Denitration in Colonic Smooth Muscle

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

By

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List of Abbreviations

ACh	Acetylcholine
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
DHP	Dihydropyridines
DSS	Dextran sulphate sodium
ENS	Enteric nervous system
IBD	Inflammatory bowel disease
ICC	Interstitial cells of Cajal
IFN-γ	Interferon-gamma
iNOS	Inducible nitric oxide synthase
IL-1β	Interleukin-1βeta
LPS	Lipopolysaccharide
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
O_2^-	Superoxide
$ONOO^-$	Peroxynitrite
PP2	4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine)
PDGF	Platelet-derived growth factor
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G

SIN-1	3-Morpholinylsydnoneiminechloride
TNBS	Trinitrobenzene sulphonic acid
TNF-α	Tumor necrosis factor-alpha
UC	Ulcerative colitis
VGCC	Voltage-gated calcium channel

Abstract

Evidence of denitration in colonic smooth muscle

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Tyrosine nitration results in altered function of smooth muscle voltage-gated L-type calcium channel. We explored the possibility that smooth muscle contains denitrase activity to allow functional recovery of the calcium channel without requiring synthesis of new channel proteins. Following peroxynitrite treatment of mouse colonic smooth muscle strips, $CaCl_2$ (1 mM)-induced smooth muscle contraction was significantly reduced by 67% ($P \leq 0.05$), which reversed by approximately 86% upon periodic washing within 2 hr period ($P \leq 0.001$). The effect of the c-Src kinase inhibitor, PP2, on muscle contraction was also restored after 2 hr post-peroxynitrite treatment consistent with the thesis that recovery from tyrosine nitration allows for tyrosine phosphorylation of the calcium channel. In addition, sodium orthovanadate prevented nitration-induced inhibition of muscle contraction by approximately 90%. Moreover, denitration of nitrated proteins was observed by western blots in smooth muscle cells over 2 hr. Since nitrotyrosine formation interferes with tyrosine kinase pathways involved in cell signaling, the presence of denitrase activity in smooth muscle cells may have profound

and important effects in restoring the function of nitrated proteins involved in cell signaling processes.

Chapter 1. Introduction

1.1 Immunomodulation of gastrointestinal motility

Motility of the gastrointestinal tract is essential for the orderly passage of food from the esophagus into the stomach, its storage and trituration in the stomach, its digestion and absorption in the small intestine, and its passage through the large intestine for the formation and ejection of stool.

Gastrointestinal motility is regulated by the coordination of input from interstitial cells of Cajal (ICC), the intrinsic nervous system (the enteric nervous system (ENS)) as well as extrinsic neuronal input on the smooth muscle. ICC network is embedded in the musculature of the gastrointestinal tract and act as an electrical pacemaker that electronically conducts signals to the underlying smooth muscle cells and defines rhythmic smooth muscle electrical activity known as slow waves. In addition to the involvement of the ICCs, the enteric nervous system generates and coordinates peristaltic activity throughout the gastrointestinal tract. Most enteric nerve cell bodies are situated in the myenteric and submucousal plexii. The myenteric plexus regulates smooth muscle activity whereas submucousal plexus regulates mucosal functions. Generally, myenteric plexus controls peristalsis and non-propagating contractions to allow digestion and absorption via the input from both excitatory and inhibitory motor neurons. The excitatory motor neurons synthesize the excitatory neurotransmitters including acetylcholine (ACh) and Substance P, which contract smooth muscle through specific receptors, whereas the inhibitory motor neurons contain inhibitory neurotransmitters including nitric oxide, vasoactive intestinal peptide and ATP to cause muscle relaxation.

Moreover, gastrointestinal motility is also regulated indirectly via modulation of the enteric neurons by sympathetic and parasympathetic systems (reviewed in [Sanders, 2008]).

The regulatory mechanisms of gastrointestinal excitation and contraction can be modulated by the immune system leading to motility disorders. Alteration in the motility of the various gut regions has been demonstrated in inflammatory diseases such as esophagitis, gastritis and inflammatory bowel disease (IBD). Inflammation stimulates immune activation leading to recruitment of macrophages and other immune cells at the site of inflammation. Activated immune cells recognize and destroy foreign and harmful agents by releasing cytokines, inflammatory mediators and reactive oxygen metabolites that cause alteration in the activity of enteric nerves and excitability of smooth muscle (reviewed in [Collins, 1996]).

Ulcerative colitis (UC) is an idiopathic inflammatory disease of the colon that decreases colonic motility through immune activation. A decrease in colonic motility has been demonstrated in isolated colonic muscle strips from both patients with ulcerative colitis and from animal models of colonic inflammation (Reddy et al., 1991 and Kinoshita et al., 2006). Attenuated colonic motility may be the result of increased levels of inflammatory cytokines such as TNF-α, IL-1β, IL-6, etc (Kinoshita et al., 2006). For example, 2-hr exposure to IL-1β significantly reduces neurokinin A-induced contraction in human colonic muscle strips (Vrees et al., 2002). Likewise, TNF-α-treated longitudinal smooth muscle colonic strips from normal mice produced significantly reduced contraction in response to carbachol when compared to the untreated tissues (Kinoshita et al., 2006). Taken together, inflammatory cytokines and reactive oxygen

metabolites appear to specifically alter the basal excitation-contraction coupling mechanisms of smooth muscle cells leading to decreased contractile function of smooth muscle.

1.2 Regulation of smooth muscle contraction

An increase in free intracellular Ca^{2+} is essential for smooth muscle contraction. Intracellular Ca^{2+} concentration may be increased by Ca^{2+} mobilization from sarcoplasmic reticulum and by the influx of extracellular Ca^{2+} through L-type voltage–gated Ca^{2+} channels. Increase in intracellular Ca^{2+} concentration occurs when excitatory neurotransmitter acetylcholine, produced by the parasympathetic and enteric neurons, activates muscarinic receptors, M2 and M3. M3 receptors are coupled via $G_{q/11}$ proteins to stimulate phospholipase C and phosphatidylinositol turnover, resulting in Ca^{2+} mobilization from sarcoplasmic reticulum and activation of protein kinase C (PKC). M2 receptors are coupled via $G_{i/o}$ proteins that increase Ca^{2+} entry through L-type Ca^{2+} channel by c-Src kinase activation (Jin et al., 2002). The mechanism by which M2 receptors modulate the activity of c-Src kinase is not entirely clear. Several studies have suggested that Src kinase activation requires $G\beta\gamma_i$ stimulation; however more recent studies have shown that $G\alpha_i$ is directly coupled to c-Src kinase. Both M2 and M3 receptors are also coupled to non-selective cation channels that allow entry of Ca^{2+} and Na^{+}, resulting in further depolarization and activation of L-type voltage-gated Ca^{2+} channels (reviewed in (Unno et al., 2006]). Increase in cytosolic calcium can be achieved by release from both sarcoplasmic reticulum and influx of extracellular Ca^{2+}. The principal molecular switch that stimulates smooth muscle contraction is Ca^{2+} binding to

calmodulin. Ca^{2+}/calmodulin complex activates myosin light chain kinase which in turn activates regulatory myosin light chain thus activating molecular motors to initiate smooth muscle contraction. Several mechanisms have been proposed to activate myosin phosphatase to dephosphorylate myosin II and cause muscle relaxation (reviewed in [Murthy, 2006]).

As mentioned earlier, smooth muscle contractions are decreased following colonic inflammation. Colitis-induced attenuation of smooth muscle contraction may involve alterations in the activity of G-proteins, changes in the structural or biochemical properties of the contractile proteins, and/or alteration in Ca^{2+} mobilization. The density and biochemical properties of the muscarinic receptors are not altered during inflammation (Shi and Sarna, 1999). Hence, it has been suggested that the reduced smooth muscle contraction during inflammation may be due to defective Ca^{2+} mobilization (Biancani et al., 1992). Although the Ca^{2+} release from the ryanodine- and IP3-sensitive intracellular stores is not altered in smooth muscle cells following colonic inflammation (Shi and Sarna, 2000), several studies have demonstrated that the decreased contraction may be due to impairment of Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels which likely contributes to the suppression of smooth muscle contractility during inflammation (Shi and Sarna, 2000; Kinoshita et al., 2003; Akbarali et al., 2000).

1.3 Voltage-gated Ca^{2+} channels

Voltage-gated Ca^{2+} channels (VGCCs) in many different cell types activate and mediate Ca^{2+} influx in response to membrane depolarization. Ca^{2+} entering the cell through VGCCs serves as the second messenger of electrical signaling, allowing

activation of intracellular events such as contraction, secretion, synaptic transmission, and gene expression. VGCCs are formed as a complex of several different subunits. They have a pore-forming α1 subunit of ~ 190-250 kDa; an extracellular glycosylated α2 subunit that is disulfide-bonded to a small transmembrane δ subunit; an intracellular β subunit; and in skeletal muscle a membrane-spanning γ subunit. Ten α1 subunits, four α2 δ complexes, four β subunits and two γ subunits have been identified. The α1 subunit consists of four membrane domains, and each domain is composed of six transmembrane (TM) helices. Each domain has a pore loop between TM5 and TM6 that serves as a selectivity filter and TM4 serves as a voltage sensor. Both the N-terminal and C-terminal ends of the α1 subunit are exposed to the cytoplasmic surface of the plasma membrane. A cytoplasmic loop between domains I and II appears to be involved in channel inactivation. Although the functional roles of the other subunits have not been entirely understood, they are proposed to contribute in assembly of VGCCs, docking of other proteins to VGCCs, movement of the channel to the membrane, and regulation of channel properties (reviewed in [Catterall, 2000]).

There are multiple types of Ca^{2+} currents as defined by their biophysical properties, physiological roles and inhibition by specific toxins. Ca^{2+} currents are designated as L-, N-, P-, Q-, R-, and T-type. Voltage-gated Ca^{2+} channels have been categorized into four families of α1 subunit. The Ca_v1 family contains four types of α1 subunits: $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$, and $Ca_v1.4$, which conduct L-type Ca^{2+} currents. L-type voltage-gated Ca^{2+} channels display high voltage-dependent activation, large single-channel conductance, slow voltage-dependent inactivation, marked regulation by cyclic adenosine monophosphate (cAMP)-dependent protein phosphorylation pathways, and

specific inhibition by dihydropyridines (DHP) (Reuter, 1977). The Ca_v2 family conducts N-type, P/Q-, and R-type Ca^{2+} currents, which initiate rapid synaptic transmission. The Ca_v3 family conducts T-type Ca^{2+} currents, which are activated and inactivated very rapidly (reviewed in [Catterall, 2000]).

Smooth muscle cells mainly express L-type Ca^{2+} channels, which belong to the $Ca_v1.2$ family, previously designated α_{1c}, of which several isoforms have been identified (Kang et al., 2004). In gastrointestinal smooth muscle, the upstroke of action potential is mediated by the influx of extracellular Ca^{2+} through $Ca_v1.2$ Ca^{2+} channels and is responsible for the initiation of smooth muscle contraction. Previous studies measuring calcium channel function in several different experimental colitis models have suggested that the impairment of Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels is responsible for decreased smooth muscle contractility during inflammation (Shi and Sarna, 2000; Kinoshita et al., 2003; Akbarali et al., 2000). Using single smooth muscle cell patch-clamp recordings in the dextran sulphate sodium (DSS) mouse colitis model, Ca^{2+} currents were reduced by 70% (Akbarali et al., 2000). Similar decreases in calcium currents were also obtained from canine acetic-acid/ethanol-induced inflammation model (Liu et al., 2001) and in the trinitrobenzene sulphonic acid (TNBS) rat colitis model (Kinoshita et al., 2003). The decrease in calcium currents can occur as a result of altered gene and protein expression or altered regulation of the channel function. Neither gene nor protein expression levels of Ca^{2+} channel α_{1c} subunit were changed in the mouse DSS or the rat TNBS-treated smooth muscle cells (Kinoshita et al., 2006), but the regulation of the Ca^{2+} channel by the protein tyrosine kinase, c-Src kinase was significantly reduced during inflammation (Kang et al., 2004).

1.4 c-Src regulation of voltage-gated calcium channels

It has been well established that the calcium channel activity is modulated by the serine/threonine kinases such as protein kinase A (PKA), protein kinase C (PKC) and protein kinase G (PKG) (reviewed in [Davis et al., 2001]). Recent evidence suggests that smooth muscle contraction and calcium influx through L-type voltage-gated Ca^{2+} channels are also regulated by the nonreceptor tyrosine kinase, c-Src kinase (Hu et al., 1998). c-Src kinase regulation of Ca^{2+} channel is coupled to activation of muscarinic receptors and growth factor receptors (Hu et al., 1998). This is supported by the fact that c-Src kinase is concentrated around cellular attachment sites to the extracellular matrix (reviewed in [Richardson and Parsons, 1995]). ACh-induced muscarinic stimulation of canine colonic smooth muscle cells increases c-Src kinase activity through muscarinic receptors (reviewed in [Singer et al., 1996]; Jin et al., 2002). Blockade of M2 and M3 receptors with 4-DAMP and AF-DX 116 respectively confirms that c-Src kinase activation is mediated through M2 receptors but not M3 receptors in colonic smooth muscle. In addition, activation of growth factor receptors through platelet-derived growth factor (PDGF) BB also increases activation of c-Src kinase and enhances Ca^{2+} currents by 43% which can be attenuated by the anti-Src antibody (Hu et al., 1998). Likewise, epidermal growth factor enhances calcium currents by 60% (Hatakeyama et al., 1996). Moreover, specific and non-specific inhibitors of Src kinase have been shown to reversibly attenuate smooth muscle contraction and Ca^{2+} currents. DiSalvo et al (1993) initially demonstrated that structurally unrelated tyrosine kinase inhibitors (Geldanomycin, tyrphostin and genistein) markedly and reversibly inhibit tonic and phasic smooth muscle contractions induced by carbachol and norepinephrine. It was

confirmed by Hatakeyama et al (1996) that genistein and tyrphostin dose-dependently inhibit Ca^{2+} currents in colonic smooth muscle cells. The specific inhibitor of c-Src kinase, PP2, also inhibits Ca^{2+} currents by about 70% in circular smooth muscle cells from rabbit distal colon (Jin et al., 2002) and PP2 inhibits Ca^{2+} currents by approximately 50% in mice colonic smooth muscle cells (Kang et al., 2004). Conversely, sodium orthovanadate, which is a tyrosine phosphatase inhibitor and therefore enhances kinase activity, augments calcium currents by 30% and induces smooth muscle contraction. The fact that basal calcium currents are attenuated by tyrosine kinase inhibitors and enhanced by phosphatase inhibitor indicates that c-Src kinase may be constitutively up-regulating calcium channel activity and the finding of high basal levels of c-Src kinase in smooth muscle is consistent with this proposal (Hatakeyama et al., 1996).

Direct association of c-Src kinase with the α_{1c} subunit of the L-type calcium channel has been demonstrated by coimmunoprecipitation with anti-Src and anti-phosphotyrosine antibodies (Hu et al., 1998). The Src kinase structure consists of a unique domain, SH3 domain, SH2 domain, a catalytic domain, and a C-terminal regulatory segment (reviewed in [Roskoski, 2004]). Src kinase is negatively regulated by phosphorylation of a conserved C-terminal tyrosine residue, Tyr^{527}, which can be autophosphorylated or phosphorylated by another tyrosine kinase known as C-terminal Src kinase (CSK). The regulatory SH2 domain interacts with phosphorylated Tyr^{527}, rendering Src kinase in a closed, inactive conformation and preventing interaction with other proteins. The intramolecular interaction between SH2 and Tyr^{527} is relatively weak and can be repelled by a protein with greater affinity to the Src SH2 domain, such as an activated growth factor receptor, resulting in a phosphorylation of Tyr^{416} in the

catalytic domain of Src molecule which is essential for activation of Src kinase (reviewed in [Superti-Furga, 1995]).

The SH2 and SH3 domains of Src kinase interact with phosphorylated tyrosine residues and proline-rich regions, respectively. Both Src SH3 and SH2 binding domains have been identified in the C-terminal tail of the α_{1c} subunit of the L-type Ca^{2+} channels. An important tyrosine residue Y^{2134}, when phosphorylated, binds to the SH2 domain of c-Src kinase. Interestingly, a single mutation does not prevent tyrosine phosphorylation, but requires mutations in both Y^{2134} and Y^{1837}. The double mutation significantly prevents the regulation of the Ca^{2+} channels by the c-Src kinase (Jin et al., 2002). In addition, a consensus sequence of PPQP (amino acids 1965-1968) in the C-terminus of α_{1c} subunit binds to the SH3 domain of Src but does not seem to be involved in the regulation of Ca^{2+} channels (Kang et al., 2007). The biophysical mechanism by which Src enhances calcium currents is not entirely understood but may involve changes in the gating behavior of the channel.

1.5 Inflammation alters Ca^{2+} channel regulation by c-Src Kinase

The mechanism by which colonic inflammation attenuates smooth muscle contraction and Ca^{2+} channel currents may involve impaired c-Src kinase regulation of Ca^{2+} channel activity. Kang et al (2004) initially demonstrated that inhibition of the Ca^{2+} channel by PP2 was reduced in smooth muscle cells from inflamed mice colon suggesting that the regulation of the Ca^{2+} channel by c-Src kinase was altered during inflammation. Further studies by Ross et al (2007) demonstrated that inhibition of c-Src kinase by PP2 has no significant effect on calcium-induced smooth muscle contraction in

inflamed colonic tissue while PP2 significantly reduced Ca^{2+} induced contractions in control colonic muscle. This raises the possibility that either c-Src kinase activity or tyrosine phosphatase activity may be altered during inflammation, resulting in decreased c-Src kinase regulation of Ca^{2+} channels. By measuring Src kinase phosphate incorporation, Ross et al (2007) suggested that c-Src kinase activity was not altered during inflammation. Thus, possible mechanisms for the decreased Ca^{2+} channel activity may reside in the inability of the c-Src kinase to phosphorylate the Ca^{2+} channel. This could occur as a result of functional modification of Ca^{2+} channel by nitration.

1.6 Nitration impairs phosphorylation of tyrosine residues in Ca^{2+} channel

Peroxynitrite ($ONOO^-$), formed from the combination of nitric oxide (NO) and superoxide (O_2^-) appears to be the major nitrating agent in vivo and has been shown to nitrate tyrosine residues in vitro (Estévez et al., 1998). During inflammation, O_2^- is produced by NADPH oxidase in neutrophils which is induced by proinflammatory stimuli and NO is synthesized by inducible nitric oxide synthase (iNOS) induced by cytokines such as TNF-α, IL-1β and IFN-γ and lipopolysaccharide (LPS) in macrophages (reviewed in [Turko and Murad, 2002]). NO is highly unstable and therefore reacts in an almost diffusion-limited manner with O_2^- to form the very reactive species $ONOO^-$, which has the potential to nitrate free and protein-associated tyrosine residues (reviewed in [Singer et al., 1996]). Nitrotyrosine formation involves a covalent modification, resulting in the addition of a nitro group onto the ortho carbon of the tyrosine residues. An association between enhanced NOS activity and nitrotyrosine formation has been demonstrated in inflammatory disorders, such as inflammatory bowel disease (Kimura et

al., 1997 and Rachmilewitz et al., 2005). This is consistent with the increased nitrotyrosine formation observed in the inflamed human colonic epithelium and many other inflamed states (reviewed in [Singer et al., 1996]). The nitration of proteins may involve altered conformation and function of the target proteins, susceptibility to proteolysis and/or impairing tyrosine phosphorylation regulatory mechanism (Irie et al., 2003). Hence, it was proposed that the nitration of Ca^{2+} channels may interfere with c-Src kinase-mediated phosphorylation and thus decrease Ca^{2+} channel activity during inflammation (Ross et al., 2007). Exposure to peroxynitrite significantly reduces Ca^{2+} channel currents and Ca^{2+}-induced smooth muscle contractions in mouse colon, consistent with decreased Ca^{2+} channel currents and Ca^{2+}-induced smooth muscle contractions observed in TNBS-induced colitis (Ross et al., 2007). Interestingly, inflammation- and peroxynitrite-induced decrease in Ca^{2+} channel currents and Ca^{2+}-induced smooth muscle contractions were not further affected by the inhibition of c-Src kinase with PP2, suggesting that peroxynitrite impairs the tyrosine phosphorylation regulatory mechanism by nitrating the tyrosine residues of Ca^{2+} channel. This is supported by the evidence that peroxynitrite treatment increases nitration and reduces tyrosine phosphorylation of Ca^{2+} channels in HEK293 cells cotransfected with human smooth muscle L-type Ca^{2+} channel and mouse c-Src kinase. Further studies confirmed that peroxynitrite decreases tyrosine phosphorylation and increases nitrotyrosine formation in endothelial cells (Kong et al., 1996; Gow et al., 1996). Thus, nitration of Ca^{2+} channels during colonic inflammation underlies decreased functional ability of Ca^{2+} channels and attenuates c-Src kinase regulation (Kang et al., 2008).

Analogous to protein phosphorylation and dephosphorylation, the process of denitration may have considerable functional significance in modification/repair of the nitrated proteins allowing proteins to regain their functional ability.

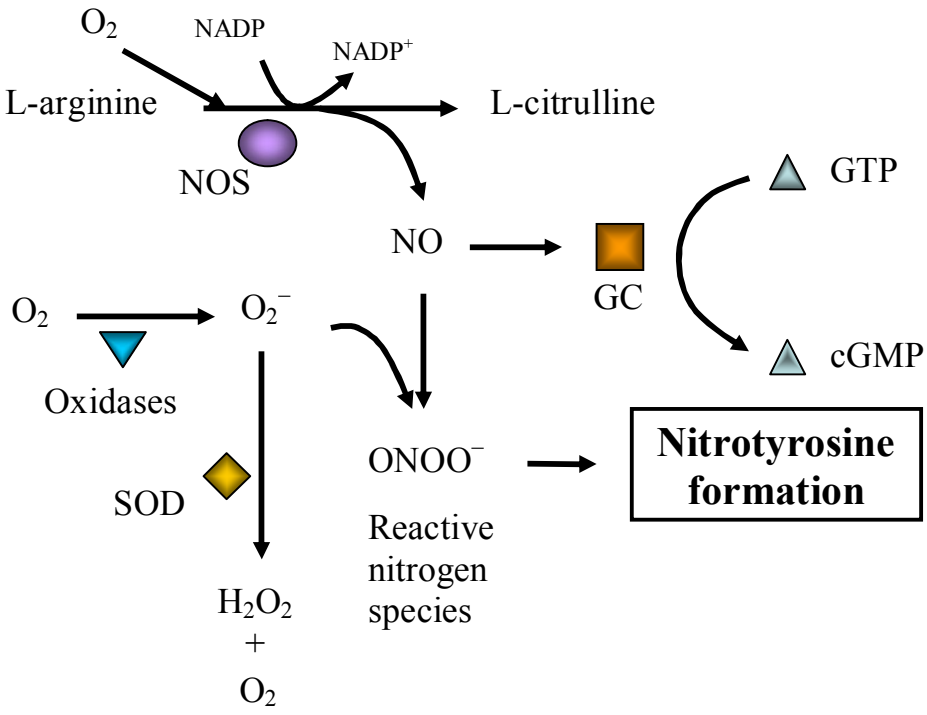

Figure 1. Schematic representation of peroxynitrite production and nitrotyrosine formation.

1.7 Evidence of denitration

Recent studies have reported the presence of an activity in lung, spleen and activated macrophages that modifies nitrotyrosine-containing proteins (Kamisaki et al., 1998). The activity is time- and protein-concentration dependent, labile to heating and trypsin treatment, regulatable, perhaps inducible and uses specific substrates, suggesting that this activity is a protein and may be a "nitrotyrosine denitrase" that removes nitro group from nitrotyrosine in proteins. Kamisaki et al (1998) examined homogenates from vascular rich tissues such as spleen, lung, liver, and kidney for an activity that modified nitrated BSA from LPS-treated rats. Incubation of nitrated BSA with extracts from rat lung or spleen reduced nitrotyrosine content without changes in the BSA levels. On the contrary, incubation with extracts from liver and kidney decreased nitrated BSA levels in the absence, but not in the presence of protease inhibitors, presumably because of protein degradation. Taken together, denitrase activity is observed in the rat lung and spleen but not in the liver and kidney, suggesting that denitrase activity may be tissue specific. Further characterization of the denitrase activity in rat spleen revealed that the denitrase activity is time- and protein-concentration dependent, is sensitive to heating and trypsin treatment and is regulatable and perhaps inducible with LPS treatment (Kamisaki et al., 1998). In addition, incubation of a mixture of nitrotyrosine-containing proteins revealed that the denitrase activity shows selectivity against specific substrates, including BSA, β-galactosidase, carbonic anhydrase, and lysozyme (Kamisaki et al., 1998). For further experiments, Murad and colleagues (2003) developed an assay strategy for denitrase to screen specific substrates in a mouse macrophage cell line, RAW264.7. Activated macrophages produce excessive amounts of nitric oxide and superoxide leading to

nitrotyrosine formation. Activated macrophages or RAW264.7 cells can survive under oxidative stressful conditions because they may contain denitrase activity to restore function of nitrated proteins. The assay strategy was to induce nitration in RAW264.7 cells with peroxynitrite, apply fractionated cell lysate to 2D gel electrophoresis, blot onto PVDF membrane, and incubate with the cell lysate from LPS-treated RAW264.7 cells, as a source of denitrase. Possible substrates for denitrase were detected with anti-nitrotyrosine antibody by immunoblots. Several proteins showed decrease in nitrotyrosine immunoreactivity and one of the proteins was found out to be mouse Histone H1.2, suggesting that activated RAW264.7 cells express denitrase activity to modify nitrated proteins. Further experiments confirmed that the nitration of recombinant and purified Histone H1.2 was significantly reduced with the treatment of RAW264.7 cell lysate. Denitrase activity behaved as an enzymatic activity for its specific substrate, Histone H1.2, because the denitration was time dependent and was sensitive to heat or trypsin treatment.

Later on, Kang et al (2008) used 1D membrane assay for denitrase activity to show that the C-terminus of the L-type Ca^{2+} channel may also be a potential target for the denitrase. For instance, after pretreatment with the cell lysates from RAW264.7 cells, nitration of the Ca^{2+} channel was significantly down-regulated. In addition, pretreatment with the cell lysates from RAW264.7 cells allowed recovery of tyrosine phosphorylation by c-Src kinase. This phenomenon confirms that native smooth muscle calcium channel is a substrate for regulation by denitrase activity.

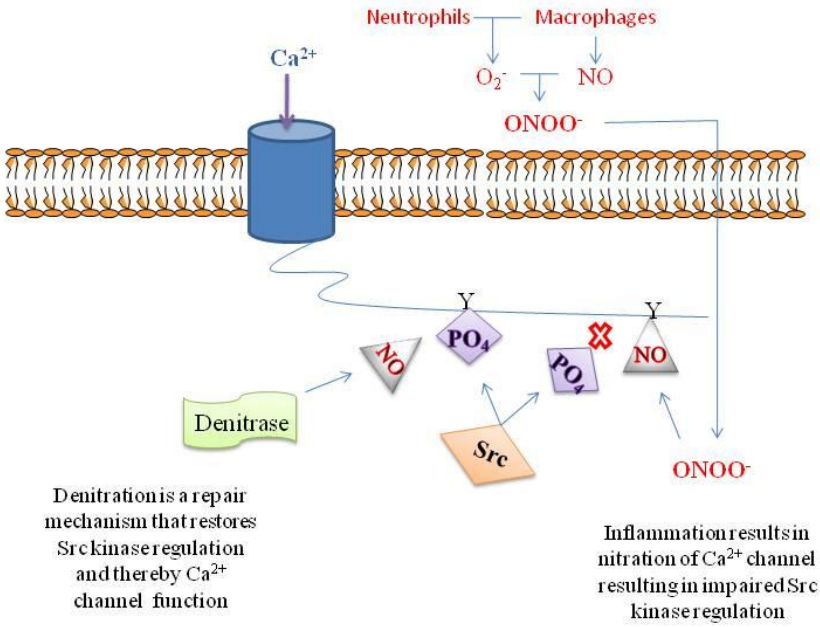

Figure 2. Schematic representation of nitration and denitration of tyrosine residues with the carboxy terminus of $Ca_v1.2$.

1.8 Objective

As illustrated above, calcium channels may be potential substrate for denitration. This would allow for functional recovery of the channel without requiring synthesis of new channel proteins. While the previous study has demonstrated this for the c-terminal fragment in vitro, it is not known whether smooth muscle cells contain denitrase activity and whether this results in functional reversal of the calcium channel. The objective of this study was to determine if a denitrase activity can be demonstrated in smooth muscle. To this end, the following methodology was employed: 1) Muscle contraction, and 2) Western blots.

We determined if calcium channel function in terms of contractile ability of the muscle is reversed following nitration, and if denitration of nitrated proteins could be observed by Western blots from smooth muscle lysates.

Chapter 2. Materials and Methods

2.1 Materials. SIN-1 chloride (3-Morpholinylsydnoneiminechloride) was purchased from Sigma-Aldrich; sodium peroxynitrite was purchased from Cayman Chemical; and Sodium orthovandate from Sigma-Aldrich. PP2 (10 mM in dimethyl sulfoxide) and mouse monoclonal anti-nitrotyrosine antibody were purchased from Calbiochem; and rabbit anti-nitrotyrosine antibody was purchased from Millipore. RIPA buffer and protein A/G beads were purchased from Santa Cruz Biotechnology, Inc. The secondary antibodies (anti-mouse IRDye 800and anti-rabbit IRDye 680) were obtained from LiCor Biosciences.

2.2 Isometric Tension Recording

a) Preparation of colonic smooth muscle strips for isometric measurement

Approximately 2-cm strips of mouse colon were suspended in the longitudinal direction in an organ bath containing 15 ml of Krebs solution. The Krebs physiological solution contained: 118 mM NaCl, 4.6 mM KCl, 1.3 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose, and 2.5 mM $CaCl_2$. Krebs solution was maintained at 37°C and bubbled continuously with carbogen (95% O and 5% CO_2) to maintain physiological pH of 7.4. Tissues were suspended under a resting tension of 1 g and equilibrated for 1 h. Preliminary experiments were conducted to examine the length-tension characteristics. Carbachol-induced colonic smooth muscle contractions were measured at various resting tensions ranging from 0.5 to 1.5g; a resting tension of 1 g was taken to be the optimal length at which the muscle developed the greatest active tension. Hence, the applied

passive tension and tissue length were kept constant for further experiments. Isometric contractions were recorded by a force transducer (model GR-FT03; Radnoti) connected to a personal computer using the Acqknowledge 382 software program (BIOPAC Systems). After equilibration in Krebs solution, tissues were incubated for 0.5-1 h in Ca^{2+}-free high-potassium solution (80 mM) in which equimolar NaCl was replaced by KCl containing 0.1 mM EGTA and changed every 15 min. Stock solutions of 100 mM EGTA were made in 0.2 N NaOH. Tissues were washed 3 times and depolarized by Ca^{2+}-free high-potassium solution (80 mM) without EGTA for 15 min. Tissues were washed again with Ca^{2+}-free high-potassium solution (80 mM) to ensure that EGTA has been removed completely and were equilibrated for 15 min.

b) Treatment with SIN-1 and peroxynitrite

To determine nitration-induced changes in smooth muscle contractions, mouse colonic strips were incubated in 3-morpholinosydnonimine (SIN-1) (500 μM) for 1 h followed by sodium peroxynitrite (150 μM) twice at 10-min interval and washed with Ca^{2+}-free high-potassium solution (80 mM). $CaCl_2$ (1 mM) induced contractile responses were recorded before and after peroxynitrite treatment. To determine whether smooth muscle contractions recovered from nitration-induced changes, colonic strips were washed with Ca^{2+}-free high-potassium solution (80 mM) every 30 min for 2 h following SIN-1 and peroxynitrite treatment. $CaCl_2$ (1 mM) induced contractions were recorded after every wash.

c) Treatment with PP2

To determine the effect of PP2, a Src kinase inhibitor, colonic strips were incubated in PP2 (10 μM) for 10 min and $CaCl_2$ (1 mM)-induced smooth muscle contractions were recorded in the presence and absence of PP2.

To determine whether PP2 was effective after 2 hrs post-treatment with SIN-1 and peroxynitrite, colonic strips were incubated in PP2 (10 μM) for 10 min and $CaCl_2$ (1 mM)-induced contractile responses were recorded.

d) Treatment with sodium orthovanadate

To determine whether sodium orthovanadate (Na_3VO_4), a tyrosine phosphatase inhibitor, prevented peroxynitrite-induced changes, colonic tissue strips were treated with Na_3VO_4 (100 μM) followed by SIN-1 (500 μM) for 1 hr and $ONOO^-$ (150 μM) twice at 10 min interval. $CaCl_2$ (1 mM)-induced contractile responses were recorded before and after pharmacological treatment.

2.3 Quantitative analysis of peroxnitrite-induced nitration of proteins in mouse colon smooth muscle by immunoblotting

Colon were excised from mice and cleansed in Krebs solution. Colon were incubated in SIN-1 (500 μM) for 1h followed by $ONOO^-$ (150 or 300 μM) 4 times at 10-min intervals in Krebs solution under aerated or non-aerated conditions. Colon were washed with Krebs solution and flash-frozen in liquid nitrogen. Tissues were washed in phosphate-buffered saline (PBS) solution and homogenized for 1 min in RIPA buffer (3 ml/g tissue) supplemented with protease inhibitors containing 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml Calpain I, 10 μg/ml Calpain II, 0.1 mM sodium orthovanadate. The

homogenization was repeated with after a 1-min rest on ice. After incubation on ice for 30 min, the cell debris was pelleted by centrifugation at 10,000 x g for 10 min at 4ºC. Protein concentration was determined by BCA Protein Assay Kit (Pierce) before immunoprecipitation of nitrated proteins. The protein samples were incubated with rabbit anti-nitrotyrosine antibody and placed on a rocker at 4ºC for 1 h followed by overnight incubation with Protein A/G beads (40 µl). Immunoprecipitated samples were centrifuged at 1,000 x g at 4ºC for 2 min, and the buffer was removed. Beads were washed three to four times with PBS at 4ºC. After last wash, 40 µl of sample loading buffer was added to the beads. Samples were run on a 10% SDS-polyacrylamide gel electrophoresis and transferred on a 0.2-mm polyvinylidene difluoride membrane at 5 V for 3 h. Membranes were blocked for 1 h at room temperature with 5% non-fat milk. Membranes were rinsed with tris-buffered saline containing 0.1% Tween-20 (TBST) for 5 min and incubated overnight at 4ºC with mouse monoclonal anti-nitrotyrosine antibody (1:500) diluted in 5% non-fat milk with 0.1% Tween-20. The membranes were rinsed with TBST for three to four times for 5 min and incubated for 1 h at room temperature with secondary antibodies, anti-mouse IRDye 800 and anti-rabbit IRDye 680, diluted (1:5,000) in 5% non-fat milk with 0.1% Tween-20. After rinsing with TBST for three to four times for 5 min, the membranes were visualized using LI-COR Odyssey Infrared imaging system (LI-COR).

For measuring the effect of denitration on colonic smooth muscle, colon were excised from mice and treated with SIN-1 and peroxynitrite as previously described. Colon were then washed every 30 min for 2 hrs with Krebs solution and flash-frozen in liquid

nitrogen. Protein samples were prepared as previously described and examined for nitrotyrosine as mentioned above.

Chapter 3. Results

3.1 $CaCl_2$-induced concentration dependent contractions in Murine colon

Initial experiments were performed to examine the cumulative addition of $CaCl_2$-induced contraction in mouse colonic smooth muscle strips. The muscle strips were initially stretched to 1 g to bring them to a condition of optimum force development, and equilibrated for 45 min in an oxygenated Krebs solution at a pH of 7.4 and 37°C. After an equilibration period of 1 hr, the muscle strips were incubated with Ca^{2+}-free high potassium (80 mM) solution containing 0.1 mM EGTA to remove extracellular and intracellular Ca^{2+} ions, and then depolarized by Ca^{2+}-free high potassium (80 mM) solution to allow opening of L-type Ca^{2+} channels. After spontaneous phasic contractions had stabilized, $CaCl_2$ was added to induce colonic smooth muscle contractions.

Cumulative addition of $CaCl_2$ (10 μM to 1 mM) produced concentration-dependent contractions in colonic tissues. Contractile responses increased from 0.016 ± 0.004 g to 0.212 ± 0.022 g ($P \leq 0.001$; $n = 5$). In some tissues, at concentrations greater than 300 μM a phasic contraction followed by a more sustained contraction was observed. Maximal contraction in response to 1 mM $CaCl_2$ was considered as 100%. $CaCl_2$ (1 mM) was chosen as a final concentration to be used for further experiments as previous studies established that in the mouse colon, 1 mM Ca^{2+} resulted in maximal contractions in high K^+ solutions. Data were analyzed by one-way repeated measures-ANOVA followed by Holm-Sidak method.

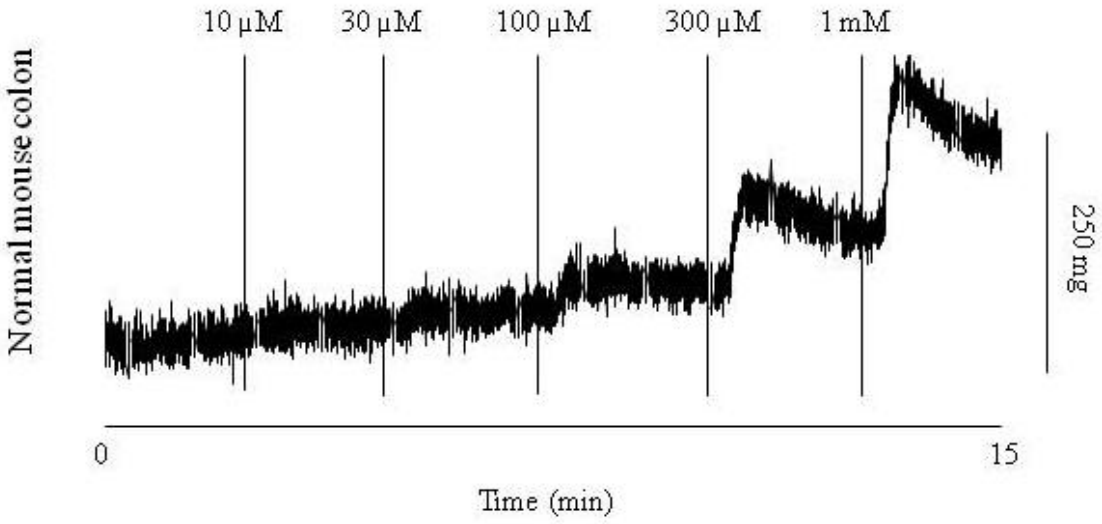

Figure 3. Representative tracing of $CaCl_2$-induced concentration-dependent contraction in isolated mouse colon.

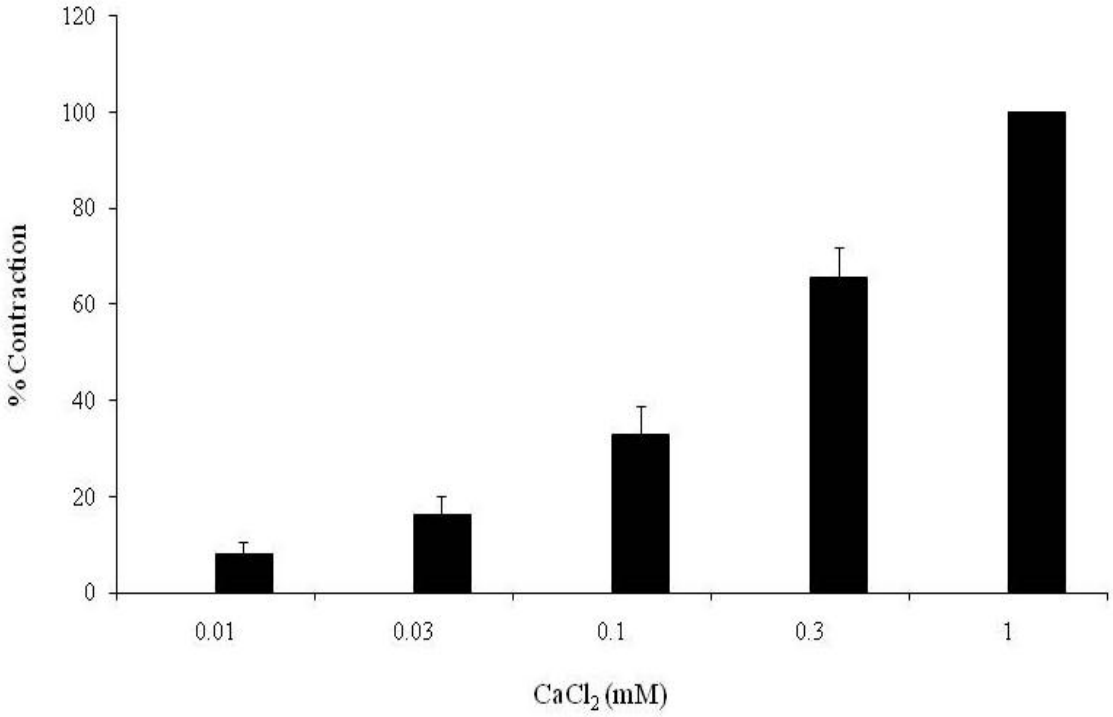

Figure 4. $CaCl_2$-induced concentration-dependent contraction in isolated mouse colon. $CaCl_2$ (1 mM)-induced contraction is considered as 100%. Bars represent the mean ± S.E.M. Data were analyzed by one-way repeated measures-ANOVA followed by Holm-Sidak method. $P \leq 0.001$, $n = 5$.

3.2 Effect of peroxynitrite on $CaCl_2$-induced smooth muscle contractions

Previous studies have shown that inflammation significantly reduces $CaCl_2$-induced smooth muscle contractions in mouse distal colon (Ross et al., 2007). To confirm whether peroxynitrite mimics inflammation-induced changes, mouse colonic smooth muscle strips were incubated with SIN-1 and peroxynitrite.

Following depolarization by Ca^{2+}-free high-potassium (80 mM) solution, $CaCl_2$ (1 mM) was added to induce contraction. Maximal contractile response to $CaCl_2$ (1 mM) was considered as 100%. Muscle strips were then incubated with SIN-1 (500 μM) for 1 h followed by sodium peroxynitrite (150 μM) twice at 10 min interval. Pre-incubation with nitrating agents significantly ($P \leq 0.05$) reduced the contractions from 0.35 ± 0.06 g to 0.12 ± 0.05 g, representing a 66.9% reduction in contraction to $CaCl_2$ (1 mM) ($n = 4$). Data were analyzed by paired *t*-test.

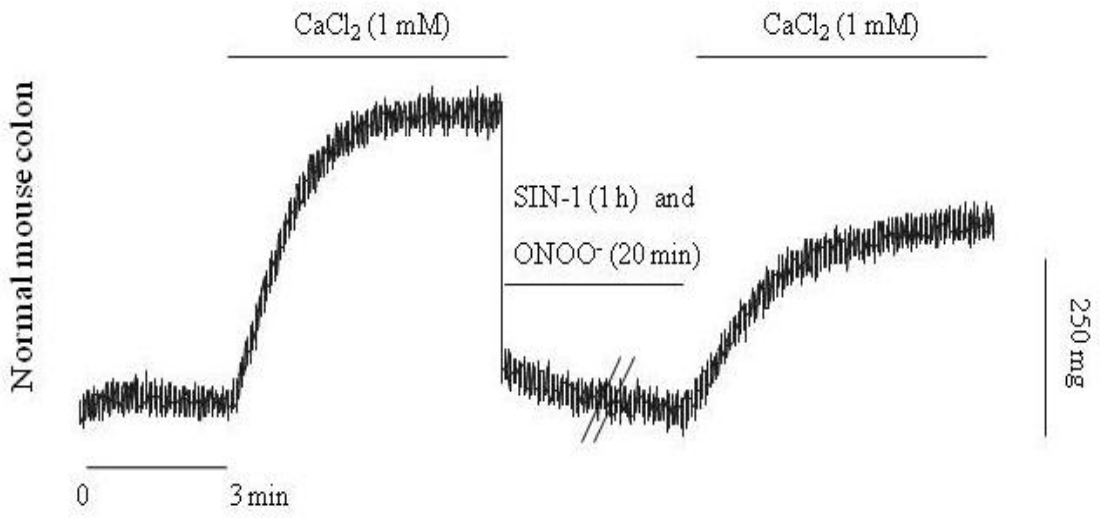

Figure 5. Representative tracing of the effect of peroxynitrite on $CaCl_2$-induced colonic smooth muscle contractions. // represents break in time.

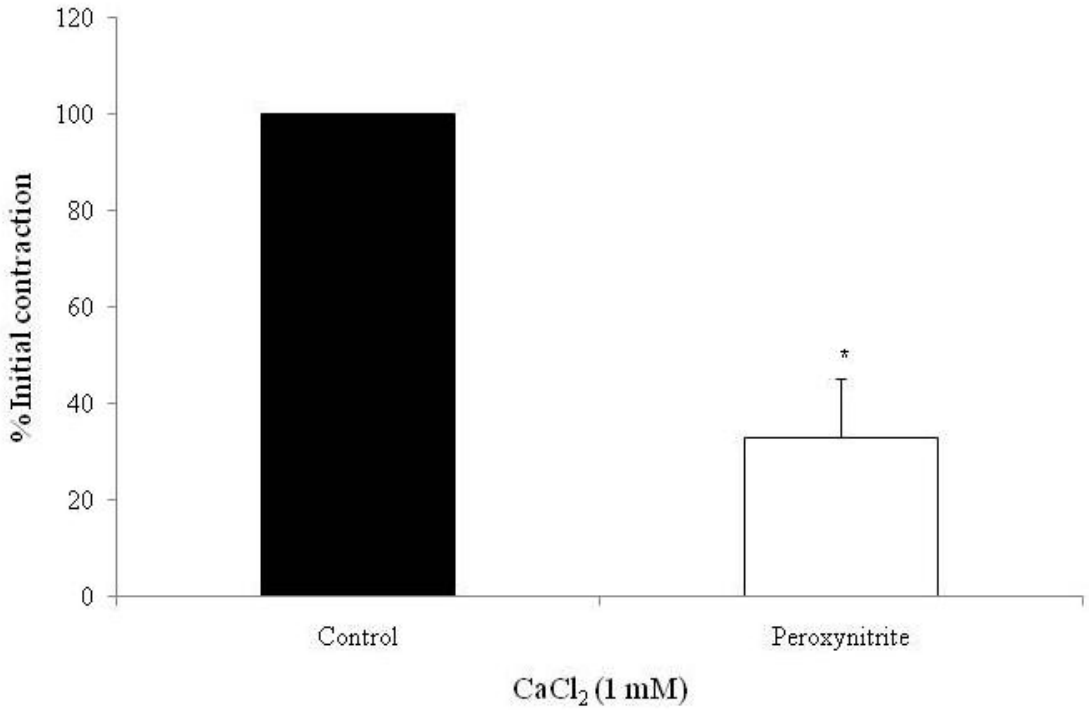

Figure 6. Graphical representation of the effect of peroxynitrite on $CaCl_2$-induced colonic smooth muscle contractions. Maximal contraction of control was considered as 100%. Bars represent mean ± S.E.M. Data were analyzed by paired *t* test, $P \leq 0.05$ versus control, $n = 4$.

3.3 Reversal of peroxynitrite-induced attenuation of $CaCl_2$-induced smooth muscle contractions

This experiment was performed to determine whether peroxynitrite-induced attenuation of colonic smooth muscle contraction was a reversible process. Following depolarization by Ca^{2+}-free high-potassium (80 mM) solution, $CaCl_2$ (1 mM)-induced maximal contractile response was considered as 100%. Pre-incubation with nitrating agents significantly ($P \leq 0.001$) reduced $CaCl_2$ (1 mM)-induced contractions by 63.4 ± 4.4%. The muscle strips were then washed every 30 min for 2 hrs with Ca^{2+}-free high-potassium (80 mM) solution, and $CaCl_2$ (1 mM) was added after every wash to measure the extent of contraction. A time-dependent recovery of smooth muscle contraction was observed from 36.6 ± 4.4% to 83.6 ± 6.9% after 2hrs ($P \leq 0.001$; $n = 9$). Data were analyzed by one-way repeated measures-ANOVA followed by Holm-Sidak method.

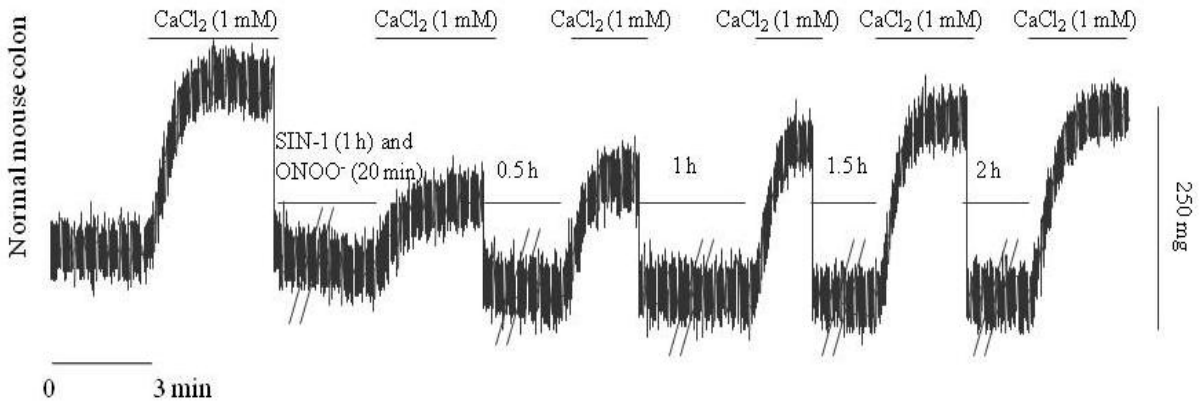

Figure 7. Representative tracing of a time-dependent recovery of peroxynitrite-induced attenuation of colonic smooth muscle contractions. // represents break in time.

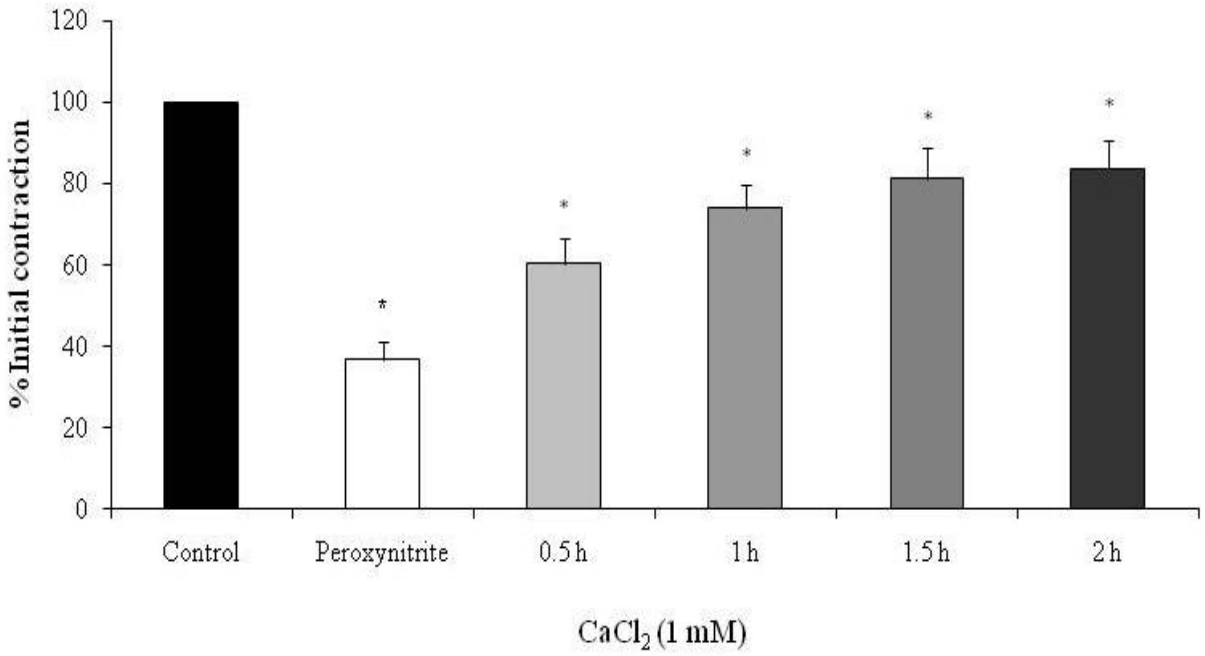

Figure 8. Recovery of smooth muscle contractions in a time-dependent manner. $CaCl_2$ (1 mM)-induced contractions were reduced following peroxynitrite treatment. However, $CaCl_2$ (1 mM)-induced contractions appeared to recover within 2 hrs. Maximal contraction of control group was considered as 100%. Bars represent mean ± S.E.M. Data were analyzed by one-way repeated measures-ANOVA followed by Holm-Sidak method. $P \leq 0.001$ versus control, $n = 9$.

3.4 Effect of c-Src kinase inhibition on Ca^{2+}-induced contractions in mouse colon

Smooth muscle calcium channels are regulated by c-Src kinase under basal conditions, which is attenuated following inflammation. To confirm whether Ca^{2+} influx-induced contraction is sensitive to the c-Src kinase inhibitor under physiological conditions, tissues were pre-incubated with PP2 (10 μM), a specific c-Src kinase inhibitor. Initial maximal contractile response to $CaCl_2$ (1 mM) was considered as 100% after depolarization of muscle strips by high-potassium (80 mM) solution. In the presence of 10 μM PP2, $CaCl_2$ (1 mM)-induced contractions were significantly ($P \leq 0.01$) reduced by 59% from 0.31 ± 0.09 g to 0.14 ± 0.06 g ($n = 4$). Data were analyzed by paired *t* test.

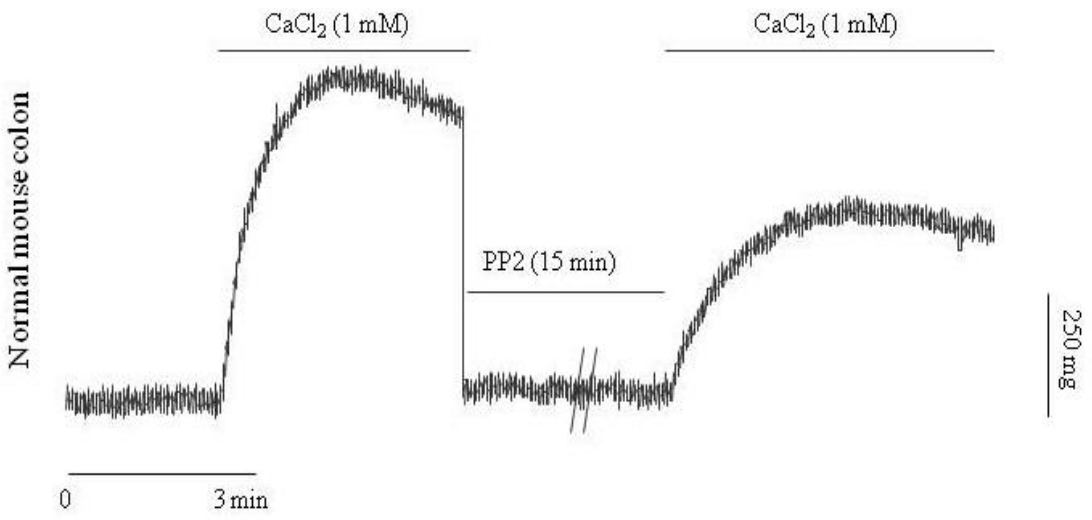

Figure 9. Representative tracing of the effect of c-Src kinase inhibitor (PP2) on the $CaCl_2$-induced contractions in isolated mouse colon. // represents break in time.

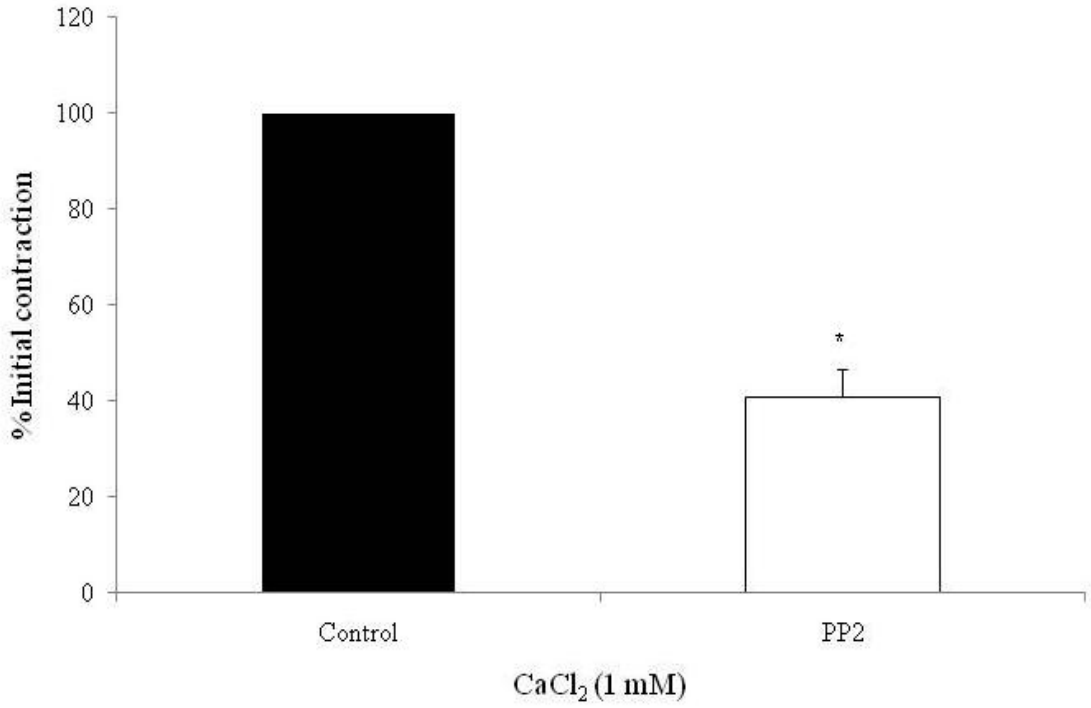

Figure 10. Effect of PP2 (10 μM), a c-Src kinase inhibitor on the $CaCl_2$-induced contractions in isolated distal colon. Maximal contraction of control group were considered as 100%. The $CaCl_2$ contractions were elicited in tissues depolarized by high-potassium (80 mM) physiological solution. Bars represent mean ± S.E.M. Data were analyzed by paired *t*-test. $P \leq 0.01$ versus control, $n = 4$.

3.5 c-Src kinase regulation of Ca^{2+} channels is restored after recovery from peroxynitrite-induced inhibition of contractions

This experiment was performed to determine whether c-Src kinase regulation of the Ca^{2+} channels is restored after recovery from peroxynitrite-induced reduction of colonic smooth muscle contractions. Initial maximal contractile response to $CaCl_2$ (1 mM) was considered as 100%. . In some tissues, incubating with the nitrating agents resulted in a maintained tone. In such cases, this tone was considered as baseline and as shown in figure 5, after preincubation with nitrating agents, $CaCl_2$ (1 mM)-induced contractions were significantly decreased from 0.22 ± 0.04 g to 0.10 ± 0.02 g, representing a 52.2% reduction. The muscle strips were then washed every 30 min for 2 hrs with high-potassium (80 mM) solution. Contractile response to $CaCl_2$ (1 mM) after 2 hrs significantly recovered to 115.2 ± 19.1%. After reversal of smooth muscle contractions, muscle strips were treated with PP2 (10 μM) for 15 min. Preincubation with PP2 resulted in a significant decrease ($P \leq 0.05$) in $CaCl_2$ (1 mM)-induced contractions by 47.6 ± 6.9% ($n = 3$). Data were analyzed by one-way repeated measures-ANOVA followed by Student-Newman-Keuls Method.

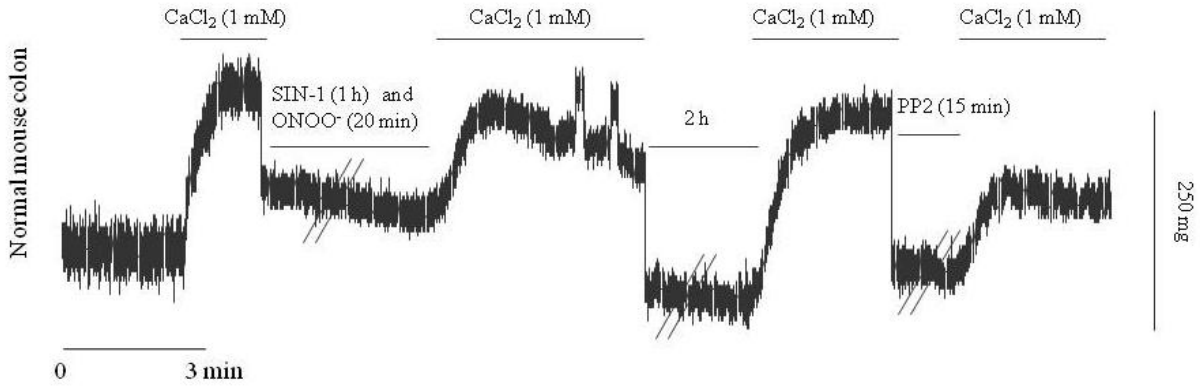

Figure 11. Representative tracing of the effect of c-Src kinase inhibitor (PP2) after recovery from peroxynitrite-induced attenuation of $CaCl_2$-induced colonic smooth muscle contractions. // represents break in time.

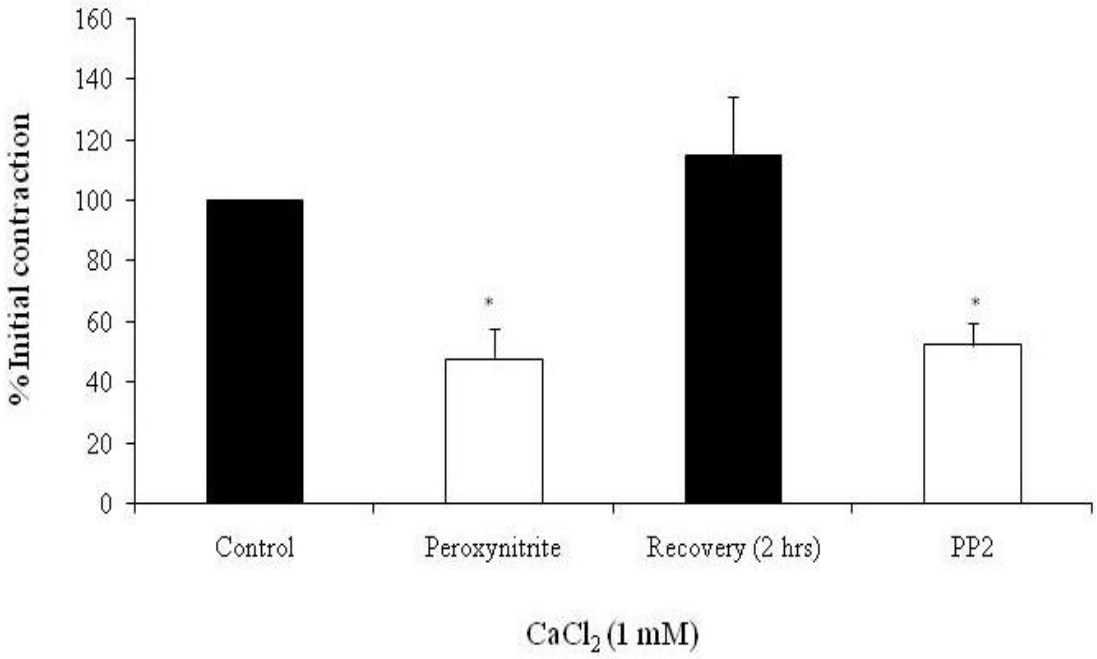

Figure 12. Graphical representation of the effect of c-Src kinase inhibitor (PP2) after recovery from peroxynitrite-induced attenuation of $CaCl_2$-induced colonic smooth muscle contractions. Maximal contraction of control group was considered as 100%. Bars represent mean ± S.E.M. Data were analyzed by one-way repeated measures-ANOVA followed by Student-Newman-Keuls Method. $P \leq 0.05$, $n = 3$.

3.6 Sodium orthovanadate prevents peroxynitrite-induced inhibition of contractions

This experiment was performed to determine whether sodium orthovanadate (Na_3VO_4), a tyrosine phosphatase inhibitor, prevented peroxynitrite-induced attenuation of smooth muscle contractions. Following depolarization by Ca^{2+}-free high-potassium (80 mM) solution, $CaCl_2$ (1 mM)-induced maximal contractile response was considered as 100%. The muscle strips were then incubated with Na_3VO_4 (100 μM) for 30 min followed by treatment with SIN-1 (500 μM) for 1 hr and $ONOO^-$ (150 μM) twice at 10-min interval. Pre-incubation with Na_3VO_4 prevented peroxynitrite-induced inhibition of colonic smooth muscle contractions by approximately 90%; and no statistically significant difference was detected by paired *t*-test (n = 14).

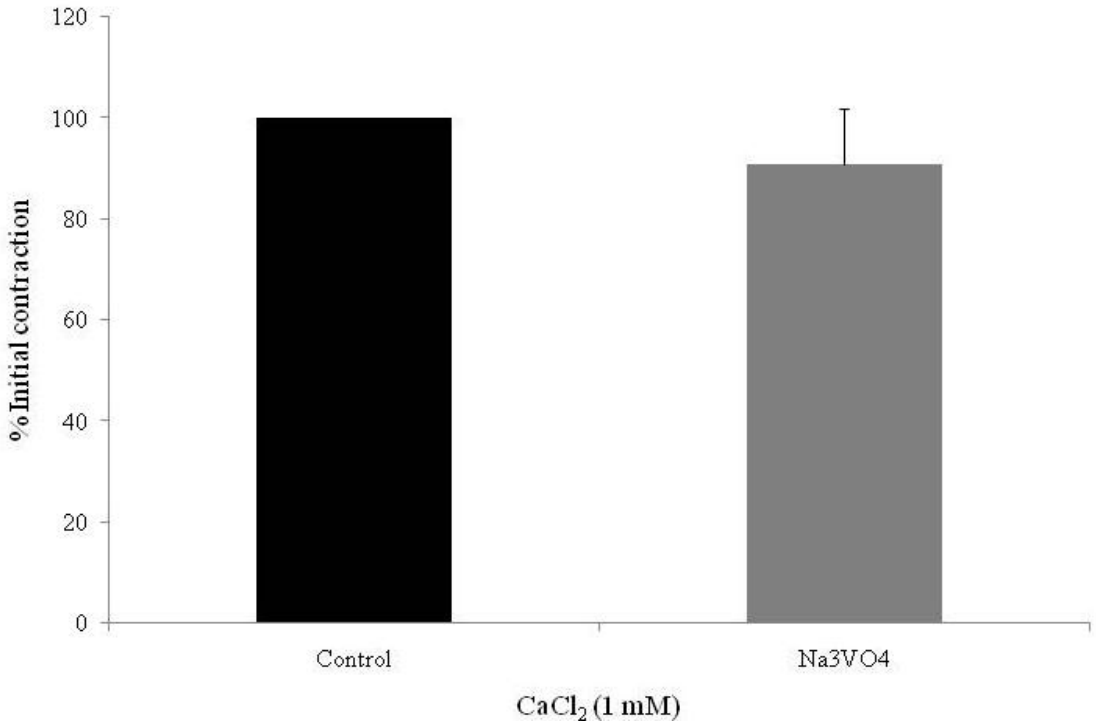

Figure 13. Graph showing the summarized data on the preventive effect of sodium orthovanadate (Na_3VO_4) on nitration-induced reduction in colonic smooth muscle contractions. Bars represent the mean ± S.E.M. Data were analyzed by paired *t*-test; *n* = 14.

3.7 Measurement of nitrated proteins under conditions involving aeration and non-aeration

The concentration of peroxynitrite that produced consistent nitration of the cellular proteins was determined by treating mouse colonic tissues with a combination of SIN-1 (500 μM) and varying peroxynitrite concentrations (150 μM – 300 μM). Mouse colonic tissues were treated with nitrating agents in Krebs solution which was not continuously aerated with carbogen. This resulted in a more alkaline solution (pH = 9.2), providing a stable environment for peroxynitrite. Following tissue homogenization, protein samples were run on SDS-PAGE gel, transferred to PVDF membrane and immunoblotted with anti-Nitrotyrosine (mouse) antibody. Several nitrotyrosine bands (molecular weight ranging from 250 kDa to 55 kDa) were evident at 300 μM peroxynitrite concentration; and therefore it was chosen as the final concentration to induce nitration of tyrosine residues for the following experiments.

In separate experiments, mouse colonic tissues were treated with peroxynitrite under physiological conditions in which the Krebs solution was continuously aerated. This led to a decreased extent of nitration, resulting in a decreased number and intensity of detectable nitrotyrosine bands (Fig. 14).

The immunoblots of nitrated proteins from mice colonic tissues were repeated several times in both aerated and non-aerated conditions ($n = 4$). However, results were inconsistent as clear bands of nitrated proteins were detected in some blots but not in others. Moreover, high intensity of nitration was observed concentrated over a wide range from 150 kDa to 250 kDa. To further establish if a better resolution could be

obtained, the following experiments were done by immunoprecipitation of the nitrated proteins.

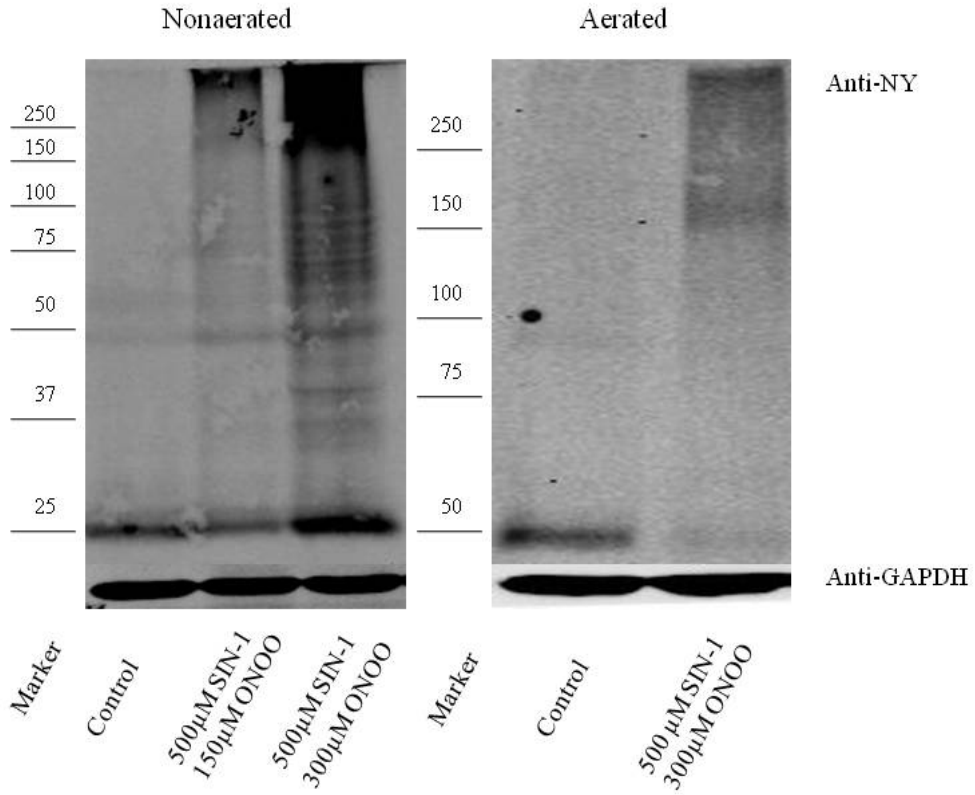

Figure 14. Western blots demonstrating the effect of increasing $ONOO^-$ concentration on colonic smooth muscle proteins under aerated and non-aerated conditions. Nitrotyrosine immunoreactivity was detected with anti-NY antibody.

3.8 Evidence of denitration in colonic smooth muscle cells

This experiment was performed to determine whether nitrotyrosine-containing proteins were modified by a denitrase enzyme in colonic smooth muscle cells. Mouse colonic tissues were treated with nitrating agents in Krebs solution under aerated and non-aerated conditions. To establish the extent of nitration, cell lysates were immunoprecipitated with anti-nitrotyrosine (rabbit) antibody. Immunoprecipitated samples were separated by electrophoresis, transferred to PVDF membrane and immunoblotted with anti-nitrotyrosine (mouse) antibody. Under non-aerated conditions, a smear of nitrotyrosine bands was detected within the molecular weight of 250 to 150 kDa, with clearer single bands being observed in some blots (e.g. Fig. 15) between 100 and 55 kDa. In order to determine a quantitative effect of nitration and denitration, intensity was measured for the range from 250 kDa – 55kDa in 3 separate blots. Under aerated conditions, the extent of nitration was reduced when compared to the tissues treated with non-aerated conditions.

To determine whether nitration of these cellular proteins was subject to denitration in colonic smooth muscle cells, the colonic tissues were washed every 30 min for 2 hrs following incubation with nitrating agents. Immunoblotting of cell lysates with anti-nitrotyrosine (mouse) antibody, after immunoprecipitation with anti-nitrotyrosine (rabbit) antibody, demonstrated marked reduction in the extent of nitration; however the difference was not significant. As shown in Fig. 16, the relative intensity was greater for the nitrotyrosine bands upon nitration and was markedly reduced after 2 hrs. The intensity of the bands was measured using the LiCor Software.

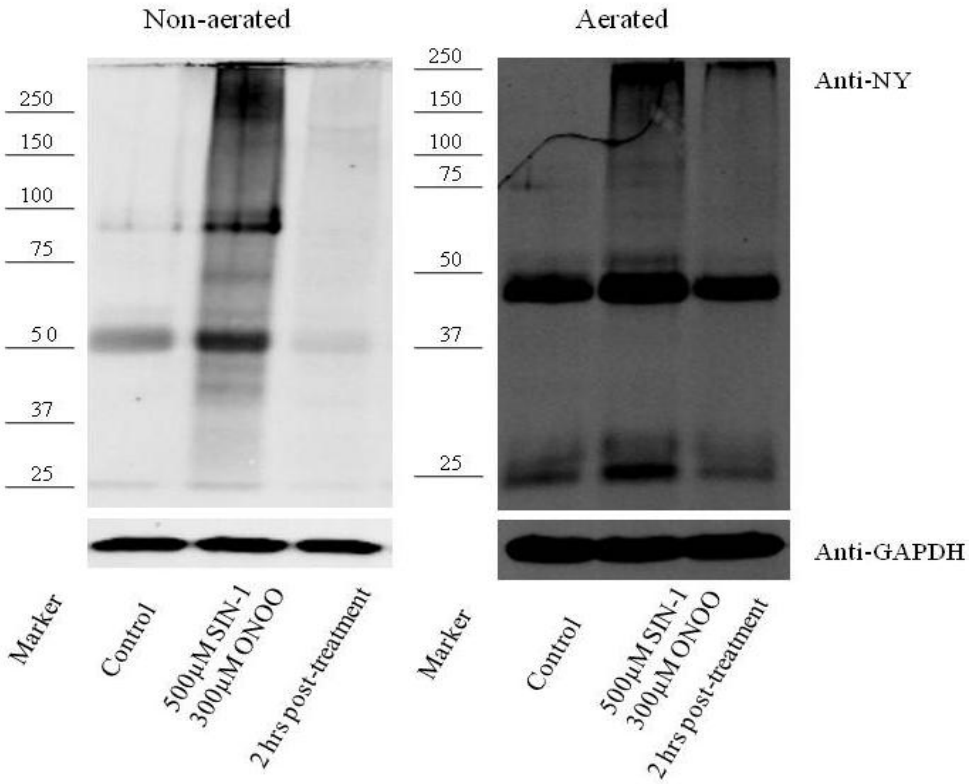

Figure 15. Nitrotyrosine formation was reduced after 2 hrs following induction of nitration by SIN-1 and $ONOO^-$ in mouse colon under aerated and non-aerated conditions. Samples were co-immunoprecipitated with anti-NY (rabbit) antibody and immunoblotted with anti-NY (mouse) antibody.

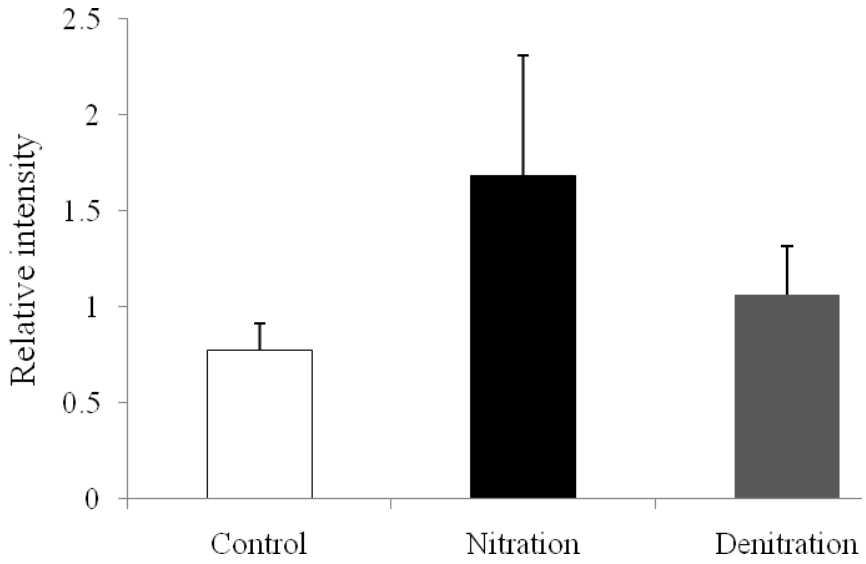

Figure 16. Graphical representation of the intensity of nitration and denitration of proteins ranging from 250 kDa to 55 kDa from smooth muscle over 2 hr period under non-aerated conditions ($n = 3$).

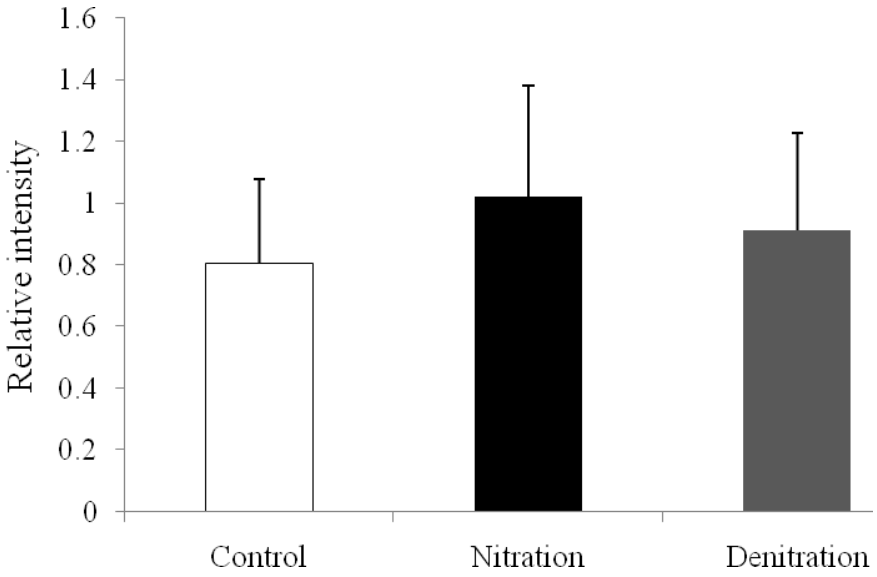

Figure 17. Graphical representation of the intensity of nitration and denitration of proteins ranging from 250 kDa to 55 kDa from smooth muscle over 2 hr period under aerated conditions ($n = 3$).

Chapter 4. Discussion

This research study presents five main findings. 1) Peroxynitrite inhibits Ca^{2+}-induced smooth muscle contractions, 2) Colonic smooth muscle contractions recover from peroxynitrite-induced attenuation in a time-dependent manner, 3) Reversal of peroxynitrite-induced attenuation of smooth muscle contractions restores the ability of c-Src kinase to regulate Ca^{2+}-induced contractions, 4) Peroxynitrite-induced inhibition of Ca^{2+}-induced contractions is prevented by sodium orthovanadate, and 5) Denitration of proteins can be observed in smooth muscle cells over a 2 hr period.

4.1 Peroxynitrite decreases Ca^{2+}-induced smooth muscle contractions

Ca^{2+}-induced smooth muscle contractions were elicited in tissues depolarized by 80 mM K^+ solution. 80 mM K^+ results in the depolarization of smooth muscle to membrane potential of approximately -10 mV in smooth muscle cells (Kang et al., in press). The depolarization by high K^+ is due to change in the equilibrium potential for K^+ (the major permeating ion at rest). This membrane potential falls within the range of Ca^{2+} channel window current. Ca^{2+} channel window current reflects the steady-state voltage-dependent activation and inactivation of Ca^{2+} channels. In other words, window current is the membrane potential range at which the Ca^{2+} channels remain open. Cumulative addition of Ca^{2+} under these conditions induces a concentration-dependent contraction in normal mouse colon. The Ca^{2+}-induced contractions can be attributed largely to calcium entry through L-type Ca^{2+} channels as these are blocked by the Ca^{2+} channel blocker, nifedipine. By using the methodology of Ca^{2+}-induced contractions, we showed that

peroxynitrite pretreatment of colonic muscle strips results in decreased Ca^{2+}-induced contractions. This is consistent with the decreased Ca^{2+} channel currents identified previously in patch clamp studies (Ross et al., 2007). Peroxynitrite is thought to be the major nitrating agent and has been associated with nitrotyrosine formation in numerous human diseases and animal models, including inflammatory bowel disease. Concentration-dependent nitrotyrosine formation of the Ca^{2+} channels by peroxynitrite has been confirmed by western blots using the anti-nitrotyrosine antibody (Kang et al, 2008). The concentration of the peroxynitrite that produced consistent nitration of the Ca^{2+} channel was determined to be 150 μM. In the present study, colonic muscle strips were incubated in both SIN-1 and peroxynitrite to induce nitration of the Ca^{2+} channels. SIN-1 simultaneously generates nitric oxide and superoxide and thus forms peroxynitrite. Constant production of peroxynitrite was used to induce nitrotyrosine formation in colonic muscle strips. Additionally, authentic peroxynitrite was used in a pulse-like manner to maximize the induction of nitration. The decrease in contractions induced by nitration could conceivably be attributed to alterations in the contractile proteins. In an earlier study, Ross et al (2007) showed that peroxynitrite treatment did not affect acetylcholine-induced contractions in the absence of extracellular calcium. Under these conditions, acetylcholine-induced contractions occur mainly due to intracellular Ca^{2+} release. Hence, nitrotyrosine formation of the Ca^{2+} channels underlies the attenuated Ca^{2+}-induced smooth muscle contractions.

4.2 Reversal of nitration-induced inhibition of smooth muscle contractions

Recent studies have reported the presence of a denitrase activity in activated macrophages that modifies nitrotyrosine-containing proteins. Denitrase activity of macrophages is selective towards specific substrates, such as Histone H1.2 and calmodulin (Irie et al., 2003; Smallwood et al., 2007). Kang et al (2008) suggested that the C-terminus of the L-type Ca^{2+} channel may be a potential target for denitrase activity. The c-terminus end of the calcium channel was treated with peroxynitrite to induce tyrosine nitration. The nitrated protein was then incubated with cell lysates from activated macrophages which significantly down-regulated the expression of nitrotyrosine as detected by the anti-nitrotyrosine antibody in Western blots. While the study of Kang et al (2008) suggested that the calcium channel may be a potential target of denitration process, we hypothesized that smooth muscle cells may also synthesize denitrase to regulate endogenous calcium channel function under oxidative conditions. The recovery of calcium-induced contractions over a 2 hr period was construed to suggest that denitration of the calcium channel may occur in smooth muscle. However, it is also possible that new Ca^{2+} channels may be synthesized to restore Ca^{2+} channel function and smooth muscle contractions. The half-life of $Ca_v1.2$ L-type Ca^{2+} channel protein is estimated to be 7 days as demonstrated by Wegerner et al (2006); however Ca^{2+}-induced smooth muscle contractions recover within 2 hrs negating the possible synthesis of new Ca^{2+} channels within this time frame. Hence, it may be possible that denitrase activity is present in smooth muscle cells and is responsible for modifying nitrated Ca^{2+} channels to restore Ca^{2+}-induced smooth muscle contractions. We tested

that nitrotyrosine formation of Ca^{2+} channels is a reversible process by studying the basal regulation of the Ca^{2+} channels by c-Src kinase.

4.3 c-Src kinase regulation of Ca^{2+} channels is restored

Smooth muscle L-type Ca^{2+} channels are regulated by c-Src kinase under basal conditions. Ca^{2+} channel currents are inhibited by approximately 50 to70% by the c-Src kinase inhibitor PP2 (Kang et al., 2007). Likewise, in the present study, we showed inhibition of Ca^{2+}-induced smooth muscle contractions by approximately 60% in the presence of PP2. Previous studies in our laboratory have shown that nitration of the tyrosine residues in the Ca^{2+} channel disables the tyrosine phosphorylation by c-Src kinase. Cotransfection of HEK-293 cells with human smooth muscle L-type Ca^{2+} channel and c-Src kinase resulted in tyrosine phosphorylation of the Ca^{2+} channel, which was prevented by nitration of tyrosine residues by peroxynitrite (Kang et al., 2007). Further experiments demonstrated that Ca^{2+} channel currents and Ca^{2+}-induced contractions are decreased by exposure to peroxynitrite but are not further affected by c-Src kinase inhibition with PP2, suggesting that nitration impairs Src-mediated regulation of the Ca^{2+} channels (Kang et al., 2007; Ross et al., 2007). In the present study, we demonstrated that after reversal of smooth muscle contractions from nitration-induced inhibition, contractile responses were inhibited by the c-Src kinase inhibitor PP2. This phenomenon indicates the presence of denitrase activity in smooth muscle cells that allowed recovery of tyrosine phosphorylation by c-Src kinase. This is consistent with the finding that nitrated C-terminus of the Ca^{2+} channel could be phosphorylated after incubation with macrophage cell lysates, suggesting that denitration restores c-Src kinase

induced tyrosine phosphorylation (Kang et al., 2007). Since nitrotyrosine formation interferes with tyrosine kinase pathways involved in cell signaling, the presence of denitrase activity in smooth muscle cells may have profound and important effects in restoring the function of nitrated proteins involved in cell signaling processes.

4.4 Sodium orthovandate prevents peroxynitrite-induced inhibition of contractions

Previous studies have shown that Ca^{2+} channel currents are enhanced by tyrosine phosphatase inhibition with sodium orthovanadate (Na_3VO_4). This is consistent with the finding that cumulative addition of Na_3VO_4 produces a concentration-dependent contraction in mouse colon. In addition, c-Src kinase inhibition with PP2 significantly reduces Na_3VO_4-induced contraction in mouse colon (Ross et al., 2007). Taken together, these data suggest that Ca^{2+} channel activity is enhanced by tyrosine phosphatase inhibition with Na_3VO_4 because phosphorylation of Ca^{2+} channel is preserved. We hypothesized that Na_3VO_4 could prevent nitrotyrosine formation by preserving phosphorylation state of the Ca^{2+} channel. As expected, preincubation with Na_3VO_4 prevented nitration-induced attenuation of Ca^{2+}-induced smooth muscle contractions by approximately 90%. This is important because Na_3VO_4 preserves the tyrosine residues from becoming nitrated; and this phenomenon suggests that phosphorylation competes with nitration.

4.5 Denitration of proteins in smooth muscle cells

Although previous experiments suggest the presence of denitrase activity in smooth muscle cells, they do not provide unequivocal evidence for such an enzymatic activity. We therefore tried to determine specific protein denitration in colonic smooth muscle by western blots. Initial experiments were conducted to determine the concentration of peroxynitrite that induced consistent nitrotyrosine formation in colonic smooth muscle. We found that 300 μM of peroxynitrite produced detectable nitrotyrosine bands; and therefore it was chosen as the final concentration to induce protein nitration in mouse colon. Protein nitration was induced in Krebs solution under non-aerated and aerated conditions. In aerated conditions, Krebs solution was continuously bubbled with carbogen (95% O and 5% CO_2) to maintain physiological pH of 7.4; whereas non-aerated conditions resulted in a more alkaline solution (pH = 9.23). The more alkaline pH resulted in a greater extent of nitrotyrosine formation in colonic smooth muscle proteins most likely due to the stable environment for peroxynitrite. In physiological conditions, peroxynitrite gets protonated (peroxynitrous acid) and rapidly decays to nitrogen dioxide and hydroxyl radical (Beckman, 1990). It should be noted that the physiological solution is bubbled with high O_2. The immunoblots were repeated several times in both aerated and non-aerated conditions; however, the results were inconsistent as nitrotyrosine bands were detected in some blots but not in others. Since this may occur as a result of low abundance of nitrated proteins, induction of nitration was maximized by immunoprecipitation of nitrated proteins. Immunoprecipitated samples showed increased intensity of detectable nitrotyrosine bands; however the extent of nitration was relatively greater under non-aerated conditions. Denitration or reduction

of the relative intensity of the nitrated proteins was observed under both non-aerated and aerated conditions after 2 hr period, providing evidence for the presence of denitrase activity. A possible limitation of this experiment is that the proteins may be susceptible to proteolysis. To confirm that the decrease in nitrated proteins was not associated with proteolysis, the quantities of the nitrated proteins should have been determined immediately and 2 hrs after protein nitration with Coomassie brilliant blue staining. Although denitrase enzyme has not yet been identified, Benhar et al (2008) has identified thioredoxins as denitrosylase enzyme that reverses S-nitrosylation of cysteine residues within a broad spectrum of proteins in basal and stimulated conditions.

4.6 Future Studies and concluding remarks

Although we demonstrated that nitration-induced attenuation of smooth muscle contractions was reversible, future studies will be required to determine whether endogenous smooth muscle L-type Ca^{2+} channels can be denitrated to further support the evidence for the presence of denitrase activity in smooth muscle cells. One possible route of exploration would be to perform 1D membrane assay of the nitrated Ca^{2+} channel for denitrase activity in smooth muscle. Nitrated Ca^{2+} channel will be immunoprecipitated from peroxynitrite-treated smooth muscle, subjected to 1D gel electrophoresis, blotted onto PVDF membrane, blocked with 5% skim milk and incubated with smooth muscle cell lysate followed by treatment with anti-nitrotyrosine antibody. This membrane assay limits the risk of artifacts by proteolysis because the Ca^{2+} channel is bound to the membrane. This assures that the nitrated tyrosine residues will stay at the same position on the membrane even if the Ca^{2+} channel is cleaved by a

protease. In addition, blocking membrane with 5% skim milk will coat the surface of the membrane with protein and therefore reduce the chance of nonspecific proteolysis. After identification of the denitrase activity in smooth muscle, further experiments will be required to constitute and characterize denitrase activity. Another important aspect is to explore if denitration occurs upon mild colonic inflammation. This could be accomplished by inducing colonic inflammation with TNBS in mice and treating nitrated proteins with inflamed smooth muscle cell lysates to determine if denitrase activity is present and if it is up-regulated or down-regulated during inflammation.

An important feature of a denitrase activity is its therapeutic potential. For instance, during inflammation when calcium channel proteins are nitrated, induction of denitrase activity would be useful to alleviate the effects of inflammation on gastrointestinal motility.

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