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**TNBS-induced chronic colitis is associated with fibrosis and modulates TGF-β1 signaling**

Loeuillard E *et al*. TNBS-induced chronic colitis in rats

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**Abstract**

**AIM:** To investigate whether targeting proteasome might reverse intestinal fibrosis in rats.

**METHODS:** Chronic colitis was induced by repeated administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) for 6 wk while control rats received the vehicle. Histological scoring of inflammation and fibrosis were performed. Fibrosis-related proteins and tight junction proteins were assessed by western blot. Proteasome activities were assessed.

**RESULTS:** TNBS rats had a higher colon weight/length ratio (*P <* 0.001) compared to control rats. Fibrosis and inflammation scores were higher in TNBS rats compared to control rats (*P <* 0.01 for both). Colon transforming growth factor (TGF)-β production tended to be higher in TNBS rats (*P <* 0.06). Fibrosis-related proteins such as phospho-p38, phospho-SMAD2-3 or peroxisome proliferator activated receptor (PPAR)γ were significantly higher in TNBS rats compared to control rats (*P <* 0.05 for both). Tight junction proteins were modified by repeated TNBS challenge: colon occludin expression rose significantly (*P <* 0.001) whereas claudin-1 expression fell (*P <* 0.001). Bortezomib inhibition significantly decreased chymotrypsin-like activity (*P <* 0.05) but had no significant effect on trypsin-like activity (*p >* 0.05). In contrast, bortezomib had no effect on other studied parameters such as fibrosis score, TGF-β signaling or tight junction expression (*p >* 0.05 for all).

**CONCLUSION**: Rats with TNBS-induced chronic colitis exhibited colon fibrosis associated with higher TGF-β signaling. Proteasome inhibition by bortezomib had no effect on fibrosis in our experimental conditions.

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**Key words:** Bortezomib; Colitis; Fibrosis; Proteasome

**Core tip:** Inflammatory bowel disease gives rise to challenging clinical conditions and many patients have to undergo surgery throughout their life due to irreversible lesions. As no specific treatment is currently available for intestinal fibrosis, we tested an antifibrotic drug which is efficient in other chronic inflammatory diseases. In the present study, we investigated whether targeting proteasome by bortezomib might inhibit intestinal fibrosis in rats with chronic colitis. We found that rats with 2,4,6-trinitrobenzene sulfonic acid-induced chronic colitis exhibited colon fibrosis associated with high expression of transforming growth factor-β signaling especially in the Akt pathway. Nevertheless, we failed to inhibit colon fibrosis by proteasome inhibition in our experimental conditions.

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**Introduction**

Intestinal fibrosis is a common consequence of inflammatory bowel diseases (IBD), giving rise to severe clinical complications such as strictures and intestinal obstructions and ultimately leading to surgery. Chronic inflammation is also able to activate wound healing responses including increased extracellular matrix (ECM) or mesenchymal cell activation that can promote intestinal fibrosis. Crohn’s disease (CD) is the main example of fibrosis process. Indeed, CD is characterized by chronic transmural inflammation from the mucosal to the deeper layers of the gut where mesenchymal cells are mainly located.

While management of inflammation by IBD therapy has progressed markedly, specific treatment for intestinal fibrosis is still unavailable and surgery is often the only remaining option. Thus, there is a great need for specific medical therapy aimed at preventing or reversing intestinal fibrosis. Antifibrotic drugs which are efficient in other chronic inflammatory diseases may be good candidate molecules.

Targeting proteasome is one potential antifibrotic strategy which might help to reverse fibrosis. Proteasome activates transcription factor nuclear factor-κB (NF-κB) by degrading the inhibitory protein IκB, with consequent induction of genes encoding adhesion molecules and cytokines. Bortezomib, which is an inhibitor of proteasome has demonstrated antifibrotic properties in skin and lung fibrosis models[1]. The nuclear receptor PPARγ has recently appeared as a molecule which is involved in fibrosis and also in proteasomal inhibition by bortezomib in lung and skin fibrosis. In the present study, we aimed to investigate its expression[1].

Animal models have been developed to better understand the mechanisms underlying intestinal fibrosis and to test therapeutic interventions. Intrarectal administration of the hapten reagent TNBS in ethanol is widely used to investigate acute intestinal inflammation[2-5] and exhibits CD-like features such as transmural inflammation. Stidham *et al*[6] developed a chronic model of intestinal inflammation-associated fibrosis by weekly exposition of TNBS with ethanol for 6 wk.

We aimed to investigate the signaling pathways involved in a chronic-intestinal inflammation-associated fibrosis model in rats and to test the potential antifibrotic effect of proteasome inhibition by bortezomib.

**MATERIALS AND METHODS**

***Animals and study design***

Animal care and experimentation complied with both French regulations and European Community regulations (Official Journal of the European Community L 358, 18/12/1986). In addition, RML and MC are authorized by the French government to use this rat model (Authorization *n* = 76-106, *n* = 76-107). Sprague-Dawley male rats (Janvier, Le Genest St Isle, France) weighing 150 g were randomized into three groups: control and control colitic (TNBS), and BTZ-treated rats (TNBS + BTZ). Rats were weighed three times a week. Water and food were provided *ad libitum*.

***Induction of colitis***

Rats were food-deprived for 24 hours prior to induction of colitis and were allowed free access to tap water throughout the study. Chronic colitis was induced by weekly administration of increasing concentrations of TNBS (15, 30, 45, 60, 60 and 60 mg) (Sigma Aldrich-Company, Saint-Quentin Fallavier, France) over 6 weeks as previously described by Stidham *et al*[6]. Colitis was induced in two groups at day 0 by intrarectal injection of TNBS, whereas the control group received the vehicle (0.25 mL of 50% ethanol). Briefly, rats were anesthetized with an intraperitoneal injection of ketamine and chlorpromazine following 24 h food deprivation. TNBS dissolved in 50% (v/v) ethanol was instilled into the colon through a canula (0.25 mL) to induce chronic colitis. After instillation of hapten, the rats were then maintained in a head-down position for a few minutes to prevent leakage of the intracolon instillate. Control groups received the same volume of the vehicle .

***Bortezomib treatment (velcade, selleckchem)***

TNBS + BTZ rats received intraperitoneal injections of bortezomib (d37, d41, d43) twice weekly at a dose of 25 mg/kg per day while the control and TNBS groups received the same amount of the vehicle.

***Material***

Phosphate Buffer Solution (PBS), phosphatase and protease inhibitor cocktail, were purchased from Sigma Aldrich-Company (Saint-Quentin Fallavier, France). Bis-Tris gels (4%-12%), Invitrolon, PVDF membranes, and Seeblue multi-colored standard were obtained from Invitrogen (Cergy Pontoise, France). Monoclonal antibody anti-PPARγ (sc-7273), monoclonal antibody anti-AKt (sc-81434), polyclonal antibody anti-p38 (sc-535), polyclonal antibody anti-p-P38 (sc-7975-R), monoclonal antibody anti-p70S6 kinase (sc-8418), polyclonal antibody anti-p-Smad2/3 (sc-11769), goat polyclonal antibody anti-COX-2 (sc-1747), and secondary antibodies IgG1 horseradish, rabbit and mouse conjugated were obtained from Santa Cruz biotechnology (Tebu, Le Perray-en-Yvelines, France). Polyclonal antibody anti-extracellular regulated kinase (ERK)1/2 (#4665), polyclonal antibody anti-p-AKt (#5473) and monoclonal antibody anti-p-ERK1/2 (#9106) were obtained from Cell Signaling technology (Ozyme, Montigny le Bretonneux, France). Polyclonal antibody anti-Occludin (711500) and monoclonal antibody anti-claudin-1 (374900) were obtained from Life Technologies Corporation (Saint Aubin, France). The proteasome inhibitor bortezomib (S1013) was obtained from Selleck chemicals (2626 South Loop West, Suite 225, Houston, TX 77054 United States, 77054). The proteasome inhibitor for proteolytic pathway activities MG 132 Z-Leu-Leu-Leu-al (c2211) and the substrate for trypsin Boc-Gln-Ala-Arg-7-amido-4-methylcoumarin hydrochloride (B4153) were obtained from Sigma-Aldrich (Saint Louis, United States). Suc-LLVY-AMC, Chymotrypsin Substrate III, and Fluorogenic (539142) were obtained from Calbiochem, Merck Chemicals (Merck KGaA, Darmstadt, Germany).

***Western blot***

Frozen colon samples were homogenized in PBS with 1% protease inhibitor cocktail (Sigma) and 1% phosphatase inhibitor cocktail (Sigma). Homogenates were centrifuged (12,000g, 15 min, 4°C) and the supernatants were collected. Protein concentration was determined using Bradford’s colorimetric method. Aliquots of supernatants containing equal amounts of protein (40 μg) were separated on 4-12% NuPAGE gel (Invitrogen) and then transferred to a nitrocellulose membrane (Hybond, GE Healthcare, UK). After blocking, membranes were incubated with specific primary antibodies at dilutions of 1:250 (for COX-2,) 1:500 (for AKt, ERK1/2, p-ERK1/2, P38 and p-P38) 1:1000 (for PPARγ, p-SMAD2/3 and P70S6K) and 1:2000 (for α-SMA). After three washes, the filter was then incubated with secondary horseradish peroxidase linked anti-goat IgG for COX-2 and ERK1/2 antibody, anti-mouse IgG for PPARγ, P70S6K and AKt antibody and anti-rabbit IgG for p-SMAD2/3, α-SMA p-ERK1/2, P38 and p-P38 antibody. To check equal loading, the blots were analyzed for β-actin expression. Immunodetection was performed using enhanced chemiluminiscence light-detecting kit (Amersham, Arlinghton Heights, IL, United States). Densitometric data were measured following normalization to the control (house-keeping gene) using an ImageScanner II densitometer (GE Healthcare) and ImageQuant TL analysis software (GE Healthcare).

***Immunoprecipitation of 4EBP1 proteins***

Regulation of expression of proteins of interest was confirmed with immunoprecipitation experiment followed by western blotting. Immunoprecipitation was performed with 10 µm cutoff ultrafiltration spin-columns (Pierce/Thermo Scientific, Rockford, IL, United States) and 50 µl (50% slurry) protein G-agarose beads (Calbiochem, EMD Chemicals, San Diego, United States). First, total protein samples were incubated overnight with antibody directed against protein of interest (polyclonal rabbit eIF4E, SantaCruz Biotechnology). Then, samples were incubated overnight with beads at 4 °C in a tube rotator. Beads were washed two times with 600 µl of ice-cold PBS. Proteins of interest were finally eluted two times with 50 µL of 5 mol/L urea solution. Eluted samples were loaded on SDS-PAGE gels as previously described[7]. Proteins were transferred onto nitrocellulose membranes. After transfer, membranes were soaked in TBS-T/BSA solution [5% (w:v) BSA] for 1 h at room temperature. Then, blots were incubated overnight at 4°C in TBS-T/BSA with anti-4EBP1 antibody (1:500). Membranes were washed 3 times for 10 min with TBS-T, incubated with swine anti-rabbit IgG HRP conjugated (1:5000, Dako, Trappes, France) in TBS-T/BSA for 1 h at room temperature, and then washed 3 times in TBS-T. Immunocomplexes were revealed using ECL detection system (GE Healthcare). To determine protein abundance, band intensities were quantified using an ImageScanner II densitometer (GE Healthcare) and ImageQuant TL analysis software (GE Healthcare).

***Proteolytic pathway activities***

Proteolytic pathway activities such as trypsin-like and chymotrypsin-like activities were evaluated as previously described[4]. Briefly, evaluation of their activities was performed by spectrofluorimetry on a microtiter plate fluorometer (Mithras LB 940, Berthold Technologies) using fluorogenic proteasome substrate in the presence or absence of specific proteasome inhibitors.

***Colon production of TGF-β1***

 Frozen colon samples were homogenized in PBS with 1% protease inhibitor cocktail (Sigma) and 1% phosphatase inhibitor cocktail (Sigma). Homogenates were centrifuged (12000 g, 15 min, 4 °C) and supernatants were collected. Protein concentration was determined using Bradford’s colorimetric method. Samples were then analyzed with a kit (MB100B) DuoSet® ELISA Development System (R and D SYSTEM, United Kingdom). Their concentrations were determined by spectrophotometry at wavelength 450 nm with spectrophotometer Metertech, Σ960, Taiwan.

***Histological studies***

Intestinal tissues were fixed in 40% formol, embedded in paraffin wax blocks, and 5 µm sections were stained with hematoxylin-eosin safran to estimate collagen content. Sections were scored by the same pathologist and samples were blinded. Epithelial necrosis, inflammatory cell infiltration and thickness of the mucosa were assessed using semi-quantitative scores which ranged from 0 to 3 for each variable (0, no inflammation; 1, very low level of inflammation; 2, moderate level of leukocyte infiltration; 3, high levels of leukocyte infiltration and vascular density, ulcerations) using Leica QWin analysis software (Leica Microsystems, Bensheim, Germany).

***Statistical analysis***

## Statistical comparisons were performed using GraphPadPrism 5. Data are expressed as mean ± SE. Body weight changes and food intake were analyzed by 2-way ANOVA for repeated measures with Turkey’s post hoc tests. All other variables were analyzed by one-way ANOVA with Bonferroni post hoc test or Kruskal Wallis test as appropriate. Differences were considered significant at P < 0.05.

**Results**

***Chronic TNBS challenges induce colon fibrosis in rats***

Colon weight/length ratio was significantly higher in TNBS rats compared to control rats (*P <* 0.05, Figure 1A). The TNBS group had distal thickening and rigidity of the colon (Figure 1B). Histological analysis of tissue sections from TNBS rats revealed inflammatory infiltrate, thickening of the mucosa and submucosa and a higher proportion of collagen deposition compared to the control group (Figure 1C). TNBS rats had a significantly higher fibrosis score (*P <* 0.01, Figure 1D) and inflammatory score (*P <* 0.01, Figure 1E).

***Chronic TNBS challenges induce TGF-β signaling***

TGF-β is a key cytokine in fibrosis processes and TNBS rats tended to have a higher production of colon TGF-β compared to control rats (*P* = 0.06, Figure 2A). TGF-β receptors interact with SMAD signaling, and phosphoSMAD2-3 expression was higher in TNBS rats compared to control rats (*P <* 0.05, Figure 2B). Non-SMAD signaling pathways such as Erk-MAPK pathway were also involved in TGF-β response. While no significant difference in phosphoERK1/ERK1 was found among groups (Figure 2C), TNBS rats had increased expression of phosphoERK2/ERK2 (*P <* 0.01, Figure 2D) and phosphop38/p38 ratio (*P <* 0.05, Figure 2E). PPARγ is a nuclear receptor and has recently been identified as a molecule involved in fibrosis. TNBS rats had a higher colon expression of PPARγ compared to control rats (*P <* 0.01, Figure 2F).

Other TGF-β-induced non-Smad signaling pathways include the Akt-mTOR pathway. TNBS rats had a higher expression of Akt compared to control rats (*P <* 0.001, Figure 3A). There was no significative change in colon P70S6K expression among groups (*P >* 0.05, Figure 3B). There was an enhanced association of 4EBP1 with eIF4E in rats with chronic TNBS compared to controls (*P <* 0.05, Figure 3C, D).

# **Chronic TNBS and altered tight junction proteins**

While chronic administration of TNBS significantly increased occludin expression (*P <* 0.001, Figure 4A) TNBS decreased claudin-1 expression (*P <* 0.001, Figure 4B).

# **Chronic TNBS and altered proteasome**

Bortezomib inhibition of proteasome significantly decreased chymotrypsin-like activity (*P <* 0.05, Figure 5A) but it had no significant effect on trypsin-like activity (*p >* 0.05, Figure 5B). In contrast, bortezomib had no effect on other studied parameters such as fibrosis score (*p >* 0.05), TGF-β signaling (*p >* 0.05 for all) or tight junction expression (*p >* 0.05 for all).

**Discussion**

We aimed to characterize a model of chronic colitis that reflects human CD. By administration of weekly doses of intrarectal TNBS, rats developed increasing fibrosis of the colonic *lamina propria.* Although repeated administration of TNBS has been widely evaluated in mice, resistance to mouse strain dependent fibrosis has led to unacceptably high mortality in mice[8,9]. By contrast, rats are more susceptible to fibrosis and we found that 7 out of 9 rats showed fibrosis with chronic TNBS-induced colitis. A rat model with chronic TNBS has already been used to investigate the feasibility of ultrasound elasticity imaging to evaluate intestinal fibrosis[6]. An appropriate animal model of intestinal fibrosis will add to understanding of the fibrosis process. The present study investigated signaling involved in rats with chronic TNBS and tested the potential of targeting proteasome as a therapeutic intervention.

TGF-β is known to play a key role in intestinal fibrosis by inducing collagen production in fibroblast and intestinal smooth-muscle cells. In an acute model of TNBS colitis, Medina *et al*[10] observed a significant increase in colon TGF-β production. In the present study, TNBS rats had a higher level of colon TGF-β production which did not reach statistical significance (*P* = 0.06). The mechanisms behind TGF-β effects have not yet been elucidated and are difficult to study in humans. TGF-β signaling involves TGF-β receptors which transduce signals to the nucleus through the phosphorylation of SMAD2 and SMAD3 proteins. We investigated the expression of these proteins and found overexpression of SMAD2/3 in rats with chronic TNBS. As non-SMAD signaling is also involved in TGF-β response, we also studied the expression of Akt/mTOR signaling. mTOR is a key regulator of protein synthesis, and plays important role in other biological processes such as cell growth, angiogenesis and autophagy. mTOR exists in two functionally distinct complexes: mTORC1 and mTORC2. mTORC1 activates p70 ribosomal protein S6 kinase (P70S6K1) and inactivates 4E-BP1 (eIF4E binding protein 1), which promotes protein translation and cell growth as well as autophagy and fibrosis. mTOR regulation is best achieved through activation of the PI3K/Akt pathway, but mTOR receives input from multiple signaling pathways. In the present study, we found that chronic TNBS upregulates Akt expression without affecting P70S6K1. As we observed an enhanced association between 4E-BP1 and eIF4E, mTORC1 does not seem to be involved in the fibrosis process in the present model.

We studied expression of PPARγ since this nuclear receptor has recently emerged as a molecule involved in fibrosis. PPARγ can be activated by nutrients[11] and regulates intestinal inflammation. In addition, proteasomal inhibition by bortezomib prevents lung and skin fibrosis after injury, in part by increasing the abundance and activity of PPARγ[1].

Occludin and claudin-1 are major tight junction proteins of gut epithelial cells. In the present study, we observed increased expression of occludin with concomitant altered expression of claudin-1. A similar alteration in junctional proteins, and reduced expression of claudin-1 with increased occludin, have been observed in eosinophilic esophagitis[12], a disease leading to sub-epithelial fibrosis. Eosinophils are a source of TGF-β in fibrosis processes[13]. To induce a pro-fibrotic phenotype, fibroblasts are exposed to TGF-β and it has been demonstrated that TGF-β-treated fibroblasts exhibit a higher expression of occludin[14]. An identical tight junction pattern has been observed in intestinal epithelial cell line Caco-2 monolayers on TNFα stimulation: down-regulation of claudin-1 with increased occludin expression[15].

Although we had assumed that proteasome inhibition might hold promise for intestinal fibrosis, no therapeutic effect of bortezomib was observed in the present study. Indeed, bortezomib inhibits chymotrypsin-like activity without affecting fibrosis. As intestinal inflammation drives intestinal fibrosis, we chose to test the therapeutic effect of bortezomib in preexisting tissue fibrosis. As proteasome inhibition by bortezomib attenuates experimental colitis[16,17], we speculated that testing the preventive effect of bortezomib might result in inhibition of colitis and subsequently partial development of fibrosis in an animal model. Analyzing fibrosis in experimental models is particularly challenging because distinguishing altered inflammation from direct antifibrosis effect is tricky[18]. In murine TNBS colitis, treatment with NF-κB antisense down-regulated fibrosis parameters[8] but it has also been reported as an anti-inflammatory agent in colitis[19]. It is thus difficult to determine whether anti-fibrosis effects are independent of the anti-inflammatory properties of tested molecules. A recent model of murine *Salmonella typhimurium* demonstrating-induced intestinal fibrosis showed that early elimination of inflammation with levofloxacin led to eradication of *Salmonella typhimurium* infection with concomitant reduced intestinal fibrosis[20].

Although bortezomib prevents development of dermal fibrosis, it had no therapeutic effect once fibrogenisis was set in motion and was unable to reverse the process[21].

In summary, repeated injections of TNBS can induce chronic colitis with fibrosis in rats. We observed an upregulation of fibrosis score with increased fibrosis-related protein expression that characterized a reliable model of intestinal fibrosis. However, our attempt to target proteasome to inhibit or reverse fibrosis process by bortezomib administration failed. Identification of relevant therapeutic targets remains crucial in this field of intestinal fibrosis where treatment needs remain unmet.

**comments**

***Background***

Intestinal fibrosis is a common consequence of inflammatory bowel diseases (IBD), giving rise to severe clinical complications. Chronic inflammation is also able to activate wound healing responses including increased extracellular matrix or mesenchymal cell activation that can promote intestinal fibrosis. While management of inflammation by IBD therapy has progressed markedly, specific treatment for intestinal fibrosis is still unavailable and surgery is often the only remaining option. Thus, there is a great need for specific medical therapy aimed at preventing or reversing intestinal fibrosis. Antifibrotic drugs which are efficient in other chronic inflammatory diseases may be good candidate molecules.

***Research frontiers***

Animal models have been developed to better understand the mechanisms underlying intestinal fibrosis and to test therapeutic interventions. Intrarectal administration of the hapten reagent TNBS in ethanol is widely used to investigate acute intestinal inflammation and exhibits Crohn’s disease (CD)-like features such as transmural inflammation.

***Innovations and breakthroughs***

This paper investigates the signaling pathways involved in a chronic-intestinal inflammation-associated fibrosis model in rats and to test the potential antifibrotic effect of proteasome inhibition by bortezomib.

***Applications***

Targeting proteasome is one potential antifibrotic strategy which might help to reverse fibrosis. Proteasome activates transcription factor nuclear factor-κB by degrading the inhibitory protein IκB, with consequent induction of genes encoding adhesion molecules and cytokines.

***Peer review***

This manuscript entitled “tnbs-induced chronic colitis is associated with fibrosis and modulates transforming growth factor-β1 and akt signaling in rats.” your manuscript provides an interesting view of the subset of patients with CD characterized by fibrotic changes. this is an essential topic as we continue to try to understand CD and target therapeutic interventions. any therapeutic intervention that moderates or reverses fibrosis will be a major step forward in our disease management. your project does a very nice job simulating fibrosis and characterizing the basic science pathways involved in the fibrotic process.

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**Figure 1 Validation of chronic 2,4,6-trinitrobenzene sulfonic acid-induced colitis.** Chronic colitis was induced by weekly intrarectal injection of TNBS for 6 wk. A: Colon weight/length (g/m) in control and TNBS groups; B: Colon macroscopy; C: Hematoxylin-eosin stained tissues in control and TNBS groups. In the TNBS sections, chronic inflammation and fibrosis led to architectural disorders, lymphotic infiltrate and fibrin deposits. (Magnification, X 2.5, X 5, X 10); D: Histological fibrosis score from 0 (no fibrosis) to 3 (severe fibrosis); E: Histological inflammation score from 0 (no inflammation) to 3 (severe inflammation). Values are means ± SE. b*P <* 0.01 *vs* control. TNBS: 2,4,6-trinitrobenzene sulfonic acid.

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**Figure 2 Colon** transforming growth factor**-β production and** transforming growth factor**-β-associated proteins in rats with chronic 2,4,6-trinitrobenzene sulfonic acid-induced colitis.** A: Colon TGF-β production; B: phosphoSMAD2-3; C: phosphor-ERK-1; D: phospho-ERK2; E: phosphop38; F: PPARγ expression in control and TNBS rats. Values are means ± SE. a*P <* 0.05 *vs* control; b*P <* 0.01 *vs* control. PPAR: Peroxisome proliferator activated receptor; TGF: Transforming growth factor; ERK: Extracellular regulated kinase; TNBS: 2,4,6-trinitrobenzene sulfonic acid.



**Figure 3 Chronic 2,4,6-trinitrobenzene sulfonic acid challenge induces Akt signaling pathway.** A: Colon Akt expression; B Colon P70S6K expression; C: eIF4E immunoprecipitation; D western blot analysis of eIF4E -4E-BP1 association. Values are means ± SE.b*P <* 0.01 *vs* control. TNBS: 2,4,6-trinitrobenzene sulfonic acid.



**Figure 4 Chronic 2,4,6-trinitrobenzene sulfonic acid alters tight junction proteins.** A:Colon occludin expression; B colon claudin-1 expression. Values represent the mean expressed as a percentage of internal β-actin control ± SE. b*P <* 0.01 *vs* control. TNBS: 2,4,6-trinitrobenzene sulfonic acid.



**Figure 5 Proteasome inhibition by bortezomib treatment inhibits chymotrypsin-like activity. A:** Chymotrypsin-like activity; B: trypsin-like activity. Values are means ± SE. a*P <* 0.05 *vs* control. TNBS: 2,4,6-trinitrobenzene sulfonic acid.