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Spermidine stimulates protein tyrosine phosphatase N2- mediated protection of intestinal epithelial barrier function

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Spermidine Stimulates Protein Tyrosine Phosphatase N2-Mediated Protection of Intestinal Epithelial Barrier Function

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology by Harrison M. Penrose

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2012
The Thesis of Harrison M. Penrose is approved and is acceptable in quality and in form for publication on microfilm and electronically:

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Co-Chair

Chair

University of California, San Diego

2012
EPIGRAPH

“I didn’t have time to write a short letter, so I wrote a long one instead.”

Mark Twain
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ABSTRACT OF THE THESIS

Spermidine Stimulates Protein Tyrosine Phosphatase N2-Mediated Protection of Intestinal Epithelial Barrier Function

by

Harrison M. Penrose

Master of Science in Biology

University of California, San Diego, 2012

Professor Kim E. Barrett, Chair

Professor Kit J. Pogliano, Co-Chair

Genome-wide association-studies (GWAS) have revealed that single nucleotide polymorphisms (SNP) in the gene locus encoding a particular phosphatase, protein tyrosine phosphatase non-receptor type 2 (PTPN2), are associated with the chronic intestinal inflammatory condition, Crohn’s disease. PTPN2 is ubiquitously expressed. However, its expression in intestinal epithelial cells (IEC) has been shown to play an important role in the protection of epithelial barrier function during periods of inflammation by acting as a negative regulator of the pro-inflammatory cytokine,
interferon-γ (IFN-γ). Therefore, agents that increase the activity of PTPN2 are of general interest as modifiers of inflammatory signaling events.

A recent study demonstrated that the small molecule, spermidine, is a selective activator of PTPN2 in vitro. Here, I describe the effects of spermidine on PTPN2 expression and activity, as well as its effect on IFN-γ signaling and barrier function in the human colonic epithelial cell line, T84. My studies revealed that spermidine increased both PTPN2 protein levels and enzymatic activity, correlating with a decrease in the phosphorylation of the signal transducers and activators of transcription (STAT)1 and 3, downstream mediators of IFN-γ signaling, upon co-administration of spermidine to IFN-γ treated cells. Additionally, spermidine protected barrier function in the setting of inflammation, restricting the decrease in transepithelial electrical resistance (TER) induced by IFN-γ in co-incubation experiments. Spermidine’s ability to increase PTPN2 levels and activity, as well as reduce IFN-γ signaling in IECs, implicate spermidine as a potential therapeutic agent for treating conditions associated with dysregulated IFN-γ signaling and a faulty mucosal barrier.
I. INTRODUCTION

**Inflammatory Bowel Diseases** Inflammatory bowel disease (IBD) is the umbrella term given to two separate but related conditions, Crohn’s Disease (CD) and Ulcerative Colitis (UC), both of which are characterized by chronic inflammation of the gastrointestinal tract [1]. CD can affect all regions of the gastrointestinal tract from mouth to anus, whereas UC is confined to the colon. CD and UC also differ from each other by the type of ulceration present (transmural vs. superficial), the location of the ulcers (ileal/colonic skip lesions vs. continuous colonic ulcerations), and the predominating cytokine (interferon-γ (IFN-γ) vs. IL-13), respectively [2]. Both subtypes of IBD are idiopathic in nature and the pathogenesis of disease is a multi-factorial event. Disease progression has been attributed to genetic predisposition, environmental triggers, and an inappropriate immune response to normally harmless commensal flora in the gut [3].

The epithelium of the gastrointestinal tract is vital for proper intestinal function. It is responsible for the absorption of water and nutrients, the maintenance of ionic homeostasis, and the establishment of a protective layer between the intestinal flora and sub-mucosa. In many cases, inflammation seen in patients with IBD is due to a dysfunctional epithelial mucosal barrier, as a decrease in intestinal barrier function is predictive of disease onset and clinical relapse in humans and mouse models [4, 5]. Both CD and UC are characterized by a decrease in epithelial barrier resistance and an increased permeability, thought to be a contributing factor to the major clinical symptom of IBD, diarrhea [6, 7]. This defective mucosal barrier coupled with a dysfunctional
immune response to commensal flora is thought to drive the development of chronic intestinal inflammation [3].

In IBD, pro-inflammatory cytokines such as IFN-γ and tumor necrosis factor-α (TNF-α) are present at high concentrations, with elevated levels of IFN-γ predominating in CD [8]. While elevated levels of TNF-α are linked to an innate immune response and NFκB activation, the high levels of IFN-γ associated with CD are due to an overactive T-cell response, predominated by a Th1 subpopulation. The Th1 responses are mediated by IFN-γ, which is produced by antigen-presenting cells (APCs) in the intestinal mucosa in response to IL-12 stimulation [9]. Both IFN-γ and TNF-α have been shown to decrease barrier function [10]. These cytokines lead to increased epithelial permeability and decreased resistance through the leak pathway, which is characterized by the activation of myosin light-chain kinase (MLCK) leading to contraction of the actinomyosin ring around the cell body. At the same time, components of the tight junction such as junctional adhesion molecule A (JAM-A) are internalized [10, 11]. Both the contraction of the actinomyosin ring and the internalization of tight junction constituents lead to an increase in gaps between adjacent epithelial cells in the monolayer, thereby compromising the barrier [10]. Resolution of the barrier occurs when concentrations of pro-inflammatory cytokines fall through decreased production, or due to increased expression and/or activity of negative regulators of cytokine signaling. One of these negative regulators of IFN-γ cytokine signaling is protein tyrosine phosphatase non-receptor 2 (PTPN2).
**The role of PTPN2 and mutations associated with CD** PTPN2 is a member of the protein tyrosine phosphatase family (PTP) whose members share a highly conserved catalytic motif. All members of the PTP superfamily utilize the same basic catalytic mechanism, in which the cysteiny1 residue in the signature motif executes a nucleophilic attack on the phosphate group in the substrate [12]. PTPs play an integral role in regulating a variety of basic cellular processes including cell growth and differentiation [13]. PTPN2, whose protein product is also referred to as T-cell protein tyrosine phosphatase (TCPTP), exists in two splice forms, a nuclear 45-kilodalton and a cytoplasmic 48-kilodalton variant that localizes to the endoplasmic reticulum via the ER localization signal at its N-terminus [14]. The ER localization signal of the 45-kilodalton variant is truncated and replaced instead with a nuclear localization sequence, allowing the 45 kDa isoform to traffic between the cytosol and the nucleus [14].

Among PTPN2 substrates are the phosphotyrosine residues on the receptors for epidermal growth factor [15] and insulin [16]. Additionally, PTPN2 negatively regulates phosphorylation of the IFN-γ receptor (IFNγR) and the IFN-γ signaling molecules, signal transducers and activators of transcription (STAT)1 and 3 [17, 18]. In the setting of inflammation, binding of IFN-γ to its receptor leads to receptor dimerization and recruitment of the JAK kinase. JAK induces phosphorylation of IFNγR tails, which is followed by STAT protein recruitment and phosphorylation. Phosphorylated STATs form homo- or heterodimers, which are able to translocate to the nucleus, bind DNA, and upregulate the expression of certain gene products [19]. Increased levels of phosphorylated STAT1 and 3 are seen in IBD, correlating with the elevated IFN-γ levels [20]. Dephosphorylation of STATs by PTPN2 leads to their inactivation and termination
STAT-induced transcription [17]. PTPN2, therefore, plays an integral role in regulating IFN-γ signaling and maintaining cellular homeostasis (Fig. 1).

Genome-wide-association-studies have revealed that individuals with single nucleotide polymorphic mutations in the PTPN2 locus have an increased susceptibility to developing CD and UC [21, 22]. Although PTPN2 expression is increased in CD intestinal biopsy specimens, the current hypothesis concerning the role of PTPN2 SNPs in IBD is that a loss of PTPN2 function may contribute to disease progression [7]. Loss of PTPN2 leads to prolonged IFN-γ induced STAT activation of transcription and increases in intestinal permeability [7]. While it has been shown that PTPN2 expression and activity is increased in response to IFN-γ, reduced PTPN2 expression leads to increased effects of IFN-γ on STAT signaling and a greater reduction in barrier function. Therefore, agents that increase the activity of PTPN2 are of general interest as modifiers of inflammatory signaling events.

**The small polyamine, spermidine, as a stimulus of PTPN2** A recent study demonstrated that the small molecule, spermidine, is a selective activator of PTPN2 *in vitro* [23]. Small molecule activation of protein tyrosine phosphatases provides new insight into the potential pharmacologic regulation of certain cellular events. By investigating the effects of spermidine on PTPN2 in intestinal epithelial cells, my studies serve not only to promote a further understanding of intestinal epithelial barrier maintenance, but also to identify a possible therapeutic agent for conditions associated with dysregulated IFN-γ signaling and intestinal barrier defects.

Spermidine belongs to a class of molecules called polyamines, which are involved
in many physiological processes including cell growth and proliferation, immunity, protein and nucleic acid synthesis, and certain cellular signaling processes (Fig. 2) [24]. In addition to spermidine, two other major polyamines that are produced in the intestine include putrescine and spermine. Polyamines are low molecular weight amines that are water-soluble and fully protonated at physiological pH [25]. They therefore exhibit polycationic character, thought to play a critical role in their biological activity, including the interaction with polyanionic macromolecules such as DNA and RNA [26].

Polyamines are derived from a precursor molecule called ornithine. Putrescine is the first polyamine synthesized following an initial decarboxylation reaction facilitated by the enzyme ornithine-decarboxylase (ODC) [25]. Spermidine is derived from putrescine, and spermine from spermidine. The formation of spermidine and spermine are derived from successive attachments of two propylamine groups by the action of the aminopropyl-transferases, spermidine and spermine synthetase [24].

In addition to de novo synthesis, polyamines are also taken up from extracellular sources. Transport is one of the main ways in which the intracellular polyamine concentration is regulated. The transport of polyamines has been well characterized in bacteria, but not in eukaryotes. In Escherichia coli (E. coli), multiple polyamine transport mechanisms have been determined, including two ATPase-dependent transporters, one particular to putrescine, another to spermidine, and a third system specific to putrescine allowing for both its uptake and release [24]. In mammals, though, gene(s) encoding the transporter(s) have not been identified. Additionally, the uptake of polyamines in lung epithelial cells was shown to occur via diffusion [25].

Polyamines are known to play an important role in many biological processes.
They have been shown to bind both DNA and RNA, leading to both increased expression of certain gene products, as well as the stabilization of mRNA transcripts [24]. Additionally, polyamines have been reported to be involved in cell signaling processes through interactions with various proteins [24]. Because of their polycationic character, polyamines interact strongly with membrane phospholipids, and therefore may play an important role in regulating the activity of membrane-linked enzymes. An activating effect on various protein tyrosine kinases and the GTPase activity of some G-proteins has been demonstrated [26, 27]. Additionally, a recent report demonstrated that the interaction of spermidine with PTPN2 stimulated the enzyme’s activity [23]. While the proposed mechanism by which spermidine increases PTPN2 activity is not defined, one current hypothesis suggests that the polycationic character of spermidine is responsible for breaking an autoregulatory intramolecular bond between the carboxy-terminal and catalytic domain in PTPN2 [23]. This hypothesis is based on previous experiments showing that activation of PTPN2 was significantly increased in response to stimulation with the positively charged cytoskeletal protein, α1-integrin [15]. This view is further supported by the fact that a truncated PTPN2, which lacks the carboxy-terminal region of the protein, has significantly greater activity than the wild-type protein [28]. Thus the carboxy-terminal sequence of PTPN2 is important for regulating its catalytic function, and keeps the phosphatase from being constitutively active. Moreover, breaking of this intramolecular bond through electrostatic interactions significantly stimulates its activity, correlating with increased dephosphorylation events.

In summary, my work here represents the first steps in understanding the effects of spermidine on PTPN2 in intestinal epithelial cells. Identifying the effects of
spermidine on PTPN2 is an important undertaking as increased activity of PTPN2 in IECs could lead to enhanced downregulation of inflammatory cytokine signaling. In addition, these findings, when applied to the setting of intestinal inflammation, could serve not only to promote a further understanding of intestinal epithelial barrier maintenance, but also to identify a possible therapeutic agent for conditions associated with intestinal barrier defects. Targets for the control of inflammation in IBD may be found by understanding the signaling pathways involved in disease progression. Moreover, researching the effects of spermidine on PTPN2 may contribute to discovering more about phosphatase function. This in turn could generate insights into the pharmacological development of PTPN2 agonists that could become clinically relevant therapies in the future.
II. MATERIALS AND METHODS

Materials Human recombinant IFN-γ (Roche, Mannheim, Germany), spermidine trihydrochloride (Sigma), spermine tetrahydrochloride (Sigma), cycloheximide (Sigma), monoclonal mouse anti-PTPN2 antibody CF-4, which detects the 45-kilodalton and the 48-kilodalton isoforms (Calbiochem, San Diego, CA), anti-phospho-STAT1 (Tyr\(^{701}\)), anti-STAT1, anti-phospho-STAT3 (Tyr\(^{705}\)), anti-STAT3 (Cell Signaling Technologies, Danvers, MA), and monoclonal mouse anti-β-Actin (Sigma) were obtained from the sources noted. Millicell culture plate inserts were purchased from Millipore Corporation (Millipore, Bedford, MA). All other reagents were of analytical grade and acquired commercially.

Cell Culture The human colonic epithelial cell line, T\(_{84}\), was used for all experiments in this study. This cell line was developed from a lung metastasis of a human colonic carcinoma [29]. T\(_{84}\) cells display the functional properties of colonic crypt epithelial cells, and are capable of forming polarized monolayers with tight junctions. Cells were grown in DMEM/F12 media with 5% newborn calf serum in 75 cm\(^2\) flasks. The cells were trypsinized and passaged at 95% confluency, and split 1:5. For in vitro experiments, T\(_{84}\) cells were seeded onto 12mm Millicell-HA culture plate inserts (filter membranes) and grown for 12-14 days before study, at which time they had stable values of transepithelial electrical resistance. IFN-γ (1000U/mL) was added basolaterally, whereas spermidine (10\(\mu\)M), spermine (10 \(\mu\)M), and cycloheximide (35.5 \(\mu\)M) were added bilaterally.
RNA Isolation and Real-Time Polymerase Chain Reaction Total RNA was isolated and DNA was removed from T84 cells using the Direct-zol RNA MiniPrep kit (Zymogen, Irvine, CA) according to manufacturer’s instructions. RNA purity and concentration were assessed by absorbance at 260 and 280 nm. Complementary DNA (cDNA) synthesis was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Fostery City, CA). Quantitative Reverse-Transcriptase Polymerase Chain Reaction was performed using MESA GREEN qPCR MasterMix Plus for SYBR Assays (Eurogentec, San Diego, CA on a StepOnePlus Real-Time PCR sytem using Step One Software v2.0 (Applied Biosystems). Measurements were performed in triplicate, human GAPDH was used as an endogenous control, and results were analyzed by the ΔΔCT method.

Preparation of Cytoplasmic Lysates On the day of the experiment, cells from inserts containing T84 monolayers were suspended in ice cold lysis buffer (50mM Tris, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 20μM NaF, 1mM EDTA, 1μg/ml antipain, 1μg/ml pepstatin, 1μg/ml leupeptin, 1mM NaVO₃, 100μg/ml phenylmethylsulfonyl fluoride), vortexed thoroughly, and subjected to lysis using a .22 gauge needle. Cells were centrifuged at 10,000 rpm for 10 min to remove insoluble material, and an aliquot was removed from each sample to determine protein content (Bio-Rad protein assay according to manufacturer’s instructions). Samples were resuspended in loading buffer (50mM Tris, pH 6.8, 2% SDS, 100 mM dithiothreitol, 0.2% bromophenol blue, 20% glycerol) and boiled for 5 min.
**Western Blotting** Samples suspended in loading buffer were loaded onto a 4-15% gradient polyacrylamide gel in order to resolve proteins. The proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was incubated in blocking buffer (5% BSA in 0.1% TBST) for 1 hr followed by overnight incubation of the membrane in blocking buffer containing primary antibody diluted 1:1000. This was followed by four 15-min washes with 0.1% TBST. After washes, secondary antibody (goat-anti-rabbit, Cell Signalling, Danvers, MA, or goat-anti-mouse IgG conjugated to horse radish peroxidase, BD Pharmigen, San Diego, CA) diluted 1:2000 was added to the membrane and incubated for 30 min. This was followed by four more 15-min washes with wash buffer. The membrane was then treated with chemiluminescent solution according to the manufacturer’s directions (Thermo Scientific, Rockford, IL) and exposed to film. Densitometric analysis of the blot was performed using Image J software (NIH).

**Immunoprecipitation** $6 \times 10^6$ T$_{84}$ cells were prepared in 75 cm$^2$ flasks. Following stimulation, cells were scraped and suspended in 1 mL of ice-cold PBS. Cells were centrifuged for 5 min at 5,000 rpm. The pellet was fully resuspended and lysed in 500 µl of ice-cold lysis buffer (5% IGEPAL CA-360, 750 mM NaCl, 250 mM Tris-Cl, pH 8.0, 1µg/ml antipain, 1µg/ml pepstatin, 1µg/ml leupeptin, 100µg/ml phenylmethylsulfonyl fluoride). Lysed cells were centrifuged for 10 minutes at 10,000 rpm to pellet DNA and small nonsoluble particles. 1 µg of monoclonal mouse anti-PTPN2 CF-4 antibody was added per sample and placed on a rotating platform at 4°C for 1 hr. This was followed by the addition of 30 µl of the 50% (v/v) protein A-sepharose beads to the cell
lysate/antibody mix and allowed to incubate on a rotating platform at 4°C for 1 hr.
Samples were then centrifuged briefly to pellet the protein A-Sepharose-antibody-antigen complexes, and the complex was washed three times with cold lysis buffer followed by three more washes with phosphatase reaction buffer (25 mM Hepes, 50 mM NaCl, 1 mM ditetheothreitol). Beads were resuspended in phosphatase reaction buffer and allowed to warm to room temperature before the initiation of the phosphatase assay.

**Phosphatase Activity Assay** Phosphatase activity was assessed using the EnzChek Phosphatase Assay Kit (Molecular Probes, Eugene OR) according to manufacturer’s instructions using the fluorescent phosphatase substrate, 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP). Upon dephosphorylation, DiFMUP fluoresces at an excitation/emission wavelength of 360/460. Therefore, the greater the fluorescence units, the larger amount of dephosphorylated DiFMUP. PTPN2 was first immunoprecipitated from whole cell lysates using anti-PTPN2 antibody (see above). Immunoprecipitates were incubated for 15 minutes with DiFMUP after which fluorescence was detected with a SpectraMax M2 Fluorescence Microplate reader using SoftMax Pro v5 Software (Molecular Devices, Sunnyvale, CA). Fluorescence was measured every 15 minutes for the first hour and every 30 minutes thereafter. Measurements were performed in triplicate. A sample from each immunoprecipitation was run on SDS-PAGE and probed for PTPN2 to account for equal protein loading. To account for any differences in overall phosphatase amounts, fluorescence activity units gathered from each assay were compared to PTPN2 densitometric values obtained from
Western blotting. Thus, values represent the specific activity of PTPN2 rather than total quantities of the enzyme.

**Small Interfering RNA Transfection** 2 x 10^6 T84 cells were seeded 3 days before transfection. For PTPN2, 100 pmol of 3 different annealed Silencer Pre-designed small interfering RNA (siRNA) oligonucleotides (Applied Biosystems, Foster City, CA) were transfected into T84 cells using the Amaxa nucleofector system (Amaxa, Gaithersburg, MD). After transfection, cells were cultured on filter membranes for 72 hours before treatment. Control siRNA SMARTpool (Upstat Biotechnology/Dharmacon, Chicago, IL) (100 pmol/transfection) was used as a negative control.

**Transepithelial Electrical Resistance** Transepithelial electrical resistance (TER) across T84 monolayers was assessed by voltohmeter (WPI, Sarasota, FL) and companion electrodes (Milipore, Bedford, MA). Measurements were calculated in Ω · cm^2 and expressed as a percentage of the baseline measurement.

**Statistical Analysis** All data are means ± SEM for a series of experiments. Statistical analysis was performed by Student’s unpaired t-test or analysis of variance (ANOVA) and Student-Newman-Keuls post-test using Graph Pad Instat 3 software (Graph Pad Software, La Jolla, CA). P values < 0.05 were considered significant.
III. RESULTS

**Spermidine shows no effect on PTPN2 mRNA transcription** To determine whether spermidine affects intestinal epithelial PTPN2 expression, T84 cells grown as monolayers on permeable supports were treated with increasing concentrations of spermidine (0.1, 1.0, 10 µM) for 24 hours. Spermidine was applied to both the apical and basolateral compartment. RT-PCR analysis revealed no increase in PTPN2 messenger RNA (mRNA) levels in cells treated with spermidine compared to untreated T84 cells (n=6) (Fig. 3).

**Spermidine increases PTPN2 protein levels in a dose dependent manner** To investigate whether spermidine affects intestinal epithelial PTPN2 protein levels, T84 cells grown as monolayers on permeable supports were treated with increasing concentrations of spermidine (0.1, 1.0, 10 µM) for 24 hours. Spermidine was applied to both the apical and basolateral compartment. Western blotting indicated that cytoplasmic PTPN2 protein levels were increased in a dose-dependent manner compared to untreated cells following a 24-hour incubation with spermidine (Fig. 4). In T84 cells treated with 10 µM spermidine, PTPN2 levels were 2.2 ± 0.3-fold greater than those found in control cells (p < 0.05, n = 4) (Fig. 4). Spermidine was also administered to T84 monolayers at concentrations of 100 µM and 1mM; however, these concentrations led to significant decreases in transepithelial electrical resistance following 24-hour incubation and were not pursued in further studies.
**Spermidine may act to reduce PTPN2 protein degradation** To further elucidate the mechanism by which spermidine increased PTPN2 protein levels, I used the ribosomal inhibitor, cycloheximide, to investigate whether spermidine prevented PTPN2 protein degradation, perhaps through post-translational modification(s). Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms. It is a small organic compound that works to inhibit protein elongation by interfering with the translocation of tRNA along the mRNA transcript, thus inhibiting amino acid addition. Previous studies employing the T84 cell line used cycloheximide at a final concentration of 35.5 µM to effectively inhibit protein synthesis [30]. Therefore, in my studies, T84 cells grown as monolayers on permeable supports were treated with cycloheximide (35.5 µM) for 30 minutes followed by the addition of media alone or media containing spermidine (10 µM) for 24 hours. Western blotting indicated an 18 ± 6% decrease in PTPN2 protein levels in T84 cells incubated with cycloheximide compared to the DMSO vehicle control (p < 0.05, n = 4) (Fig. 5). Co-administration of spermidine to cycloheximide-treated T84 cells restored PTPN2 to levels greater than those seen in the vehicle control (Fig. 5).

Moreover, PTPN2 proteins levels were on average 26 ± 6% higher in cycloheximide-spermidine co-incubations compared to cycloheximide-treated T84 cells, indicating that spermidine appears to prevent PTPN2 degradation post-translationally (p < 0.01, n = 4) (Fig. 5).

**Spermidine increases PTPN2 activity in intestinal epithelial cells** Next, I investigated whether spermidine alters PTPN2 enzymatic activity in T84 cells. Following incubation with or without spermidine (10µM) for 30 minutes, PTPN2 was immunoprecipitated
from T84 whole cell lysates. Data acquired from the activity assays demonstrated that spermidine exhibited a stimulatory effect on PTPN2 activity for the first 45 minutes of the phosphatase activity assay, followed by a decline to basal levels thereafter (Fig. 6). PTPN2 activity in immunoprecipitates incubated with spermidine peaked at 45 minutes, and was $1.9 \pm 0.2$-fold greater than PTPN2 activity in immunoprecipitates from unstimulated T84 cells ($p < 0.01, n = 3$) (Fig. 6). These data implicate spermidine as a bona fide stimulus of PTPN2 activity in vitro in the intestinal cell line, T84.

Spermidine attenuates IFN-γ signaling in intestinal epithelial cells

Having determined that spermidine stimulates PTPN2 activity, and given our laboratory’s interest in PTPN2 regulation of IFN-γ signaling, it was of interest to establish whether spermidine had any effect on IFN-γ signaling and the phosphorylation state of the IFN-γ signaling molecules, STAT1 and 3. A pathophysiologically relevant dose of IFN-γ (1000 U/mL) was added basolaterally to T84 monolayers [7]. Western blot analysis revealed that IFN-γ induced STAT1 phosphorylation following 30 minutes of treatment compared to untreated cells ($p < 0.001, n = 5$) (Fig. 7). Co-administration of spermidine (10 µM) for this time significantly attenuated IFN-γ-induced phosphorylation of STAT1 by $26 \pm 7\%$ ($p < 0.01, n = 5$) (Fig. 7). Likewise, IFN-γ induced STAT3 phosphorylation following 30 minutes of treatment compared to untreated cells ($p < 0.01, n = 4$) (Fig. 8). Co-administration of spermidine (10 µM) for this time attenuated IFN-γ-induced phosphorylation of STAT3 by $26 \pm 7\%$ ($p < 0.05, n = 4$) (Fig. 8). The decrease in STAT1 and 3 phosphorylation observed in cells co-administered spermidine with IFN-γ, coupled with the observed
spermidine-induced increase in PTPN2 phosphatase activity (Fig. 6), suggests that the increase in PTPN2 activity results in downregulation of IFN-γ signaling through a decrease in STAT1 and 3 phosphorylation.

**Spermidine attenuates IFN-γ signaling in a PTPN2-dependent manner** Next, I wanted to investigate whether the attenuation of STAT1 phosphorylation observed in T84 cells co-administered spermidine with IFN-γ was reversed by PTPN2 knockdown. T84 cells were transfected with either PTPN2-specific siRNA or nonspecific control siRNA, seeded on permeable supports, and allowed to grow for 72 hrs. Nonspecific effects of the siRNA transfections were not observed, as shown by equivalent levels of the loading control, β-actin, and total STAT1 in each experiment. Following the addition of IFN-γ (1000 U/mL) to cell monolayers, Western blotting revealed that IFN-γ induced STAT1 phosphorylation following 30 minutes of treatment and that this level was increased in the PTPN2 knockdown samples (n = 3) (Fig. 9). As expected, a decrease in STAT1 phosphorylation was observed in control siRNA transfected cells co-administered spermidine (10μM) with IFN-γ (Fig. 9). Interestingly, the decrease in STAT1 phosphorylation caused by spermidine was not observed in PTPN2 knockdown cells compared to control siRNA-treated cells (Fig. 9). Together, these data indicate that PTPN2 is likely the target by which spermidine reduces IFN-γ-induced STAT1 phosphorylation. While these data did not achieve statistical significance, they show a meaningful trend. The lack of significance is likely due to a low number of replicates, and the study should be repeated.
**Activation of PTPN2 is specific for the chemical nature of spermidine** There are other polyamines that have similar chemical structures to spermidine. Therefore, it was of interest to determine whether or not other structurally similar polyamines had any effect on IFN-γ signaling events in T\textsubscript{84} cells. One of these chemically relevant polyamines is spermine (Fig. 10). As mentioned earlier, spermine is produced by the addition of a propylamine group to spermidine, facilitated by the enzyme spermine transferase. To study the effects of spermine on IFN-γ signaling, IFN-γ (1000 U/mL) was added basolaterally to T\textsubscript{84} monolayers. Western blot analysis revealed that IFN-γ induced STAT1 phosphorylation following 30 minutes of treatment (p < 0.01, n = 5) (Fig. 11). Co-administration of spermine had no significant effect on IFN-γ-induced phosphorylation of STAT1, revealing that activation of PTPN2 seems to be specific for the chemical nature of spermidine (Fig. 11).

**Spermidine protects intestinal epithelial barrier function in the setting of inflammation** Finally, it was of interest to determine whether spermidine exhibited a protective effect on IEC barrier function following challenge with IFN-γ. A conventional way to determine barrier function in a cell model that establishes tight junctions is by measuring transepithelial electrical resistance (TER). Decreases in TER correlate with increases in paracellular permeability and a ‘leaky’ barrier. IFN-γ has been reported to decrease TER in T\textsubscript{84} cells by as much as 40% as early as 24 hours after treatment [31]. To determine whether spermidine protects barrier function in the setting of inflammation, IFN-γ (1000 U/mL) was added basolaterally to T\textsubscript{84} cells once they had established a stable monolayer in which the baseline TER before treatment was ≥ 1000 Ω · cm\textsuperscript{2}. 
Following 24-hour treatment with IFN-γ, TER decreased on average by $35 \pm 5\%$ ($p < 0.001, n = 4$) (Fig. 12). Co-administration of spermidine (10 μM) for this time lessened the effects of IFN-γ. Following a 24-hour co-incubation, TER was reduced by only $20 \pm 4\%$, and was significantly higher than TER in IFN-γ-treated cells ($p < 0.05, n = 4$) (Fig. 12).
IV. DISCUSSION

PTPN2 has been shown to play an essential role in regulating inflammatory cytokine signaling by inactivating the IFN-γ signaling molecules, STAT1 and 3. This view is supported by the fact that PTPN2 knockout mice die within 3-5 weeks from systemic inflammation characterized by elevated levels of IFN-γ and TNF-α [32]. IFN-γ has been demonstrated to increase PTPN2 expression and activity; moreover, activated PTPN2 alleviates IFN-γ-induced STAT1 and 3 activation [7]. These results indicate that PTPN2 is involved in a negative feedback mechanism that is ultimately responsible for limiting IFN-γ-induced effects.

Both STAT1 and 3 have been shown to stimulate very different biological effects in response to IFN-γ. STAT1 is associated with mediating IFN-γ-dependent changes in the immune response, cell proliferation, as well as in antiviral effects [33]. Conversely, STAT3 is more closely associated with anti-apoptotic effects, which have been associated with colon cancer pathogenesis [34]. It can therefore be seen that tight regulation of STAT signaling is critical in the maintenance of intestinal epithelial cell homeostasis. PTPN2 is a key regulator of STAT1 and 3 phosphorylation, and therefore plays an integral role in limiting their activity.

SNPs in the PTPN2 locus have been associated with CD and it is thought that these mutations could potentially be linked with its loss of function. While this has not been formally proven, it is thought that loss of PTPN2 function may contribute to IBD progression, due in part to prolonged IFN-γ-induced STAT activation of transcription and subsequent increases in intestinal permeability [7]. Therefore, agents like spermidine that
increase PTPN2 activity are of general interest as modifiers of inflammatory signaling events.

My experiments with spermidine were designed to examine its effects on PTPN2 expression and enzymatic activity, as well as inflammatory cytokine signaling. Using the human colonic epithelial cell line, T84, as my model I have shown that spermidine increases both PTPN2 protein levels and enzymatic activity. Importantly, I demonstrated that the increase in enzymatic activity observed in an in vitro activity assay correlates with downregulation of IFN-γ-induced STAT1 and 3 phosphorylation in a cell setting. Moreover, the attenuation of IFN-γ signaling is specific for the chemical nature of spermidine and occurs through a PTPN2-dependent mechanism. Finally, I have shown that spermidine protects intestinal epithelial barrier function in cells challenged with IFN-γ.

My initial experiments characterized the effect of spermidine on PTPN2 expression. RT-PCR data revealed that spermidine had no effect on the levels of PTPN2 mRNA. However, interestingly, PTPN2 protein levels were increased in a dose-dependent manner compared to untreated cells following 24-hour incubation with the polyamine. This implies that spermidine may be stabilizing PTPN2 at the protein level. In an effort to further elucidate the mechanism by which spermidine increased PTPN2 protein levels, I studied spermidine’s effect on PTPN2 following translational inhibition using the ribosomal inhibitor, cycloheximide. Following 24-hour treatment with cycloheximide, PTPN2 protein levels were significantly reduced compared to the vehicle control. The addition of spermidine alleviated this decrease. The results of this experiment suggest that the effect of spermidine on PTPN2 protein does not involve
protein synthesis. While it cannot be concluded definitely from this particular experiment, the increase in PTPN2 protein may be due to spermidine physically interacting with PTPN2 and shielding it from ubiquitination, thus preventing its degradation. This speculation could be tested by measuring the amount of ubiquitin associated with PTPN2 following stimulation with or without spermidine. Additionally, the increase in PTPN2 protein could also be an indirect result of spermidine interfering with the activity of the proteasome itself, or other effects on protein degradative pathways.

Spermidine also significantly increased PTPN2 enzymatic activity. Following incubation with spermidine, PTPN2 activity in immunoprecipitates was 1.9 times greater than that of PTPN2 immunoprecipitated from untreated cells. These data fall in line with a previous report implicating spermidine as a stimulus of PTPN2 [23]. Here, again, the mechanism by which spermidine increases PTPN2 activity is not fully understood; however, it is thought that the polycationic character of spermidine alleviates an autoregulatory bond between the c-terminus and catalytic domain of the phosphatase. The idea that electrostatic interactions may mediate PTPN2 activity is supported by previous work that established an increase in activity of the enzyme in response to increased ion concentration. In this in vitro study, it was demonstrated that increasing the KCl concentration to 100 mM stimulated the activity of TC45, the 45 kDa PTPN2 variant, about 76-fold [28]. Moreover, the increase in PTPN2 activity determined in the pull-down phosphatase assay was also observed in a cell setting. In my studies, co-administration of spermidine to IFN-γ-treated cells attenuated IFN-γ induced phosphorylation of STAT1 and 3. These data suggest that spermidine is effectively taken
up by T84 cells, either passively through diffusion and/or via a transport system, and is capable of interacting with and activating PTPN2, leading to increased dephosphorylation events. It is important, though, not to rule out the possibility that the effect of spermidine on PTPN2 activity may be due to a spermidine metabolite, because spermidine can be oxidized in serum-containing media [35]. Additionally, it can be speculated that the decrease in the phosphorylation levels of STAT1 and 3 are resultant of an overall increase in PTPN2 amount, rather than a spermidine-induced effect on PTPN2 activity. However, it is important to remember that increases in PTPN2 protein by spermidine were observed following 24 hours of treatment. Phospho-STAT1 and 3 levels in cells treated with IFN-γ and spermidine were determined following 30 minutes of treatment. The decrease in STAT1 and 3 phosphorylation observed in IFN-γ-treated cells co-administered spermidine at this time point indicates that it is likely due to a spermidine-induced effect on PTPN2 activity rather than an increase in total phosphatase amount. It would be interesting to look at the effect of spermidine on PTPN2 protein levels following 30 minutes of treatment. Increases in PTPN2 protein levels following 30 minutes of treatment with spermidine may then suggest that the decrease in STAT phosphorylation at this time is due to an overall increase in PTPN2 concentration and not activity.

Next, because there are other cellular protein tyrosine phosphatases present in intestinal epithelial cells, it was of interest to determine whether spermidine’s ability to reduce STAT1 phosphorylation was occurring through a PTPN2-dependent mechanism. Following PTPN2 knockdown using small interfering RNA (siRNA), IFN-γ-induced STAT1 phosphorylation was no longer attenuated by spermidine. Importantly, STAT1 phosphorylation was reduced by spermidine in cells treated with a non-specific control
siRNA. As expected, phospho-STAT1 levels were increased in PTPN2 knockdown cells, reinforcing PTPN2 as the cellular phosphatase responsible for regulating IFN-γ-induced STAT1 phosphorylation. Moreover, attenuation of phospho-STAT1 observed in T84 cells co-administered spermidine with IFN-γ was reversed in response to PTPN2 knockdown. Although further experiments are needed, together these data indicate that PTPN2 is the cellular phosphatase by which spermidine limits IFN-γ signaling.

It was also of interest to determine whether downregulation of IFN-γ-induced STAT1 phosphorylation was specific for the chemical nature of spermidine. Spermidine is the precursor compound to spermine, a structurally similar polyamine, which is formed by the addition of a propyl amine group to spermidine. Spermine contains an extra amine group, and, therefore a greater net positive charge, which could play a role in the activation of PTPN2. Spermine was unable to reduce STAT1 phosphorylation in IFN-γ-treated cells. To a certain degree, the results of this experiment were surprising since the structures of both spermidine and spermine are so similar. The chemical nature of spermine may not have influenced PTPN2 activity in the same manner as spermidine for at least two reasons. First, the increased molecular weight and increased polycationic character of spermine may decrease its ability to diffuse across the membrane. More importantly, the size and structural nature of spermine may prevent it from directly interacting and influencing PTPN2 in the same manner as spermidine. The results of this experiment are encouraging as to the possible specificity of spermidine for in vivo use. Additionally, they shed light onto the chemical nature of PTPN2 activation and may serve as a starting point for those interested in the pharmacological design of more potent activators of this phosphatase.
Finally, I validated spermidine as a possible protective agent in conditions associated with a leaky epithelial barrier. Following 24-hour treatment with IFN-γ, TER measurements were reduced on average by 35%, which agrees with previously reported data demonstrating the effects of IFN-γ on T84 monolayers [31]. Co-administration of spermidine during this time significantly attenuated the IFN-γ-induced drop in TER. The mechanism by which spermidine protects barrier function certainly may be due to its increase in PTPN2 protein levels and enzyme activity, and, therefore, subsequent decreases in IFN-γ signaling. However, additional pathways could also be involved. Previous reports have suggested an important role for polyamines in the regulation of tight junction protein expression as well as intestinal epithelial barrier integrity under various physiological conditions [36]. In addition to downregulation of IFN-γ signaling, therefore, spermidine may activate other cellular mechanisms responsible for increasing barrier function in the setting of inflammation. It would be of interest to study the effects of spermidine on barrier function in PTPN2-deficient cells that are also subject to IFN-γ challenge. Any protective effect of spermidine under these conditions would suggest that it targets additional protective mechanisms.

In summary, Crohn’s Disease and Ulcerative Colitis are chronic inflammatory diseases of the intestines that cause significant morbidity and severely affect the quality of life. These two conditions are characterized by an elevated inflammatory cytokine profile and dysfunctional epithelial barrier. It is therefore of particular interest to develop therapeutic agents that could target the pathogenic mechanisms underlying these inflammatory conditions. Today, there has been success in the treatment and management of Crohn’s Disease through the use of anti-TNF-α antibodies; however,
approaches to control elevated IFN-γ signaling are still being pursued. PTPN2, a non-receptor type cellular phosphatase, is present in intestinal epithelial cells and has been demonstrated to regulate IFN-γ signaling through the dephosphorylation of the IFN-γ signaling molecules, STAT1 and 3. SNPs in the PTPN2 gene locus have been associated with a greater susceptibility to Crohn’s Disease, and it is thought that loss of functional PTPN2 may contribute to disease progression. Discovering or developing agents that increase PTPN2 protein levels and/or activity are of general interest as modifiers of inflammatory cytokine signaling. My experiments have shown that the small polyamine, spermidine, is capable of both increasing PTPN2 protein levels and stimulating its enzymatic activity in vitro. I have shown that this increase in enzyme activity translates to downregulation of IFN-γ signaling in a cell setting, and that regulation of IFN-γ signaling by spermidine seems to be occurring in a PTPN2-dependent manner. Importantly, I have further shown that spermidine protects barrier function following challenge with IFN-γ. These data implicate spermidine as a bona fide stimulus of PTPN2 and protective agent of intestinal epithelial barrier function. Moreover, small molecule activation of protein tyrosine phosphatases provides new insight into the potential pharmacologic regulation of certain cellular events and acts a starting point for those interested in developing more potent PTPN2 activators.
V. FIGURES
Figure 1 | PTPN2 Regulates IFN-γ Signaling in Intestinal Epithelial Cells

IFN-γ binds to its receptor causing receptor dimerization and phosphorylation. This leads to recruitment and phosphorylation of the signaling protein, JAK. JAK-induced phosphorylation of IFN-γ receptor tails is followed by STAT protein recruitment and phosphorylation. Phosphorylated STATs form homo- or heterodimers, translocate to the nucleus, bind DNA, and activate transcription of certain gene products. PTPN2 dephosphorylates JAK and STAT proteins in the cytoplasm. PTPN2 also dephosphorylates STAT dimers in the nucleus, thus promoting their nuclear export. This terminates STAT-induced transcription and subsequent increases in IEC permeability.
Figure 2 | The Structural Formula of Spermidine
Figure 3 | The Effect of Spermidine on PTPN2 mRNA Levels in T₈₄ Cells

Spermidine (SPD) had no effect on PTPN2 mRNA levels following 24 hour incubation with the respective concentrations compared to untreated controls (n = 6). PTPN2 mRNA expression was normalized to the housekeeping gene GAPDH. Measurements were performed in triplicates. Data are expressed as a percentage of the control ± SEM.
Figure 4 | The Effect of Spermidine on PTPN2 Protein Levels in T<sub>84</sub> Cells

Spermidine (SPD) increased PTPN2 protein levels in T<sub>84</sub> cells in a dose-dependent manner. Cytoplasmic PTPN2 protein in spermidine-treated T<sub>84</sub> cells is shown by a representative Western blot in the upper panel. β-Actin was used throughout as a loading control. The relative protein level was assessed by densitometry (n = 4). Data are expressed as a percentage of the control ± SEM. (*P < 0.05 compared to untreated control, using Anova and the Student-Newman-Keuls post-hoc test).
Figure 5 | The Effect of Spermidine on PTPN2 Does not Require Protein Synthesis

Following translational inhibition by the ribosomal inhibitor, cycloheximide (CHX), PTPN2 protein levels were decreased. Co-administration of spermidine (SPD) restored PTPN2 protein levels to those of the vehicle control. Cytoplasmic PTPN2 protein levels in CHX and SPD-treated T84 cells are shown by a representative Western Blot in the upper panel. β-Actin was used throughout as a loading control. The relative protein level was assessed by densitometry (n = 4). Data are expressed as a percentage of the vehicle control ± SEM. (*P < 0.05 compared to control, ##P < 0.01 compared to CHX, using Anova and the Student-Newman-Keuls post-hoc test).
Figure 6 | The Effect of Spermidine on PTPN2 Enzymatic Activity

Following incubation +/- spermidine (SPD, 10µM) for 30 minutes, PTPN2 was immunoprecipitated from whole cell lysates and the activity assay was initiated by incubating immunoprecipitated PTPN2 with the fluorescent phosphatase substrate, DiFMUP. A sample from each immunoprecipitation was run on SDS-PAGE and probed for PTPN2 to account for equal protein loading. Fluorescence activity units were compared to PTPN2 protein levels to account for any differences in overall phosphatase amounts (n = 3). Error bars represent ± SEM. (*P < 0.05, **P < 0.01 compared to the respective untreated time point, using the Student’s unpaired t-test).
PTPN2 Phosphatase Activity in Fluorescence
Units Compared to Arbitrary Units of PTPN2
**Figure 7 | The Effect of Spermidine on STAT1 Phosphorylation**

IFN-γ (1000 U/mL) induced STAT1 phosphorylation following 30 minutes of treatment. Co-administration of spermidine (10 µM) for this time attenuated IFN-γ-induced phosphorylation of STAT1 on average by 26 ± 7% as shown by a representative Western Blot in the upper panel. β-Actin was used throughout as a loading control. The relative protein level was assessed by densitometry (n = 5). Data are expressed as a percentage of the control ± SEM. (**P < 0.001 compared to untreated control, ##P < 0.01 compared to IFN-γ-treated cells, using Anova and the Student-Newman-Keuls post-hoc test).
**Figure 8 | The Effect of Spermidine on STAT3 Phosphorylation**

IFN-γ (1000 U/mL) induced STAT3 phosphorylation following 30 minutes of treatment. Co-administration of spermidine (10 µM) for this time attenuated IFN-γ-induced phosphorylation of STAT3 on average by 26 ± 7% as shown by a representative Western Blot in the upper panel. β-Actin was used throughout as a loading control. The relative protein level was assessed by densitometry (n = 4). Data are expressed as a percentage of the control ± SEM. (**P < 0.01, *P < 0.05 compared to untreated control, using Anova and the Student-Newman-Keuhls post-hoc test).
Cytoplasmic P-STAT3 Levels Compared to T-STAT3 & Actin and Normalized to Untreated

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**Figure Caption:**

Bar graph showing the comparison of cytoplasmic P-STAT3 levels normalized to untreated samples. The graph includes untreated (UNT) and treated conditions with different inhibitors or conditions, indicated by different symbols and error bars. The x-axis represents different samples or conditions, and the y-axis represents the level of expression.

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**Legend:**

- β-Actin
- PTEN
- Total STAT3
- pSTAT3

**Images:**

Images depicting Western blot results for different samples, indicating the expression levels of various proteins under different conditions.
Figure 9 | Spermidine Attenuates IFN-γ Signaling in a PTPN2 Dependent Manner

T₈₄ cells were transfected with either PTPN2-specific siRNA or nonspecific control siRNA. IFN-γ (1000 U/ml) induced STAT1 phosphorylation following 30 minutes of treatment and this level was increased in the PTPN2 knockdown samples. A decrease in STAT1 phosphorylation was observed in control siRNA transfected cells co-administered spermidine (10µM) with IFN-γ. The decrease in STAT1 phosphorylation by spermidine was not realized in co-administration studies in PTPN2 knockdown cells as shown by a representative Western Blot in the upper right panel. β-Actin was used throughout as a loading control. The relative protein level was assessed by densitometry (n = 4). Data are expressed as a percentage of the control ± SEM.
Figure 10 | The Structural Formula of Spermine
Figure 11 | Activation of PTPN2 is Specific for the Chemical Nature of Spermidine

IFN-γ (1000 U/mL) induced STAT1 phosphorylation following 30 minutes of treatment. Co-administration of spermine (SPR, 10 µM) for this time showed no significant decrease in IFN-γ-induced phosphorylation of STAT1 as shown by a representative Western Blot in the upper panel. β-Actin was used throughout as a loading control. The relative protein level was assessed by densitometry (n = 4). Data are expressed as a percentage of the untreated control ± SEM. (**P < 0.01 compared to untreated cells, using Anova and the Student-Newman-Keuhls post-hoc test).
Figure 12 | Spermidine Protects Intestinal Epithelial Barrier Function in the Setting of Inflammation

IFN-γ (1000 U/mL) was added basolaterally to T84 monolayers that had stable TER values of ≥ 1000 Ω · cm². Following 24 hours of treatment, TER values decreased by 35% of their initial baseline measurements. Co-administration of spermidine for this time reduced the effects of IFN-γ on barrier function. Error bars are representative of ± SEM. (***(P < 0.001 compared to untreated cells; #P < 0.05 compared to IFN-γ-treated cells, using Anova and the Student-Newman-Keuls post-hoc test).
Figure 13 | Spermidine’s Proposed Mode of Action

A) PTPN2 protects epithelial barrier function during periods of inflammation by acting as a negative regulator of the pro-inflammatory cytokine, IFN-γ, through the dephosphorylation of STAT  

B) Spermidine increases PTPN2 protein levels and activity, thus amplifying PTPN2’s protective effect during periods of inflammation
REFERENCES


