, urinary bladder function, and blood pressure, and also significant reduction in MLC$_{20}$ phosphorylation in response to contractile agonist. *Changes in the expression and/or activity of Ca$^{2+}$ mobilization and MLCK regulation pathways lead altered muscle contraction.*

1.1.2. MLCP: The [Ca$^{2+}$]$_i$ transient responsible for the activation of MLCK is rapidly and efficiently dissipated by Ca$^{2+}$ extrusion from the cell and uptake into sarcoplasmic Ca$^{2+}$ stores. This leads to rapid decline in MLCK activity. MLCK activity is also regulated by several kinases such as Ca$^{2+}$/calmodulin-dependent protein kinase II, p21-activated protein kinase 1 (PAK1) or AMP-dependent kinase (38, 69). Phosphorylation of MLCK by these kinases decreases the affinity for Ca$^{2+}$/CaM and inhibits the activity. However, agonist-induced MLC20 phosphorylation and contraction are maintained in the absence of elevated [Ca$^{2+}$]$_i$ and MLCK, and mechanisms responsible for this are largely correlated to inhibition of MLCP. After phosphorylation of MLC20 by MLCK, MLC20 is dephosphorylated by MLCP, the activity of which is regulated by Ca$^{2+}$-independent mechanisms often referred as Ca$^{2+}$-sensitization. The sustained contraction does not reflect Ca$^{2+}$ sensitization to low levels of Ca$^{2+}$, because complete inhibition of MLCP in the absence of Ca$^{2+}$ induces smooth muscle contraction. This contraction induced by MLCP inhibitors in the absence of Ca$^{2+}$ is blocked by high concentrations of non-specific kinase inhibitors such as staurosporine, implying participation of Ca$^{2+}$-independent MLCK kinases, probably ZIP kinase. Intracellular pathways linking receptor activation and Ca$^{2+}$-independent contraction have been investigated in several laboratories and identification of pathways was greatly facilitated by the use of highly selective, cell permeable Rho kinase inhibitor, Y27632 (69). The pathways in Ca$^{2+}$-independent contraction involve G protein
activation, MLC20 phosphorylation by a Ca2+-independent protein kinase and inhibition of MLC phosphatase via Rho kinase and/or protein kinase C (PKC). MLCP holoenzyme is heterotrimer consisting of a 37-KDa catalytic subunit of type 1 phosphatase (ppicδ), a 110- to 130-KDa regulatory subunit, known as myosin phosphatase target subunit I or MYPT1, and a 20-kDa subunit of unknown function (29). Selectivity of PP1 catalytic subunit for myosin is conferred by the targeting subunit and this subunit potentiates dephosphorylation of MLC by the catalytic subunit. MYPT1 is abundantly expressed in smooth muscle and alternative splicing of two exons gives rise to four major MYPT1 isoforms that differs in the presence of a central insert and/or a leucine zipper motif at the C-terminal end of the protein (LZ+ and LZ− isoforms). Phosphorylation of MYPT1 in its C-terminus half at Thr696 or Thr853 by Rho kinase or ZIP kinase fosters dissociation and inhibits activity of the catalytic subunit and, thus MLC20 dephosphorylation (68, 90, 91). MLCP catalytic activity is also dependent on an endogenous phosphorylation-dependent inhibitor, CPI-17 (PKC-potentiated PP1 inhibitory protein of 17 kDa), which is preferentially expressed in smooth muscle. Phosphorylation of CPI-17 at Thr38 by PKC, and probably by other kinases such as Rho kinase, ZIPK or ILK, greatly augments the inhibitory effect (69, 89–91, 98). CPI-17 is phosphorylated in response to contractile agonists and the phosphorylation is correlated with Ca2+-independent contraction. Thus, MLCP activity is tightly regulated by Rho kinase/MYPT1 and PKC/CPI-17 pathways and these pathways have significant impact on MLC20 phosphorylation and muscle contraction. Changes in the expression and/or activity of Rho kinase/MYPT1 and PKC/CPI-17 pathways lead altered muscle contraction.
1.2. Mechanisms involved in the regulation of acto-myosin interaction by thin-filament associated proteins

Contraction is initiated by MLC\textsubscript{20} phosphorylation, however, it can be modulated by other mechanisms, one of which involves the thin-filament associated proteins (15, 28, 59, 67). In smooth muscle, the predominant thin-filament protein actin exists as polymerized elongated filament (F-actin) and accounts for 30-50\% of total non-collagenous protein. Associated with actin are tropomyosin, caldesmon, calponin and smoothelin.

1.2.1. Tropomyosin. Tropomyosin is a coiled-coil \alpha-helix and cooperates the contractile filament interaction by virtue of its spanning seven-actin monomers. It has been shown that binding of myosin to actin leads to movement of tropomyosin and this process is facilitated by phosphorylation of myosin suggesting a possible participation of tropomyosin in acto-myosin interaction (28, 67). Tropomyosin also facilitates inhibition of acto-myosin ATPase activity by caldesmon.

1.2.2. Caldesmon. Caldesmon is a highly conserved protein that binds actin, myosin, tropomyosin and calmodulin and exists as smooth muscle-specific h-caldesmon and non-muscle l-caldesmon. Caldesmon binds to myosin via N-terminal domain and tethers to actin in conjunction with C-terminal actin binding domain. Binding of caldesmon to actin causes inhibition of acto-myosin ATPase activity and suggested to play a role in suppressing contractile tone. Phosphorylation of caldesmon by ERK1/2 or PKC and binding of Ca\textsuperscript{2+}/CaM to the C-terminal domain of caldesmon in response to contractile agonist appear to reverse the inhibitory actions of caldesmon (6, 27, 67).
1.2.3. **Calponin.** Calponin is an actin-binding protein and exists as smooth muscle-specific basic h1-calponin, cardiac muscle predominant neutral h2-calponin, and acidic calponin that is not tissue-specific (12, 28, 67). The role of calponin in the regulation of smooth muscle contraction appears to be distinct and tissue-specific. In the urinary bladder and vas deferens of calponin knockout mice, a faster shortening velocity was noticed consistent with the hypothesis that calponin is an inhibitor of acto-myosin ATPase activity. In contrast, in tonic vascular smooth muscle suppression of calponin caused a decrease in agonist-induced contraction, suggesting a stimulatory effect of calponin on smooth muscle contraction. Calponin was also shown to acts as scaffold tethering ERK1/2 and PKC pathways to regulate smooth muscle contraction.

1.2.4. **Smoothelin.** Smoothelin is abundantly expressed actin-binding protein and exists as visceral smooth muscle-specific smoothelin-A and vascular smooth muscle-specific smoothelin-B (72, 84). Although smoothelin has been used as a marker for contractile phenotype of smooth muscle, knowledge on its role in the regulation of smooth muscle contraction is limited. A role for smoothelin was suggested recently using smoothelin knockout mice, which demonstrated decreased contraction in response to contractile agonists, however, the exact mechanism by which smoothelin regulates muscle contraction is not known.

Thus, the importance of thin-filament associated proteins in the regulation of contraction appears to vary and tissue-specific. *Changes in the expression thin-filament associated proteins may lead to altered muscle contraction* (99).
1.3. Mechanisms involved in the regulation of cytoskeletal remodeling

In smooth muscle, the non-muscle isoform of actin (e.g., β-actin and γ-cytoplasmic actin) are associated with cytoskeleton. Studies demonstrating the remodeling of cytoskeleton in response to contractile agonist raise the possibility that actin cytoskeleton is dynamic and the cytoskeleton remodeling may regulate smooth muscle contraction (67). However, much remains to be determined regarding the organization of the contractile filaments with respect to the overall cytoskeleton and the mechanism by which the cytoskeleton remodeling regulates acto-myosin interaction. Several hypotheses have proposed which include increase in the strength of connections between membrane adhesion junctions and actin filaments and facilitating the transmission of force.

2. INFLAMMATION

Inflammatory bowel diseases are multifactorial in nature and thought to occur as a result of complex interactions between the immune system and environmental factors. Ulcerative colitis and Crohn’s disease are the two main inflammatory bowel diseases (IBD) that are associated with intestinal inflammation. Patients with IBD have altered bowel habits due to changes in mucosal secretory and absorptive functions as well as colonic motility. Although the causes of IBD are not fully understood, studies in patients and using animal models suggest that there is an initial immunological insult that is followed by a dysregulated immune response. Intestinal homeostasis is coordinated by the responses of different cell types, including both immune and non-immune cells such as epithelial cells, enteric neurons, interstitial cells of Cajal (ICC) and smooth muscle cells. The interaction between immune and nonimmune cells is amplified during inflammation due to release of many proinflammatory cytokines (e.g.,
interleukin (IL)-1β and tumor necrosis factor (TNF-α) both by immune cell and non-immune cell, and contribute to the amplification of tissue injury.

2.1. Animal models

A number of animal models of gastrointestinal inflammation have been developed to characterize the mechanisms underlying pathogenesis of inflammatory bowel diseases. Although often suspected for not mimicking all the features of IBD due to its multifactorial nature, it is generally considered that many mouse models of intestinal inflammation resemble aspects of IBD in humans and provided important insights into the mechanisms that control intestinal homeostasis and regulate intestinal inflammation, albeit with some limitations (7, 40, 46, 50, 57, 61-63). To date, there are more than 50 mouse models of intestinal inflammation. The most widely used animals models are parasitic infection with Trichinella spiralis, and the hapten 2,4,6-trinitrobenzene sulphonic acid (TNBS) and dextran sodium sulphate (DSS) models of colitis (50, 57, 61-63, 92, 100-103). Intracolonic instillation of a solution of TNBS in ethanol causes transmural inflammation due to a delayed type hypersensitivity reaction which involves a T-helper (Th)1 immune response, whereas the immune response to nematode infection involves Th2 cytokine production. Acute DSS colitis has a Th1 type cytokine profile, but more chronic model involves features of both Th1 and Th2 immune responses.

2.2. Colonic dysmotility

Both in vivo and in vitro studies in patients and animal models of colitis support the idea that colitis is accompanied by a decrease in the contractility of smooth muscle from the inflamed area (16, 27, 40-49, 79, 90). Measurements of in vivo motility patterns in dogs during acetic acid-induced ileitis, and in rats with acute DSS-induced colitis demonstrated that inflammation
decreased the frequency of migrating motor complexes and tone. Since the giant migrating contractions are responsible for movements of the faecal pellets, it was suggested that inflammation reduces propulsive motility (62, 71, 78). Similar decrease in pellet propels in vitro in distal colon was observed in TNBS-induced colitis. A decrease in the spontaneous contractile activity, and receptor- and non-receptor-mediated contractions were also observed during inflammation (27). The changes in motility are regardless of the model used to induce inflammation that is administration of different chemical compounds such as TNBS, DSS and acetic acid. Many studies were performed in animal models of gastrointestinal inflammation in order to elucidate the mechanisms underlying gut dysmotility during inflammatory processes. These studies have generated a number of concepts that provide insight into how the immune and motor systems of the gut interface and also clearly demonstrated how the inflammation of the mucosa causes colonic dysmotility. The mechanisms underlying the colonic dysmotility are complex and involve multiple mechanisms such as changes in enteric neurotransmission (17-22, 25, 63, 82), afferent sensory input (5, 8, 65), interstitial cells of Cajal (87), and abnormalities of the effector itself, the enteric smooth muscle intrinsic smooth muscle contractility (1-4, 31, 32, 35-37, 57, 93-96). In addition, studies in animal models of inflammation have suggested that changes in enterochromaffin cell number and 5-HT release and reuptake result in colonic dysmotility (49, 50, 55).

2.2.1. Enteric Nervous system. The neurons and the glia of the myenteric plexus, which is located between the longitudinal and circular smooth muscle layers of the muscularis externa, and the submucosal plexus, which is located in the submucosa constitute the enteric nervous system (9-11, 26). Because of the importance of ENS in gut functions, several studies have focused on changes in ENS and structure and function in patients with IBD or in animal models
of gut inflammation (7-9, 24, 30, 47-56, 64, 73). The nature of the changes in the ENS in IBD appear to be dependent on the disease (Crohn's vs. ulcerative colitis), the region of the intestinal wall and whether or not the tissue was from a site of active inflammation. Both the structure and function of the enteric nervous system (ENS) are substantially altered in enteric inflammation. These include a significant decrease in the number of myenteric and submucosal neurons during colitis, ganglion cell and axonal degeneration and necrosis, and impaired release of neurotransmitters acetylcholine, noradrenaline, and nitric oxide. Because of their projections to the mucosal layer, where inflammation is centered, AH neurons, which serve as primary afferent and interneuronal functions, appear to be more susceptible to inflammation (51, 52). Studies in patients with IBD and in animal models of inflammation revealed that the immunoreactivity of vanilloid receptor TRPV1, an integrator of noxious/inflammatory stimuli on sensory nerves that contributes to inflammation-induced sensory dysfunction, is greatly increased in colonic nerve fibers (10, 24, 60). Many of these changes in the structure and function are implicated in altered sensory, secretory and motor function of the intestine during inflammation. Inflammatory cytokines and infiltrating immune cells are responsible for many of the changes to the ENS in inflammation. The extent of damage to ENS function depends on the animal model and species, probably due to differences in the pathogenesis of inflammatory conditions. Studies also indicate that neuronal changes are not limited to the region of inflammation, but can occur in regions proximal or distal to the site of inflammation (71). Thus, plasticity of ENS is a feature of intestinal inflammation and several potentially relevant mechanisms underlying ENS plasticity have also been studied including the role of neurotrophins and enteric glia.
2.2.2. Interstitial cells of Cajal (ICC). ICC are mesenchymal cells with small cell bodies and several elongated processes and express c-kit, the proto-oncogene that encodes the receptor tyrosine kinase, kit. ICC present between longitudinal and circular muscle layers (ICC-MY) are spontaneously active and generate slow wave depolarization in different parts of the GI tract, and are important as pacemakers. Changes in the number of ICC and ultrastructure were reported in animal models of colitis and in patients with Crohn’s disease and ulcerative colitis (87). The reduction in ICC number was associated with an inhibition of spontaneous contractions of colon. However, more studies of colonic pacemaker activity in IBD are needed for further insight on the role of ICC in colonic dysmotility.

2.2.3. Smooth muscle. The smooth muscle cells of the gastrointestinal tract are the final effectors of force development and work. Smooth muscle cells are capable of synthesizing and secreting a variety of pro- and anti-inflammatory mediators, including cytokines, chemokines, growth factors, and cell-adhesion molecules that contribute to the maintenance and resolution of the inflammatory response in the gut (88, 96, 97). Thus, smooth muscle cells act as both source and target of these mediators. Substantial changes to smooth muscle structure and function have also been shown in intestinal inflammation. Studies have shown that inflammatory mediators either decrease or increase smooth muscle contractility and the response appears to be tissue- and cytokine-specific (1-4, 85, 92-96). Inflammation induced by helminth infection resulted in hypercontractility of longitudinal muscle via IL-4 and IL-13, whereas inflammation induced by TNBS or DSS resulted in hypocontractility of circular muscle via IL-1β, TNF-α or ICAM-1. Several targets in the signaling pathways mediating contraction that are affected by inflammatory cytokines have been identified and the changes in the expression or activity of these targets are
linked to altered muscle contraction. The myogenic mechanisms for decreased contraction include changes signaling pathways that lead to altered Ca\(^{2+}\) mobilization and MCL\(_{20}\) phosphorylation (1-4, 13, 14, 31, 32, 34-37, 74-77, 93-96).

**Changes in Ca\(^{2+}\) mobilization.** Changes in Ca\(^{2+}\) mobilization is implicated in the dysmotility of colon from ulcerative colitis patients and in animal models of colitis (1-4, 35-37, 39, 93-96). Studies in both TNBS- and DSS-colitis have provided evidence for the decrease in voltage-dependent Ca\(^{2+}\) currents, and the decrease in Ca\(^{2+}\) currents has been attributed to either decrease in protein expression or altered regulation of the channel. Decrease in expression was due to activation of transcription factor, NF-kB and altered regulation was due to inhibition of tyrosine phosphorylation by Src kinase (35-37, 85-86, 93, 96,). In addition to the effect on Ca\(^{2+}\) channels, inflammation enhances the ATP-sensitive K\(^{+}\) channels expression and currents (34). Decrease in Ca\(^{2+}\) currents and increase in K\(^{+}\) currents may act cooperatively to decrease smooth muscle contraction. In addition to Ca\(^{2+}\) and K\(^{+}\) channel functions, SERCA2 expression was reported to be decreased in colonic muscle during colitis, suggesting that reduced SR Ca\(^{2+}\) uptake can also disrupt intracellular Ca\(^{2+}\) mobilization in smooth muscles from inflamed colon (4). Studies on human colonic and esophageal sphincter smooth muscle suggest a role for endogenous H\(_{2}\)O\(_{2}\) in the regulation of Ca\(^{2+}\) mobilization and SERCA2 activity in acute inflammation or upon exposure to IL-1\(\beta\) (13-14). Studies also demonstrated that m3 receptor binding and G\(_{q}\) protein expression were not altered by exposure to IL-1\(\beta\), whereas PLC-\(\beta\) activity was inhibited. The inhibitory effect of IL-1\(\beta\) on PLC-\(\beta\)1 activity was attributed to up-regulation of RGS4 (regulator of G protein signaling), since G\(_{\alpha_q}\)-GTP activity is rapidly terminated by RGS4. Inhibition of PLC-\(\beta\)1 activity leads to decrease in IP3 generation and IP3-
dependent $\text{Ca}^{2+}$ release. Analysis of signaling pathways necessary for upregulation of RGS4 suggests that colonic smooth muscle employ multiple signaling pathways such as PI3-kinase/Akt, MAP kinases, and NF-kB, which is an immediate early nuclear transcription factor that is activated in response to proinflammatory cytokines (31-33).

**Changes in $\text{MLC}_{20}$ phosphorylation.** Previous studies also demonstrated that colonic inflammation impairs signal transduction pathways related to MLC20 phosphorylation. Levels of MLC20 phosphorylation are regulated by the balance between MLCK activity and MLCP activity. Phosphorylation of CPI-17, an endogenous inhibitor of MLCP, by PKC and MYPT1, a targeting subunit of MLCP, by Rho kinase in response to acetylcholine is essential for inhibition of MLC phosphatase and maintenance of sustained MLC$_{20}$ phosphorylation and contraction. Both IL-1β and TNF-α down regulates expression of CPI-17, which leads to inhibition of MLC20 phosphorylation and contraction (31, 32, 74-77). CPI-17 expression was also reduced in smooth muscle from patients with ulcerative colitis. These studies suggest that CPI-17 down regulation might contribute to the decreased muscle contraction in inflammatory bowel diseases. The mechanisms involved in the down-regulation of CPI-17 remain unknown.

As discussed in previous section, acto-myosin interaction and thus, muscle contraction are modulated the thin-filament associated proteins. The involvement of molecular components of contractile apparatus, particularly thin-filament associated proteins in colonic inflammation has not been addressed. Changes in the expression of caldesmon, calponin or tropomyosin that regulate the acto-myosin interaction could probably underlie the altered smooth muscle contraction.
It is our hypothesis that the expression of thin filament-associated proteins is altered during inflammation and contributes to the decreased contraction of colonic smooth muscle.

The aim of the present study was to determine the changes in the expression of actin isoforms and actin-associated proteins such as caldesmon, calponin, tropomyosin, and smoothelin-A in colonic smooth muscle isolated from mouse colon following TNBS- and DSS-induced colitis.
CHAPTER 2: MATERIALS AND METHODS

1. Materials

1.1 Chemicals and antibodies. Collagenase CLS type II and soybean trypsin inhibitor were obtained from Worthington, Freehold, NJ; Western blotting, and protein assay kit, Tris-HCl Ready Gels were obtained from Bio-Rad Laboratories, Hercules, CA; antibodies to α-actin, h-caldesmon, h1-calponin, α-tropomyosin, smoothelin-A were obtained from Santa Cruz biotechnology, Santa Cruz, CA; RNAqueous™ kit was obtained from Ambion, Austin, TX; Effectene Transfection Reagent, QIAEX®II Gel extraction Kit and QIAprep®Spin Miniprep Kit were obtained from QIAGEN Sciences, Maryland; PCR reagents were obtained from Applied Biosystems, Roche. 2, 4, 6 trinitrobenzene sulphonic acid (TNBS) and all other chemicals were obtained from Sigma, St. Louis, MO.

1.2 Animals. C57BL/6J mice were obtained from Charles River and housed in pairs in microfilter-isolated cages for 7 days before use with access to food and water. Animal care and treatment were conducted in accordance with the Institutional guidelines of the Virginia Commonwealth University. Institutional Animal Care and Use Committee at the Virginia Commonwealth University approved all the experimental protocols.
2. Methods

2.1 Induction of colitis. For TNBS-induced colitis, 6- to 8-weeks old male adult mice were fasted for 24 h before the induction of inflammation. Colonic inflammation was induced by intraluminal administration of single dose of TNBS (2.5% in 50% ethanol (v/v)). Mice were briefly anesthetized with isoflurane and 100 µl of TNBS solution was injected via a catheter advanced to 3 cm proximal to the anus via 1 ml syringe fitted with a catheter, and the mouse was kept in a vertical position with the head downwards for 1 min after the administration of the hapten, in order to distribute the agents within the entire colon. Age-matched mice treated with vehicle only served as controls (57, 85, 86). Each group of mice was euthanized 3 days after the induction of inflammation. For DSS-induced colitis, 6- to 8-weeks old male adults were administered 5% w/v DSS (MW = 36 000–50 000 Da, MP Biomedicals LLC, Solon, OH, USA) ad libitum in the drinking water for 5 days (35). All Mice were checked daily for loss of body weight, stool consistency and the presence of gross bleeding. Colonic tissue from mice treated with TNBS or DSS exhibited typical histological characteristics of colitis (35, 57, 85, 86).

2.2 Collection of tissue. Mice were euthanized by CO2 inhalation followed by decapitation. Two- to three centimeter-long segments of the distal colon (starting from ~0.5 cm oral to the pelvic flexure) were obtained, immediately examined with the naked eye for macroscopic lesions, opened along the mesenteric border, cleaned, and pinned flat in a Petri dish with Sylgard base. The mucosal/submucosal layers were separated by microdissection. The muscularis propria, which included both the circular and longitudinal muscle layers were then quick frozen in liquid nitrogen and broken into small particles with a chilled pestle for protein and RNA extractions. The tissue particles were homogenized on ice in phosphate-buffered saline
supplemented with protease inhibitors for protein extraction. For isolation of dispersed muscle cell, colon was emptied of its contents and placed in a cold smooth muscle buffer with the following composition: NaCl 120 mM, KCl 4 mM, KH₂PO₄ 2.6 mM, CaCl₂ 2.0 mM, MgCl₂ 0.6 mM, HEPES (N-2-hydroxyethylpiperazine-N’ 2-ethanesulfonic acid) 25 mM, glucose 14 mM, and essential amino mixture 2.1% (pH 7.4) (31, 32, 57).

2.3 Preparation of dispersed colonic smooth muscle cells. Smooth muscle cells from colonic smooth muscle were isolated by sequential enzymatic digestion of muscle strips, filtration, and centrifugation as described previously (31, 32, 57, 70). Colonic muscle were incubated for 20 min in a smooth muscle buffer at 31°C containing 0.1% collagenase (300 U/ml) and 0.01% soybean trypsin inhibitor (w/v). The tissue was continuously gassed with 100% oxygen during the entire isolation procedure. The partly digested tissues were washed twice with 50-ml of collagenase-free smooth muscle buffer and the muscle cells were allowed to disperse spontaneously for 30 min in collagenase-free medium. Cells were harvested by filtration through 500 μm Nitex and centrifuged twice at 350 g for 10 min to eliminate broken cells and organelles. The cells were counted in a hemocytometer and it is estimated that 95% of the cells excluded trypan blue. The experiments were done within 2-3 h of cell dispersion.

2.4 Quantitative real-time RT-PCR analysis (qRT-PCR). Total RNA was isolated from colonic muscle strips with TRIzol® reagent (Invitrogen) and then treated with TURBO DNase (Ambion). RNA from each preparation was reversely transcribed using the SuperScript™ II system containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (DTT), 0.5 mM deoxynucleoside triphosphates (dNTP), 2.5 μM random hexamers and 200 units of reverse transcriptase in a 20 μl reaction volume. The reactions were carried out at room
temperature for 10 min and at 42°C for 50 min, and terminated by heating at 70°C for 15 min (31, 32, 57). Quantitative RT-PCR was performed on cDNA samples using specific primers designed based on known sequences in mouse and TaqMan gene expression master mix. The procedure is based on the time point during PCR cycling when amplification of PCR product is first detected rather than by the amount of PCR product accumulated after a fixed number of cycles. The target gene copy number is quantified by measuring threshold cycle parameter (Ct), defined as fractional cycle at which the fluorescence generated by cleavage of probe passes a fixed threshold above the base line, and by using a standard curve to determine the starting copy number. The primers are designed to satisfy the requirements for use of the $2^{-\Delta\Delta C_T}$ quantification method. GAPDH expression was chosen to normalize the expression of the target genes. The GAPDH amplicon was utilized to normalize and calculate the quantitative expression of various genes. Thus, a quantitative comparison between samples from control and TNBS- or DSS-treated animal was calculated with the $2^{-\Delta\Delta C_T}$ method by normalizing the $\Delta C_T$ counts to GAPDH expression. Expression of the target gene mRNA was divided by the GAPDH mRNA. Final results are expressed as fold difference in expression in TNBS- or DSS-treated samples relative to control samples. All PCR reactions were performed in an ABI StepOne Plus PCR (Applied Biosystems, Foster City, CA). PCR conditions were optimized on the gradient thermal cycler. For each cDNA sample, real-time PCR was conducted in a 20 μl reaction volume containing TaqMan gene Expression Master Mix (Applied Biosystems, Foster City, CA). The following time and temperature profile was used for the real-time PCR reactions: 95 °C for 5 min; 40 cycles of a series consisting of 15 s at 94°C, 30 s at 52°C, 30 s at 72°C; and a final extension of 5 min at 72°C. The optimal annealing temperatures were determined empirically for each primer
set. The sequences of specific primers are listed in the results section. The ABI sequence-detection software and Microsoft Excel were used to calculate the quantities of the mRNAs.

2.5 Western blot Analysis. Frozen colonic tissues (100 mg) were pulverized while immersed in liquid nitrogen using a mortar. The muscle powder was collected into a glass tube and homogenized in buffer containing 20% glycerol, 50 mM tris-HCl (pH 6.8), 0.5% (volume per volume) Tween-20 and protease inhibitors (0.5 mM phenylmethanesulfonyl fluoride, 2 μM pepstatin, 2 μM antipain and 0.1 mg/ml trypsin inhibitor). The homogenate was centrifuged (38,000 g) for 15 minutes using a Sorvall centrifuge and the supernatant was collected. After gently mixing the samples in SDS (final concentration, 1%), they were boiled for 4 minutes and centrifuged at 10,000 rpm for 15 minutes. The supernatant was collected and the protein concentration was determined using the DC protein assay kit (BioRad Laboratories, Hercules, California). Equal amounts (40 μg) of total protein from control and TNBS- and DSS-treated mice colonic muscle strips were loaded on 10% SDS-PAGE gels, using the buffer solution composed of 25 mM Tris, 192 mM glycine, 0.1% SDS and transferred to nitrocellulose membranes with Towbin buffer (25 mM tris, 192 mM glycine and 20% (v/v)) methanol. Membranes were subsequently blocked in blocking buffer (composition: 50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, and 5% dried milk powder) for 90 min at room temperature. Next, membranes were incubated overnight at 4°C with primary antibodies [α-actin (diluted 1:500), γ-actin (diluted 1:1000), h-caldesmon (diluted 1:1000), h1-calponin (diluted 1:1000), α-tropomyosin (diluted 1:1000), and smoothelin-A (diluted 1:1000); all dilutions in blocking buffer]. After three washes with TBS-Tween 20 (0.1% TBST, containing 50 mM Tris-HCl, 150
mM NaCl, and 0.1% Tween 20) of 10 min each, membranes were incubated with horseradish peroxidase-labeled secondary antibodies (dilution 1:1000-3,000 in blocking buffer) at room temperature for 90 min, followed by an additional three washes with 0.1% TBST. Bands were subsequently visualized on film using SuperSignal Femto maximum sensitivity substrate kit (Pierce, USA) and analyzed by densitometry (31, 32, 70). All bands were normalized to β-actin expression.

2.6 Immunohistochemistry. Colonic tissue sections from control and TNBS-treated animals were processed for calponin, caldesmon, tropomyosin and smoothelin-A immunoreactivities using an on-slide immunostaining technique. After macroscopic observation, full thickness specimens from control and inflamed tissue and by immersion in 4% paraformaldehyde, washed in PBS (3 × 10 min), cryoprotected in PBS-sucrose (30%), and embedded for cryostat sectioning in optimal cutting temperature compound. Cross sections (8-10μm) were cut and the slides containing the sections incubated for 1 h at room temperature with blocking solution containing 5% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) in PBST (0.3% Triton X-100 in 0.1 M PBS, pH 7.4). The samples were then incubated in primary antibodies diluted in PBST containing 5% normal donkey serum overnight at 4 °C. The antibodies (Santa Cruz, CA) were used at 1:500 (caldesmon), 1:200 (calponin), 1:500 (tropomyosin) and 1: 500 (smoothelin) dilutions. After rinsing (3 × 10 min with 0.1 M PBS), the tissue sections were incubated with fluorescence-conjugated species-specific secondary antibody Alexa 594 (1:200, Molecular Probes, Eugene, OR) for 2 h at room temperature. Following wash, the slides were coverslipped with Citifluor (Citifluor Ltd., London). Control sections incubated in the absence of primary or secondary antibody were also processed and evaluated for specificity or background staining levels. In the absence of primary antibody, no positive immunostaining for caldesmon, calponin,
tropomyosin and smoothelin was observed. The specificity of primary antibodies was examined by western blot analysis.

2.7 **Measurement of contraction in dispersed smooth muscle cells.** Contraction in freshly dispersed colonic smooth muscle cells was determined by scanning micrometry as previously described (32, 70). An aliquot (0.4 ml) of cells containing approximately $10^4$ cells/ml was treated with 100 μl of medium containing different concentrations of acetylcholine (ACh; 10 pM to 1 µM) for 30 s and the reaction was terminated with 1% acrolein at a final concentration of 0.1%. Acrolein kills and fixes cells without affecting the cell length. The resting cell length was determined in control experiments in which muscle cells were incubated with 100 μl of 0.1% bovine serum albumin without the ACh. The mean lengths of 50 muscle cells treated with various agonists was measured by scanning micrometry and compared with the mean lengths of untreated cells. The contractile response was expressed as the percent decrease in mean cell length from control cell length.

3. **STATISTICAL ANALYSIS**

The results were expressed as means ± SE of $n$ experiments and analyzed for statistical significance using Student’s $t$-test for paired and unpaired values. Each experiment was done on cells obtained from different animals. Five mice of each group were studied; qRT-PCR and western blot were performed in duplicate. Differences among multiple groups were tested by using ANOVA and checked for significance using Fisher's protected least significant difference test. A probability of $p < 0.05$ was considered significant.
Table 1. RT-PCR TaqMan primers for the amplification of thin-filaments associated proteins
### Table 1

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<th>Aaaay ID</th>
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<th>Product size</th>
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<td>Cald1</td>
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<td>Mm00449973- m1</td>
<td>Applied Biosystems</td>
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<tr>
<td>GAPDH</td>
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Table 2. RT-PCR SYBRgreen primers for the amplification of thin-filaments associated proteins
Table 2

<table>
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<th>Reverse 3'→5'</th>
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<td>GTACGCGCGGATGCAT</td>
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<tr>
<td>IL-1B</td>
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<td>GCTTGGGATCCACACTCTCCAGCT</td>
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<td>TNF-α</td>
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<td>TTGGTTGTGGCTACGACGTTGG</td>
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<td>GAPDH</td>
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<td>GGAGTTGCTGTGGAAGTTCG</td>
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Table 3. Western blot antibodies for thin-filaments associated proteins
Table 3

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<td>Caldesmon (H-300)</td>
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<tr>
<td>Tropomyosin (FL-284)</td>
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<td>SANTA CRUZ</td>
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<td>Smoothelin (C-20)</td>
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CHAPTER 3: RESULTS

In the present study, we analyzed the changes in the expression of α-actin, γ-actin, caldesmon, calponin, tropomyosin and smoothelin-A in smooth muscle of colon isolated from control mice and TNBS- and DSS-induced colitic mice by qRT-PCR and Western blot.

For qRT-PCR specific primers were designed based on known sequences in mouse (Table 1 and 2). The accuracy of the designed primers was evaluated with gel-electrophoresis. Melt curve analyses showed single peaks for all samples. The PCR efficiencies were calculated from standard curves. These findings validated the design of primers and satisfied the requirements for use of the $^{\Delta \Delta \text{CT}}$ quantification method, described previously in the Materials and Methods section. GAPDH expression was chosen to normalize the expression of the contractile proteins. The GAPDH amplicon was utilized to normalize and calculate the quantitative expression of various genes. Thus, a quantitative comparison between inflamed and normal tissue was calculated with the $^{\Delta \Delta \text{CT}}$ method by normalizing the ΔCT counts to GAPDH expression.

For western blot analysis equal amounts of protein were subjected to electrophoresis and transferred to nitrocellulose or polyvinylidene difluoride membranes. Membranes were incubated with antibodies to α-actin, γ-actin, h-caldesmon, calponin, tropomyosin or smoothelin-A with appropriate dilutions (Table 3). Following ECL protocol as described in the Methods, protein
bands of the expected size were analyzed by densitometry and all bands normalized to β-actin expression.

For immunohistochemistry tissue sections were incubated in primary antibodies followed by fluorescence-conjugated species-specific secondary antibody. Control sections incubated in the absence of primary or secondary antibody showed no positive staining. The specificity of antibodies used was confirmed by western blot analysis.

3.1 Evaluation of colonic inflammation

Macroscopic examination of the distal colon rectum 5 days after TNBS administration revealed multiple mucosal erosions and ulceration which were in sharp contrast with the appearance of the normal colon excised from control animals with intact smooth mucosa. IL-1β, as a vital proinflammatory cytokine, plays an important role in colitis (86, 93, 97). The release of this cytokine in turn increases the expression of other cytokines and positively contributes to the progress of inflammatory bowel disease and experimental colitis in animal models of inflammatory bowel disease. Previous studies demonstrated that IL-1β or TNF-α acting on muscle cells alters the expression of signaling proteins involved in the regulation of contraction (31, 32, 93). Therefore, we tested whether the expression of IL-1β increases in smooth muscle tissue during TNBS- and DSS-induced colitis. Expression of IL-1β and TNF-α was measured by qRT-PCR using specific primers (IL-1 β: forward primer, TACCTGTGGCCTTGGGCCTCAA; reverse primer, GCTTGGGATCCACACTCTCCAGCT and TNF-α forward primer, ATGGCCCAGACCCTCACACTCAG; reverse primer, TTGGTGGTTTGCTACGACGTGGG). Indeed, the expression of both IL-1β and TNF-α was significantly increased (IL-β: 10-fold and 75-fold with TNBS and DSS, respectively, and TNF-α:
3-fold and 4-fold with TNBS and DSS, respectively, p<0.05, n=3-5) in inflammatory colonic tissue in colitis compared to control tissue (Figs. 1 and 2). These results strongly suggested that TNBS-treatment results in the generation of proinflammatory cytokines.
Figure 1. Expression of IL-1β in the colonic muscle after TNBS or DSS treatment. Total RNA isolated from smooth muscle strips of control and TNBS or DSS treated mice using RNAqueous prep kits (Ambion, Austin, Tx) and was reverse transcribed using 2 µg of total RNA using qScript cDNA prep kits (Quanta, Gaithersburg, MD). The cDNA was amplified with specific primers for IL-1β. The sequences of specific primers are listed in Table 1. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure RNA levels of IL-1β. For each cDNA sample, real-time PCR was conducted in a 20-μl reaction volume containing Quantitect SYBRgreen PCR Mastermix (Qiagen, Mississauga, ON). Real-time PCR reactions were performed in triplicate. Results are expressed as fold differences in IL-1β gene expression in TNBS- or DSS-treated colon relative to that in vehicle-treated colon. Values represent the means ±SEM of 5 separate experiments. **p<0.001 versus control.
Figure 1

IL-1β mRNA (fold)

Control | TNBS

Control | DSS

**
Figure 2. Expression of TNF-α in the colonic muscle after TNBS or DSS treatment. Total RNA isolated from smooth muscle strips of control and TNBS or DSS treated mice using RNAqueous prep kits (Ambion, Austin, Tx) and was reverse transcribed using 2 μg of total RNA using qScript cDNA prep kits (Quanta, Gaithersburg, MD). The cDNA was amplified with specific primers for TNF-α. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure RNA levels of TNF-α. For each cDNA sample, real-time PCR was conducted in a 20-μl reaction volume containing Quantitect™ SYBRgreen PCR Mastermix (Qiagen, Mississauga, ON). Real-time PCR reactions were performed in triplicate. Results are expressed as fold differences in TNF-α gene expression in TNBS- or DSS-treated colon relative to that in vehicle-treated colon. Values represent the means ±SEM of 3 separate experiments. **p<0.05 versus control.
3.2 Changes in the expression of thin-filament associated proteins in TNBS-colitis mice.

3.2.1 Actins. Six actin isoforms (α-skeletal, α-cardiac, and α-vascular; γ-enteric and γ-cytoplasmic; and β-cytoplasmic) have been described and are products of separate genes (28, 67). The α-vascular and γ-enteric actin isoforms are smooth muscle specific and associated with the contractile proteins, whereas β- and γ-cytoplasmic actins are non-muscle isoforms and associated with non-contractile cytoskeleton. Expression of α- and γ-actin was measured in colonic muscle of control and TNBS-colitic mice. The differences in the expression levels were analyzed by qRT-PCR using specific primers (α-actin: forward primer, CGCTGTCAGGAACCCTGAGA; reverse primer, CGAAGCCGCTTACAGA and γ-actin: forward primer, GTCCCTGCATGTATGTTGCTA; reverse primer, GGTACGGCCGATGCAT). The results showed that expression of α-actin and γ-actin was 2-fold higher (p<0.05, n=5) in colonic muscle cells from TNBS mice compared to control mice (Figs. 3 and 4). Protein expression of α-actin protein was examined by western blot analysis using an antibody that recognizes selectively α-actin. Results showed expression of one protein band with ~45kDa and the molecular weight of the protein band corresponds to the expected molecular weight of α-actin isoform (42 kDa). Comparing the densities of protein bands revealed 3-fold higher expression in muscle from TNBS mice compared to control mice (Fig. 3) (p<0.05). α-actin immunoreactivity was also observed from colonic tissue sections of both control and TNBS-treated mice and the immunoreactivity was predominant in muscularis propria and intensity of immunoreactivity appears to be greater in tissues from TNBS mice compared to control mice (Fig. 3)
3.2.2. **Caldesmon.** Two caldesmon isoforms (h-caldesmon and l-caldesmon) have been described. The h-caldesmon is smooth muscle specific, whereas l-caldesmon is non-muscle isofrom (6). Expression of h-caldesmon was measured in colonic muscle of control and TNBS-colitic mice. The differences in the expression levels were analyzed by qRT-PCR using specific primers (assay ID: Mm00513995-m1, Applied Biosystems). The results showed that expression of h-caldesmon was 7-fold higher (p<0.001) in colonic muscle cells from TNBS mice compared to control mice (Fig. 5). Protein expression of h-caldesmon was examined by western blot analysis using an antibody that recognizes selectively h-caldesmon. Results showed expression of one protein band with ~100 kDa and the molecular weight of the protein band corresponds to the expected molecular weight of h-caldesmon isoform. Comparing the densities of protein revealed 2.5-fold higher (p<0.05) expression in muscle from TNBS mice compared to control mice (Fig. 5). h-caldesmon immunoreactivity was observed from colonic tissue sections of both control and TNBS-treated mice and the immunoreactivity was predominant in muscularis propria and intensity of immunoreactivity appears to be greater in tissues from TNBS mice compared to control mice (Fig. 5).

3.2.3. **Calponin.** Three calponin isoforms (h1-acidic, h2-neutral and basic calponin) have been described. The h2-acidic is smooth muscle specific, whereas h2-neutral and basic calponin are non-muscle isoforms (12). Expression of h2-calponin was measured in colonic muscle of control and TNBS-colitic mice. The differences in the expression levels were analyzed by qRT-PCR using specific primers (assay ID: Mm00487032-m1, Applied Biosystems). The results showed that expression of h2-calponin was ~4-fold higher (p<0.001) in colonic muscle cells from TNBS mice compared to control mice (Fig. 6). Protein expression of
h-calponin also examined by western blot analysis using an antibody that recognizes selectively h2-calponin. Results showed expression of one protein band with 35 kDa and the molecular weight of the protein band corresponds to the expected molecular weight of h2-calponin isoforms. Comparing the densities of protein revealed 3-fold higher (p<0.001) expression in muscle from TNBS mice compared to control mice (Fig. 6). h1-calponin immunoreactivity was observed from colonic tissue sections of both control and TNBS-treated mice and the immunoreactivity was predominant in muscularis propria and intensity of immunoreactivity appears to be greater in tissues from TNBS mice compared to control mice (Fig. 6).

3.2.4. Tropomyosin. Two tropomyosin isoforms (α- and β-tropomyosin) have been described (28, 67). Expression of α-tropomyosin was measured in colonic muscle of control and TNBS-colitic mice. The differences in the expression levels were analyzed by qRT-PCR using specific primer (assay ID: Mm00437172-g1, Applied Biosystems). The results showed that expression of α-tropomyosin was 2-fold higher in colonic muscle cells from TNBS mice compared to control mice (Fig. 7). Protein expression of α-tropomyosin was examined by western blot analysis using an antibody that recognizes both isoforms. Results showed expression of one protein band with ~35 kDa and the molecular weight of the protein band corresponds to the expected molecular weight of α-tropomyosin. Comparing the densities of protein revealed a 2.5-fold higher (p<0.05) expression in muscle from TNBS mice compared to control mice (Fig. 7). A tropomyosin immunoreactivity was also observed from colonic tissue sections of both control and TNBS-treated mice and the immunoreactivity was predominant in muscularis propria and intensity of immunoreactivity appear to be greater in tissues from TNBS mice compared to control mice (Fig. 7).
3.2.5. Smoothelin. Two smoothelin isoforms (Smoothelin-A and -B) have been described. Expression of smoothelin-A is specific to visceral smooth muscle, whereas smoothelin-B is specific to vascular smooth muscle (72, 84). Smoothelin-A was measured in colonic muscle of control and TNBS-colitic mice. The differences in the expression levels were analyzed by qRT-PCR using specific primers (assay ID: Mm00449973-m1, Applied Biosystems). The results showed that expression of smoothelin-A was 2-fold greater in colonic muscle cells from TNBS mice compared to control mice (Fig. 8). Protein expression of smoothelin-A was also examined by western blot analysis using an antibody that recognizes both isoforms. Results showed expression of one protein band with 60 kDa and the molecular weight of the protein band corresponds to the expected molecular weight of smoothelin-A. Comparing the densities of protein revealed 2-fold higher expression in muscle from TNBS mice compared to control mice (Fig. 8). Smoothelin-A immunoreactivity was observed from colonic tissue sections of both control and TNBS-treated mice and the immunoreactivity was predominant in muscularis propria and intensity of immunoreactivity appears to be greater in tissues from TNBS mice compared to control mice (Fig. 8)
Figure 3. Expression of α-actin in the colonic muscle after TNBS treatment. (A) mRNA expression. Total RNA isolated from smooth muscle strips of control and TNBS treated mice using RNAqueous prep kits (Ambion, Austin, Tx) and was reverse transcribed using 2 µg of total RNA using qScript cDNA prep kits (Quanta, Gaithersburg, MD). The cDNA was amplified with specific primers for α-actin. Quantitative real-time polymerase chain reaction (qRT-PCR) was used measure RNA levels of α-actin. For each cDNA sample, real-time PCR was conducted in a 20-µl reaction volume containing Quantitect SYBRgreen PCR Mastermix (Qiagen, Mississauga, ON). Real-time PCR reactions were performed in triplicate. Results are expressed as fold differences in α-actin gene expression in TNBS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments. *p<0.05 versus control. (B) Protein expression. Colonic muscle tissue lysates containing equal amounts of total proteins were separated with SDS-PAGE and expression of α-actin was analyzed using selective antibody. Results are expressed as fold differences in α-actin protein expression in TNBS-treated colon relative to that in vehicle-treated colon. Values represent the means ±SEM of 5 separate experiments. *p<0.05 versus control. (C) Immunohistochemistry. Representative photomicrographs for α-actin staining in control and TNBS-treated colon. Note increase in the staining for α-actin in tissue sections from TNBS-treated animals.
**Figure 3**

*α-Actin mRNA (fold)*

- Control: 1.0
- TNBS: 3.0

*α-Actin protein (fold)*

- Control: 1.0
- TNBS: 3.0

**Images:**
- Control 50 μm
- TNBS 10 μm
- Control 45 kDa
- TNBS 45 kDa
Figure 4. Expression of γ-actin in the colonic muscle after TNBS treatment. Total RNA isolated from smooth muscle strips of control and TNBS treated mice using RNAqueous prep kits and was reverse transcribed using 2 µg of total RNA. The cDNA was amplified with specific primers for γ-actin. Quantitative real-time polymerase chain reaction (qRT-PCR) was used measure RNA levels of γ-actin. For each cDNA sample, real-time PCR was conducted in a 20-µl reaction volume containing Quantitect™ SYBRgreen PCR Mastermix. Real-time PCR reactions were performed in triplicate. Results are expressed as fold differences in γ-actin gene expression in TNBS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments.
Figure: 4

The graph shows the expression levels of γ-Actin mRNA (fold change) between control and TNBS groups. The control group shows a 1-fold increase, while the TNBS group shows a 2-fold increase with some variability indicated by error bars.
Figure 5. Expression of h-caldesmon in the colonic muscle after TNBS treatment. **(A)** mRNA expression. Total RNA isolated from smooth muscle strips of control and TNBS treated mice using RNAqueous prep kit and was reverse transcribed using 2 µg of total RNA using qScript cDNA prep kits. The cDNA was amplified with specific primers for h-caldesmon. Quantitative real-time polymerase chain reaction (qRT-PCR) was used measure mRNA levels of h-caldesmon. For each cDNA sample, real-time PCR was conducted in a 20-µl reaction volume containing Quantitect™ TaqMan PCR Mastermix. Real-time PCR reactions were performed in triplicate. Results are expressed as fold differences in h-caldesmon gene expression in TNBS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments. **p<0.001 versus control. (B) Protein expression.** Colonic muscle tissue lysates containing equal amounts of total proteins were separated with SDS-PAGE and expression of h-caldesmon was analyzed using selective antibody. Results are expressed as fold differences in h-caldesmon protein expression in TNBS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments. *p<0.05 versus control. **(C)** Immunohistochemistry. Representative photomicrographs for h-caldesmon staining in control and TNBS-treated colon. Note increase in the staining for h-caldesmon in tissue sections from TNBS-treated animals.
Figure 5: Bar graphs showing the expression levels of caldesmon mRNA and protein in control and TNBS-treated samples. The upper graph displays a significant increase in caldesmon mRNA expression, while the lower graph shows a similar trend in protein expression. The images on the right depict immunohistochemical staining for caldesmon in control and TNBS-treated tissue samples, with a marked difference in staining intensity.
**Figure 6. Expression of h1-calponin in the colonic muscle after TNBS treatment.**

**(A) mRNA expression.** Total RNA isolated from smooth muscle strips of control and TNBS treated mice using RNAqueous prep kit and was reverse transcribed using 2 µg of total RNA using qScript cDNA prep kits. The cDNA was amplified with specific primers for h1-calponin. Quantitative real-time polymerase chain reaction (qRT-PCR) was used measure mRNA levels of h1-calponin. For each cDNA sample, real-time PCR was conducted in a 20-µl reaction volume containing Quantitect™ TaqMan PCR Mastermix. Real-time PCR reactions were performed in triplicate. Results are expressed as fold differences in h1-calponin gene expression in TNBS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments. **p<0.05 versus control.**

**(B) Protein expression.** Colonic muscle tissue lysates containing equal amounts of total proteins were separated with SDS-PAGE and expression of h1-calponin was analyzed using selective antibody. Results are expressed as fold differences in h1-calponin protein expression in TNBS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments. **p<0.001 versus control.**

**(C) Immunohistochemistry.** Representative photomicrographs for h1-calponin staining in control and TNBS-treated colon. Note increase in the staining for h-calponin in tissue sections from TNBS-treated animals. Scale bar = 200 µm.
Figure: 6

Graph 1: Calponin mRNA (fold)
- Control: 1
- TNBS: 4

Graph 2: Calponin protein (fold)
- Control: 1
- TNBS: 4

Images:
- Control 100 μm
- TNBS 100 μm

Western Blot:
- Control
- TNBS 35 kDa

β-actin
Figure 7. Expression of α-tropomyosin in the colonic muscle after TNBS treatment. (A) mRNA expression. Total RNA isolated from smooth muscle strips of control and TNBS treated mice using RNAqueous prep kit and was reverse transcribed using 2 μg of total RNA using qScript cDNA prep kits. The cDNA was amplified with specific primers for α-tropomyosin. Quantitative real-time polymerase chain reaction (qRT-PCR) was used measure mRNA levels of α-tropomyosin. For each cDNA sample, real-time PCR was conducted in a 20-μl reaction volume containing Quantitect™ TaqMan PCR Mastermix. Real-time PCR reactions were performed in triplicate. Results are expressed as fold differences in α-tropomyosin gene expression in TNBS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments. *p<0.05 versus control. (B) Protein expression. Colonic muscle tissue lysates containing equal amounts of total proteins were separated with SDS-PAGE and expression of α-tropomyosin was analyzed using selective antibody. Results are expressed as fold differences in α-tropomyosin protein expression in TNBS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments. *p<0.05 versus control. (C) Immunohistochemistry. Representative photomicrographs for α-tropomyosin staining in control and TNBS-treated colon. Note increase in the staining for h-calponin in tissue sections from TNBS-treated animals.
Figure 8. Expression of smoothelin-A in the colonic muscle after TNBS treatment. (A) *mRNA expression.* Total RNA isolated from smooth muscle strips of control and TNBS treated mice using RNAqueous prep kit and was reverse transcribed using 2 µg of total RNA using qScript cDNA prep kits. The cDNA was amplified with specific primers for smoothelin-A. Quantitative real-time polymerase chain reaction (qRT-PCR) was used measure mRNA levels of smoothelin-A. For each cDNA sample, real-time PCR was conducted in a 20-µl reaction volume containing Quantitect™ TaqMan PCR Mastermix. Real-time PCR reactions were performed in triplicate. Results are expressed as fold differences in smoothelin-A gene expression in TNBS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments. (B) *Protein expression.* Colonic muscle tissue lysates containing equal amounts of total proteins were separated with SDS-PAGE and expression of smoothelin-A was analyzed using selective antibody. Results are expressed as fold differences in smoothelin-A protein expression in TNBS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments. (C) *Immunohistochemistry.* Representative photomicrographs for smoothelin-A staining in control and TNBS-treated colon. Note increase in the staining for h-calponin in tissue sections from TNBS-treated animals.
3.3. Changes in the expression of thin-filament associated proteins in DSS-colitis mice.

To determine whether the up-regulation of thin filament associated proteins observed in the colonic muscle of TNBS-induced colitis is also observed in another model of colitis, mice were treated with DSS for 5 days and the expression of caldesmon, calponin, tropomyosin and smoothelin-A were analyzed by qRT-PCR and western blot in colonic smooth muscle isolated from control and DSS-treated mice. Analysis of mRNA and proteins expression results revealed higher expression of calponin (~2-fold, Fig. 10), caldesmon (3- to 4-fold; p<0.001, Fig. 9), tropomyosin (2-3-fold, Fig. 11), and smoothelin-A (~2-fold, Fig. 12) in colonic muscle cells from DSS mice compared to control mice. These results suggest that inflammation induced by either TNBS or DSS treatment had similar effect on the expression thin-filament associated proteins.
Figure 9. Expression of h-caldesmon in the colonic muscle after DSS treatment. (A) mRNA expression. Total RNA isolated from smooth muscle strips of control and DSS treated mice using RNAqueous prep kit and was reverse transcribed using 2 μg of total RNA using qScript cDNA prep kits. The cDNA was amplified with specific primers for h-caldesmon. Quantitative real-time polymerase chain reaction (qRT-PCR) was used measure mRNA levels of h-caldesmon. For each cDNA sample, real-time PCR was conducted in a 20-μl reaction volume containing Quantitect™ TaqMan PCR Mastermix. Real-time PCR reactions were performed in triplicate. Results are expressed as fold differences in h-caldesmon gene expression in DSS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments. **p<0.05 versus control. (B) Protein expression. Colonic muscle tissue lysates containing equal amounts of total proteins were separated with SDS-PAGE and expression of h-caldesmon was analyzed using selective antibody. Results are expressed as fold differences in h-caldesmon protein expression in DSS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments. **p<0.05 versus control.
Figure: 9

- **Caldesmon mRNA (fold)**
  - Control: 1.0
  - DSS: 4.0

- **β-actin**
  - Control: 1.0
  - DSS: 5.0

**100 kDa**
Figure 10. Expression of h1-calponin in the colonic muscle after DSS treatment. (A) mRNA expression. Total RNA isolated from smooth muscle strips of control and DSS treated mice using RNAqueous prep kit and was reverse transcribed using 2 µg of total RNA using qScript cDNA prep kits. The cDNA was amplified with specific primers for h1-calponin. Quantitative real-time polymerase chain reaction (qRT-PCR) was used measure mRNA levels of h1-calponin. For each cDNA sample, real-time PCR was conducted in a 20-µl reaction volume containing Quantitect™ TaqMan PCR Mastermix. Real-time PCR reactions were performed in triplicate. Results are expressed as fold differences in h1-calponin gene expression in DSS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments. *p<0.05 versus control. (B) Protein expression. Colonic muscle tissue lysates containing equal amounts of total proteins were separated with SDS-PAGE and expression of h1-calponin was analyzed using selective antibody. Results are expressed as fold differences in h1-calponin protein expression in DSS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments. *p<0.05 versus control.
Figure: 10

Calponin mRNA (fold)

Control  DSS

Control  DSS

β-actin
Figure 11. **Expression of α-tropomyosin in the colonic muscle after DSS treatment.**  

**mRNA expression.** Total RNA isolated from smooth muscle strips of control and DSS treated mice using RNAqueous prep kit and was reverse transcribed using 2 μg of total RNA using qScript cDNA prep kits. The cDNA was amplified with specific primers for α-tropomyosin. Quantitative real-time polymerase chain reaction (qRT-PCR) was used measure mRNA levels of α-tropomyosin. For each cDNA sample, real-time PCR was conducted in a 20-µl reaction volume containing Quantitect TaqMan PCR Mastermix. Real-time PCR reactions were performed in triplicate. Results are expressed as fold differences in α-tropomyosin gene expression in DSS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments. *p<0.05 versus control.  

**Protein expression.** Colonic muscle tissue lysates containing equal amounts of total proteins were separated with SDS-PAGE and expression of α-tropomyosin was analyzed using selective antibody. Results are expressed as fold differences in α-tropomyosin protein expression in DSS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments. *p<0.05 versus control.
Figure 12. Expression of smoothelin-A in the colonic muscle after DSS treatment. (A) mRNA expression. Total RNA isolated from smooth muscle strips of control and DSS treated mice using RNAqueous prep kit and was reverse transcribed using 2 µg of total RNA using qScript cDNA prep kits. The cDNA was amplified with specific primers for smoothelin-A. Quantitative real-time polymerase chain reaction (qRT-PCR) was used measure mRNA levels of smoothelin-A. For each cDNA sample, real-time PCR was conducted in a 20-µl reaction volume containing Quantitect™ TaqMan PCR Mastermix. Real-time PCR reactions were performed in triplicate. Results are expressed as fold differences in smoothelin-A gene expression in DSS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments. (B) Protein expression. Colonic muscle tissue lysates containing equal amounts of total proteins were separated with SDS-PAGE and expression of smoothelin-A was analyzed using selective antibody. Results are expressed as fold differences in smoothelin-A protein expression in DSS-treated colon relative to that in control colon. Values represent the means ±SEM of 5 separate experiments. *p<0.05 versus control.
Figure: 12

- **Smoothelin-AmRNA (fold)**
  - Control: 1
  - DSS: 2

- **Smoothelin-A protein (fold)**
  - Control: 1
  - DSS: 3

- **Western Blot**
  - Control: 60 kDa
  - DSS: 60 kDa

- **β-actin**
3.4. Effect of inflammation on muscle contraction.

To determine whether changes in the expression of thin-filament proteins was associated with changes in agonist-induced contraction, freshly dispersed colonic muscle cells from control mice and TNBS-colitis mice were treated with different concentrations of acetylcholine and decrease in muscle cell length was measured by scanning micrometry. Basal muscle cell lengths were similar in control (95±4 μm) and TNBS-colitis (91±5 μm) and ACh caused contraction that was concentration-dependent in muscle cells from both control and TNBS-colitis mice (Fig. 13). However contraction in response to ACh was significantly lower in TNBS-colitis mice compared to control mice. The maximal response to 0.1 μM ACh was also significantly lower in TNBS-colitis mice (32±2% decrease in cell length) compared to control mice (20±3% decrease in cell length) (Fig. 13). These results are consistent with the previous studies in several animal models of inflammation.
Figure 13. Effect of TNBS treatment on ACh-induced muscle contraction. Contraction of dispersed muscle cells from colon was measured by scanning micrometry in response to different concentrations of ACh. Cells were treated with ACh for 30s and contraction was expressed as percent decrease in cell length from basal cell length: basal length of muscle cells from control mice 125±4 µm; basal control length of muscle cells from TNBS-colitis mice 118±5 µm. ACh caused contraction that was concentration-dependent in both inflamed and control. Contraction was significantly attenuated in cells from TNBS-colitis mice. The maximal response to 0.1 µM of ACh was 32±2% decrease in cell length in control mice and 20±3% decrease in cell length in TNBS-colitis mice. Values represent the means ±SEM of 5 separate experiments. **p<0.05 significant inhibition of contraction compared to control mice.
CHAPTER 4: DISCUSSION

Altered motility is induced by colonic inflammation, and this abnormality may lead to the diarrhea or constipation characteristic of IBD. It has been suggested that many factors, including changes in the function of ENS, ICC and smooth muscle are associated with altered colonic motility (1-4, 17-22, 25, 35-37, 63, 82, 93-96). The smooth muscle cells of the gastrointestinal tract are the final effectors of force development and work, and thus, it is important to understand the changes in expression of contractile proteins, which constitutes the main contractile apparatus of smooth muscle. In the present study, we examined the effects of colonic inflammation on the contractile properties of colonic smooth muscle by two well-characterized animal models of colitis such as TNBS (hapten)- and DSS-induced mouse models and three different approaches to measure the changes in the expression such as qRT-PCR, western blot and immunohistochemistry. Biochemically, the colonic inflammation induced by TNBS or DSS was characterized by an increased IL-1β expression, which is indicative of inflammation that occurs in these animal models of colitis, as well as in human IBD (7, 56).

Our main findings are: 1) expression of thin filament proteins involved in acto-myosin interaction and contraction such as α-actin, γ-actin is increased, 2) expression of thin-filament associated proteins involved in the regulation of acto-myosin interaction and contraction such as tropomyosin, smoothelin, calponin and caldesmon is increased, 3) contraction induced by acetylcholine in isolated smooth muscle cells is decreased by colitis, 4) The changes in the
expression of contractile proteins is observed at both protein and mRNA levels and the extent of changes in the expression of contractile proteins is similar in both TNBS- and DSS-induced colitic models. The present investigation of the response of the contractile proteins to inflammation may provide further insights into the mechanism by which inflammation modifies contractile proteins and suggests that inflammation may contribute to the regulation of the expression of genes responsible for synthesis of smooth muscle contraction proteins. Chronic inflammation of the colon also induces both hyperplasia and hypertrophy of the muscle leading to alterations in the cellular architecture (57). Increase in the expression of contractile proteins, major components of both contractile apparatus and the cytoskeletal structure of smooth muscle could contribute to the inflammation-induced changes in smooth muscle cellular architecture. Although TNBS and DSS are shown to mediate inflammation via distinct inflammatory mediators, several studies demonstrated similar decrease in spontaneous and agonist-mediated colonic muscle contraction in both models (16, 27, 40-49, 79, 90). In addition, alterations in Ca\(^{2+}\) handling and signal transduction pathway involved in muscle contraction are similar in both models (1-4, 13, 14, 34-37, 74-77, 93-96).

Contraction of smooth muscle is determined by the stimulation of ATPase activity of the myosin II, the main molecular motor for contraction, upon its interaction with actin. Stimulation of ATPase activity is greatly facilitated by the phosphorylation of 20-kDa myosin light chains (MLC20) by MLC kinase (28, 29, 38, 67, 69, 81, 89-91). A high correlation between MLC\(_{20}\) phosphorylation and muscle contraction has been demonstrated previously. The levels of MLC20 phosphorylation are regulated by opposing activities of MLC kinase and MLC phosphatase, whose activities, in turn, are regulated by feedback mechanism such as inhibition of
MLCK via CaMKII or AMP kinase, and activation or inhibition MLCP via telokin and CPI-17, respectively (38, 69, 89, 90). Previous studies have demonstrated that either exposure of cultured muscle cells in vitro to IL-1β or TNF-α or during inflammation in vivo has specific effects on the expression and activity of various targets in the signaling pathways mediating contraction and relaxation (14, 31, 32, 93-96). Colonic inflammation induced by TNBS or DSS also alters expression and/or activity of Ca2+ channels, K+ channels and SERCA2 suggesting an imbalance in Ca2+ mobilization mechanisms (1-4, 35-7, 39, 85-86, 94).

Contraction of smooth muscle is determined by mechanisms that regulate the availability of actin to interact with myosin via the action of inhibitory actin binding proteins such as caldesmon and calponin (6, 12, 28, 67). These thin-filament associated proteins are capable of stabilizing actin filaments. Caldesmon is a highly conserved, actin and myosin binding protein and exists in two isoforms: expression of heavy isoform (h-caldesmon) is specific to smooth muscle cells, whereas light isoform (l-caldesmon) is expressed in both non-muscle and dedifferentiated smooth muscle cells. Caldesmon inhibits acto-myosin interaction and the inhibitory is reversed by binding of Ca2+/CaM or by phosphorylation via ERK1/2 (6, 28, 67). Calponin is an actin binding protein and exists in three isoforms: expression of h1 calponin (basic calponin) is specific to smooth muscle, whereas h2 calponin (neutral calponin) and acidic non-muscle. Calponin inhibits acto-myosin ATPase activity interaction and the inhibition is reversed by phosphorylation via ERK1/2 or PKC. Thus caldesmon and calponin are important regulators of smooth muscle contraction (12, 28, 67). Although studies suggest that tropomyosin is necessary for full inhibition of acto-myosin activity by caldesmon, the exact mechanism of action is unclear (28, 67). The data presented in the current study reveal an association between increases in the expression of thin-
filament associated proteins, especially, caldesmon, calponin and tropomyosin in response to inflammation and decreased muscle contraction. Caldesmon and calponin inhibit muscle contraction and actin-activated ATPase activity, and tropomyosin enhances the caldesmon-induced inhibition of actomyosin ATPase. The low force in the presence of high levels of MLC_{20} phosphorylation was attributed to the suppression of acto-myosin ATPase activity and force generation by the high levels of caldesmon and calponin. The cytoskeleton modifications due to increased expression of thin-filament associated proteins may also have an effect on smooth muscle contraction. However, evidence for the cytoskeleton remodeling on cell contraction is lacking. Thus, our study in combination with previous studies proposes that in colitis, several mechanisms result in attenuated muscle contraction. First, a lower expression and/or activity of L-type Ca^{2+} channels result in decrease in intracellular Ca^{2+} and inhibition of Ca^{2+}/CaM-dependent MLCK activity (1-4, 35-37, 96). Second, a lower expression of CPI-17, an endogenous inhibition of MLCP, results in stimulation of MLCP activity (31, 74-76). Third, a greater expression of caldesmon and calponin results in attenuation of acto-myosin interaction. Further investigations might reveal whether the proposed mechanisms are dependent on each other or occur independently, and whether a singular mechanism is sufficient to produce altered muscle contraction. Smoothelin is an actin binding protein and exists in two isoforms: expression of smoothelin-A is selective to visceral smooth muscle, whereas expression of smoothelin-B is selective to vascular smooth muscle. Expression of both isoforms is abundant in contractile smooth muscle cells and limited in non-contractile proliferative smooth muscle cells. Recent studies using transgenic mice suggest expression of smoothelin-A is important slow wave activity and agonist-mediated contraction of gastrointestinal smooth muscle (72, 84). The increase in the expression of smoothelin-A in colitis suggests that it may be a compensatory
mechanism to offset the inhibitory effects exerted by increased caldesmon and calponin on smooth muscle contraction.

The results also suggest that up-regulation of thin-filament associated proteins is due to changes in the transcriptional regulation of these proteins, probably via the direct effect of proinflammatory cytokines on the transcription factors. Previous studies demonstrated that exposure of cultured muscle cells to IL-1β or TNF-α caused change in the transcriptional regulation of several proteins involved in smooth muscle contraction (31, 32, 93-96). The decrease in contraction on exposure of smooth muscle to IL-1β or TNF-α was reversed by inhibitor of nuclear factor-kappa B (NF-κB), suggesting that the cytokine effects are mediated mainly by NF-κB. Future studies on the animal models combined with studies on the smooth muscle cultures exposed to proinflammatory cytokines should provide valuable information on the direct effects of cytokines on the smooth muscle contractile proteins.

The final common pathway in the regulation of colon motility is the contractility of colonic muscle. This can be evaluated in vitro using isolated muscle cell preparation. Our studies in isolated muscle cells demonstrate that contraction induced by acetylcholine is decreased in both model of colitis. Clearly, changes in muscle contraction observed in vitro should be extrapolated into the in vivo situation with caution. Consideration must be given to the important roles mediated by enteric nerves, the ICC, and other endocrine and paracrine factors. In addition, the roles of circular and longitudinal muscle differ in terms of their contribution to peristaltic activity and their response to inflammation. However, muscle contraction is an acceptable approach and important determinant of evaluating how inflammatory and immune cells affect colonic motility.
In summary, the results of the present study have demonstrated colitis-augmented the expression of $\alpha$-actin, $\gamma$-actin, tropomyosin and smoothelin and the that the decrease in the contraction of smooth muscle isolated from colitis mouse colon is attributable to the increase in thin-filament associated proteins. Whether the changes in the expression of smooth muscle markers and the decrease of contraction are the cause or the consequence of disturbed GI tract motility remains to be determined. The changes smooth muscle contractile proteins, assessed here in inflammation, appear appropriate to associate to the functional alterations of the smooth muscle cell, and this was not studied before. A relatively minor role under physiological condition may a determining factor contributing to dysfunction of smooth muscle in diseases. In deed, changes in the expression of contractile proteins were demonstrated in various pathophysiological situations. Future studies of the mechanisms involved in the up-regulation of these proteins might provide more understanding of the molecular pathways involved in the expression of contractile protein regulation. Targeting these pathways in the regulation of colonic muscle contraction would be interesting. Clearly, a comprehensive appraisal of the enteric muscle contractile proteins and signal transduction pathways, in addition to the evaluation of enteric neurons and ICC, may contribute to further characterization of mechanisms underlying gastrointestinal motility disorders.
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Vita
Vita

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