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Regulation of transient receptor potential cation channel subfamily V1 protein synthesis by the phosphoinositide 3-kinase/Akt pathway in colonic hypersensitivity

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Abstract

The transient receptor potential cation channel subfamily V member 1 (TRPV1), also known as the capsaicin receptor or vanilloid receptor 1 (VR1), is expressed in nociceptive neurons in the dorsal root ganglia (DRG) and participates in the transmission of pain. The present study investigated the underlying molecular mechanisms by which TRPV1 was regulated by nerve growth factor (NGF) signaling pathways in colonic hypersensitivity in response to colitis. We found that during colitis TRPV1 protein levels were significantly increased in specifically labeled colonic afferent neurons in both L1 and S1 DRGs. TRPV1 protein up-regulation in DRG was also enhanced by NGF treatment. We then found that TRPV1 protein up-regulation in DRG was regulated by activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway both in vivo and in vitro. Suppression of endogenous PI3K/Akt activity during colitis or NGF treatment with a specific PI3K inhibitor LY294002 reduced TRPV1 protein production in DRG neurons, and also reduced colitis-evoked TRPV1-mediated visceral hypersensitivity tested by hyper-responsiveness to colorectal distention (CRD) and von Frey filament stimulation of abdomen. Further studies showed that TRPV1 mRNA levels in the DRG were not regulated by either colitis or NGF. We then found that an up-regulation of the protein synthesis pathway was involved by which both colitis and NGF caused a PI3K-dependent increase in the phosphorylation level of eukaryotic translation initiation factor 4E-binding protein (4E-BP)1. These results suggest a novel mechanism in colonic hypersensitivity which involves PI3K/Akt-mediated TRPV1 protein, not mRNA, up-regulation in primary afferent neurons, likely through activation of the protein synthesis pathways.

Keywords

TRPV1; protein synthesis; PI3K/Akt; NGF; visceral hypersensitivity

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Conflict of Interest: none

INTRODUCTION

Visceral hypersensitivity is a complex pathophysiological paradigm manifested by heightened sensory responses to visceral organ stimulation (Qiao, 2014). The transient receptor potential cation channel subfamily V member 1 (TRPV1) is a nonselective cation channel that is expressed in small to medium sized unmyelinated C-fiber sensory neurons presumably nociceptors and detects extremely high temperature and painful stimuli in target tissues such as skin and visceral organs (De Schepper et al., 2008; Ji et al., 2002; Jones et al., 2005). In response to peripheral/visceral irritation or ligand stimulation, the expression level and activity of TRPV1 are elevated in primary afferent neurons (Winston et al., 2007; Zhu and Oxford, 2007; Zhuang et al., 2004). In terms of visceral dysfunction, TRPV1 receptor antagonist, such as JNJ-17203212 (Wiskur et al., 2010), capsazepine (van den Wijngaard et al., 2009), SB-705498 (van den Wijngaard et al., 2009), JYL1421 (Miranda et al., 2007), and BCTC (Vermeulen et al., 2013) can reduce the visceral hypersensitivity to intracolonic mechanical and chemical stimulation in acute inflammatory, chronic post-inflammatory, or stress-induced rodent model of colonic hypersensitivity. Animals deficient in TRPV1 exhibit reduced responses of primary sensory afferent fibers to mechanical distension of the colon (Jones et al., 2005), and also prevented the development of visceral hypersensitivity and pain-associated behaviors post dextran sulfate sodium (DSS)-induced inflammation (Lapointe et al., 2015). The role of TRPV1 as hallmark of sensory activity and inflammatory pain has been extensively reviewed for their sensitization by pro-inflammatory factors and their ability to be activated by nociceptive signals (Lapointe and Altier, 2011). Nerve growth factor (NGF) is one of the potent stimuli that can evoke TRPV1 activity in the sensory neurons in response to inflammation (Zhu and Oxford, 2007; Zhuang et al., 2004).

A number of intracellular molecules in NGF-induced signaling cascade has been investigated for their roles in mediating sensory activity through the regulation of neuropeptides/transmitters production and ion channel activation (Obata and Noguchi, 2004; Obata et al., 2004; Yu et al., 2012b). In addition to the mitogen-activated protein kinases (MAPKs), the phosphoinositide 3-kinase (PI3K)/Akt pathway is recently revealed as an important mediator in nociceptive transmission. The PI3K/Akt pathway participates in sensory hypersensitivity in animal models of visceral and somatic pain evoked by chemical or nerve injuries (Choi et al., 2010; Kay et al., 2013; Pezet et al., 2008; Sun et al., 2006; Xu et al., 2007; Xu et al., 2011). The PI3K/Akt pathway regulates sensory hypersensitivity by increasing the production of neuropeptides and sodium channels in the primary afferent neurons (Liang et al., 2013; Qiao et al., 2013) and also increases the activity of glutamate receptor ion channels in the spinal cord contributing to spinal central sensitization (Choi et al., 2010; Pezet et al., 2008). There are two levels of regulation governed by the PI3K/Akt pathway: 1) transcriptional regulation of mRNA production by phosphorylating transcriptional factors such as nuclear factor kappa-B (NF κ B) (Dan et al., 2008), p-catenin (Masckauchan et al., 2005), and cAMP response element-binding protein (CREB) (Kay et al., 2013), and 2) translational regulation of protein production by activation of mammalian target of rapamycin (mTOR), p70S6 kinase (p70^{S6K}) and eukaryotic translation initiation factor 4E-binding protein (4E-BP)1 (Gingras et al., 1999; Xu et al., 2011).

The interplay between the PI3K/Akt pathway and TRPV1 expression/activity in the NGF-regulated signaling cascades has been suggested but not thoroughly understood. TRPV1 activity is regulated at multiple levels: transcriptional and/or translational up-regulation of TRPV1 can increase the membrane density of channels thus enhancing the overall activity of the channel (El Andaloussi-Lilja et al., 2009); post-translational modifications of TRPV1 such as phosphorylation at serine/threonine residues potentially by protein kinase A and protein kinase C (identified in Chinese-hamster ovary cells) are implicated in receptor sensitization (Por et al., 2013). Following acute NGF treatment, or peripheral stimulation with capsaicin, TRPV1 sensitization is mediated by the PI3K/Akt pathway (Zhu and Oxford, 2007; Zhuang et al., 2004). It is not documented how the NGF- and inflammation-regulated PI3K/Akt pathway contributes to TRPV1 activity.

The present study utilizes an *in vivo* model of inflammatory pain that exhibits colonic hypersensitivity in response to trinitrobenzene sulfonic acid (TNBS) colitis to investigate the mechanism of action of the PI3K/Akt pathway in the regulation of TRPV1 expression and visceral hypersensitivity. A number of publications have suggested an association of TRPV1 expression with colitis and colonic hypersensitivity. In TNBS colitis, TRPV1 immunoreactivity in the thoracolumbar (TL) and lumbarsacral (LS) DRG is significantly increased (Miranda et al., 2007). TRPV1 protein level is also specifically increased in neuronal tracing dye-labeled colonic afferent neurons in acetic acid-induced visceral pain (Winston et al., 2007). In patients with irritable bowel syndrome (IBS), it demonstrates a 3.5-fold increase in the density of TRPV1 immunoreactive fibers in the colon biopsies (Akbar et al., 2008). Taken together, these observations indicate an up-regulation of TRPV1 protein during colonic hypersensitivity. Our studies on the mechanisms and pathways by which TRPV1 protein synthesis is regulated by the PI3K/Akt pathway provide insights into the understanding of the signaling mechanisms underlying visceral pain process.

MATERIALS AND METHODS

Experimental animals

Adult male Sprague-Dawley rats (~200 g) (Harlan Sprague Dawley, Inc.) were used in the current studies. All experimental protocols involving animal use were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University (IACUC# AM10315). Animal care was in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and National Institutes of Health guidelines. All efforts were made to minimize the potential for animal pain, stress or distress.

Induction of colonic inflammation

To induce inflammation in the distal colon, animals were anesthetized and TNBS was instilled into the lumen of the colon 6 cm proximal to the anus at a dose of 90 mg/kg (1.5 mL/kg of 60 mg/mL solution in 50 % EtOH) through a syringe-attached polyethylene catheter as described previously (Qiao and Grider, 2007). Animals that received similar volume of 50 % EtOH enema served as control. All colonic instillations were performed under isoflurane anesthesia (2.5 %, SurgiVet, Smiths Medical PM, Inc. Waukesha, WI).

Euthanasia of animals was performed at 3 or 7 days after intracolonic instillation of TNBS or vehicle. To ensure exposure of the distal colon to TNBS and vehicle, rats were held head-down by lifting up the tail for 1 min.

Retrograde labeling of colonic afferent neurons

Colonic afferent neurons were specifically labeled by injecting conventional neuronal tracing dye to the distal colon as described previously by us (Qiao and Grider, 2007). Briefly, the rat distal colon was exposed with a lower abdominal incision under anesthesia (2.5 % isoflurane) and in a sterile environment. Fast Blue (FB, 4 %, weight/volume; Polysciences, Inc. Warrington, PA) was injected into 10 sites (4 μ L per site) in the muscular wall of the descending colon 5–7 cm away proximal to the external anal sphincter to label colonic afferent neurons innervating this area. Sterilized Hamilton syringe (10 μ L size) was used for injection. Injections into the lumen, major blood vessels, or overlying fascial layers were avoided. The incision was closed with 4-0 sutures, and the rats were allowed to survive until the harvest of the tissues. FB injection was performed 4 days prior to TNBS instillation to ensure proper transport of the tracing dye to the DRG. These animals further underwent intracardiac perfusion and tissues were used for immunohistochemistry only.

Tissue harvesting

To collect tissues used for immunohistochemistry, intracardiac perfusion was performed to euthanize animals under anesthesia (3–4 % isoflurane). The oxygenated Krebs buffer (pH 7.4) (95 % O₂, 5 % CO₂) was first used for perfusion which was followed by 4 % paraformaldehyde. After perfusion, the spinal cord and DRG were quickly removed and postfixed for 6 h. Tissue was then rinsed in phosphate buffered saline (0.1 M PBS, pH 7.4) and placed in ascending concentrations of sucrose (20 %) for cryoprotection. L1 and S1 DRGs were collected and sectioned parasagittally at a thickness of 20 μ m. Tissues from control and experimental animals were handled in an identical manner. To collect tissues used for western blot, real-time PCR, and acute culture, DRGs were freshly dissected out and either homogenized or processed for culture.

Laser capture microdissection (LCM) of DRG neurons

The DRGs that contain retrograde tracer-labeled colonic afferent neurons were freshly frozen for cryostat sectioning. Sections (10 μ m thickness) were mounted on Arcturus PEN Membrane Glass Slides (Thermo Fisher Scientific), and subject to LCM on a Veritas LCM System (Arcturus) equipped with multi-band filters. The microdissected cells were captured into CapSure HS LCM caps, lysized and spanned into a fresh tube for RNA extraction with Arcturus PicoPure RNA isolation kit (Thermo Fisher Scientific).

Immunostaining

DRG sections from control and experimental animals were processed for on-slide immunostaining. Sections were incubated for 1 h at room temperature with blocking solution containing 3 % normal donkey serum (Jackson ImmunoResearch, West Grove, PA) in PBST (0.3 % Triton X-100 in 0.1 M PBS, pH 7.4). Samples were then incubated overnight at 4 °C in primary rabbit antibody against phospho-Akt (1:500, Cell Signaling

Technology, Inc.), or rabbit antibody against TRPV1 (1:1000, Millipore-Chemicon International Inc., CA), which was diluted in PBST containing 5 % normal donkey serum. After rinse (3×10 min with 0.1 M PBS), tissues were incubated with fluorescence-conjugated species-specific secondary antibody Cy3 (1:500, Molecular Probes, Eugene, OR) for 2 h at room temperature. For double staining, mouse antibody for phospho-Akt (1:300, Cell Signaling Technology, Inc.) was used in combination with rabbit antibody for TRPV1 or TrkA (1:750, Santa Cruz Biotechnology) to examine colocalization. Following wash, the slides were coverslipped with Citifluor (Citifluor Ltd., London). Immunostaining in the absence of primary or secondary antibody was assessed for background evaluation. The specificity of the antibodies was also evaluated with western blot in the present study or pre-absorption assay published by us previously (Xia et al., 2012).

DRG cells with visible nucleus were counted with a Zeiss fluorescent photomicroscope by two investigators independently in a blind fashion to minimize any potential bias. Cells were examined in 6 to 10 sections randomly chosen from each L1 and S1 DRGs. Data from both sides of DRG for each segment in the same animal were pooled and averaged as one independent experiment (n). The number of FB-labeled cells were counted for normalization of phospho-Akt and TRPV1 immunoreactivity in these cells. The results were expressed as percentage. To avoid double counting, we chose every third section in the serial cutting for each antibody used.

Western blot

DRG homogenates in solubilization buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 100 mM NaF supplemented with protease inhibitor cocktail (P8340, 1:100, Sigma-Aldrich) and phosphatase inhibitor cocktail 1 (P2850, 1:100, Sigma-Aldrich) were subject to centrifugation at 20,200 g for 10 min at 4 °C. The protein concentration in the supernatant was determined using Bio-Rad DC protein assay kit. Protein extracts were then separated on a 10 % SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5 % milk in Tris-buffered saline for 1 hour and then incubated with specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibody. The bands were identified by enhanced chemiluminescence (ECL). For internal loading control, the same membrane was stripped and re-probed with antibodies against non-phosphoprotein or β -actin. The antibodies used in this study were rabbit anti-phospho-Akt (1:1000, Cell Signaling Technology, Inc.), rabbit anti-Akt (1:1000, Cell Signaling Technology, Inc.), rabbit anti-TRPV1 (1:1000, Millipore-Chemicon), rabbit anti-phospho-4E-BP1 (1:1000, Cell Signaling Technology, Inc.), rabbit anti-4E-BP1 (1:1000, Cell Signaling Technology, Inc.), and mouse anti- β -actin (1:3000, Sigma). The ECL-exposed films were digitized, and densitometric quantification of immunoreactive bands was performed using the software FluorChem 8800 (Alpha Innotech, San Leandro, CA).

Quantitative real-time PCR

Total RNA was extracted using a RNA extraction kit RNAqueous (Ambion, TX). RNA concentration was determined spectrophotometrically. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ABI). Following reverse

transcription, quantitative real-time PCR was performed on StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, ABI) under a condition of 40 cycles of 95°C for 15s and 60°C for 1 min. The primers for TRPV1 mRNA detection were the pre-designed Taqman probe (Applied Biosystems). The level of TRPV1 mRNA was normalized against the expression of the internal control β -actin (using Taqman probe) in the same sample that was calculated with 2^{-Ct} method and expressed as fold changes (2^{-Ct} -fold).

DRG explants culture

The segment-matched DRG pairs were freshly isolated. L1 and S1 DRGs were used. After peeling off the surrounding membrane that wrapped the ganglion, these DRGs were acutely cultured in Dulbecco's Modified Eagle Medium (DMEM) for 2–4 hours before treatment. To study the effect of NGF, one DRG explant was treated with NGF (50 ng/mL) for designated time points. The contralateral DRG served as control. To study the role of the PI3K/Akt pathway in NGF action, one DRG was pre-treated with the PI3K inhibitor LY294002 (5 μ M) for 30 min, followed by NGF treatment; the contralateral DRG served as control by receiving vehicle (the same amount of DMSO as the amount for LY294002) pre-treatment for 30 min followed by NGF treatment. The dose of LY294002 used here for the in vitro study was selected according to a study on the IC₅₀ of the compound (Ballou et al., 2007), and was also validated in our previous studies using this dose in cultured neurons to effectively block the activity of Akt (Kay et al., 2013; Liu et al., 2015).

Assessment of visceral hyperalgesic sensitivity

Hyperalgesic assessment was conducted on day 7 post colitis induction. Animals were first subject to abdominal withdrawal test in response to electronic von Frey filaments (IITC Life Science Inc., CA) stimulation to examine referred hyperalgesia (Laird et al., 2001). Animals then were subject to hyperalgesic responsiveness test to CRD. The von Frey test was conducted prior to CRD due to its non-invasive nature. All behavioral studies were performed in a blind manner.

The electronic von Frey filaments (IITC Life Science Inc., CA) stimulation of the abdomen was performed to assess abdominal withdrawal behavior. Briefly, the rat with lower abdominal area shaved was placed in an individual chamber provided by the manufacture (IITC Life Science Inc., CA) and allowed to acclimate for 30 minutes. The von Frey filaments were applied to the shaved area with gradually increased force (gram) until abdominal withdrawal. Each animal was tested for five times in at least 20-min intervals. The shaved area and size were kept similar on each animal and the stimulated point was randomly chosen within the shaved area. The minimum force (gram) that caused abdominal withdrawal was recorded and averaged in each animal.

CRD stimulation was performed to assess animal hyperalgesic responses by observing abdominal withdrawal reflex (AWR) described previously (Al-Chaer et al., 2000). Under light anesthesia (1.5–2 % isoflurane), a 5–6-cm distention balloon was placed into the descending colon and the balloon catheter was securely fixed onto the rat tail with tapes. The rat was then placed in an acrylic cage and allowed to wake up and adapt to the environment for 30 min. CRD balloon catheter was connected to a portable sphygmomanometer to

gradually induce a constant pressure at three different levels of 20, 40, and 60 mmHg. The CRD was performed three times at 3-min intervals for each rat with each measurement lasting 20s under each designated pressure. The AWR scores were recorded according to the published score criteria (Al-Chaer et al., 2000) that were: 0, no behavioral response to CRD; 1, immobile during the CRD; 2, a mild abdominal muscle contraction but no lifting of the abdomen; 3, a strong abdominal muscle contraction and lifting of the abdomen, but no lifting of the pelvic structure; and 4, body arching and lifting of the pelvic structure.

Drug treatment

To specifically desensitize the colonic afferent neurons that express TRPV1, resiniferatoxin (RTX) was dissolved in 10 % Tween-80, 10 % ethanol, and normal saline and administered intracolonic (i.c. - into the lumen of the colon) at a dose of 250µg/kg/2 mL 10 days prior to test (3 days prior to TNBS instillation) as shown in our publication (Xia et al., 2012). To completely desensitize all primary afferent neurons that express TRPV1, we injected RTX intraperitoneally (i.p) at a dose of 300 µg/kg one week prior to induction of inflammation as shown in our publication (Qiao and Vizzard, 2004). The dosage chosen, route of injection, and the effectiveness of these treatments in inhibiting TRPV1 in the DRG have been sufficiently discussed in our previous publications (Qiao and Vizzard, 2004; Xia et al., 2012) and were also validated in this study (see Figure 7). The PI3K inhibitor LY294002 (~2.4 µg/rat/daily, or ~80 µg/kg body weight) was delivered to the L4-L5 spinal cord region via an intrathecal (i.t.) catheter (No. 0007740, Alzet) connected to an osmotic-pump (Alzet 2001) as described in our previous publication (Xia et al., 2012). This procedure was done under anesthesia (2.5 % isoflurane). TNBS treatment was performed on the same day following catheter/minipump implantation. The inhibitor stock was stored in DMSO solution and diluted with saline which was used as vehicle control.

Data analysis

The results from each study were presented as mean $\pm$ SD. Comparison between control and experimental groups was made by using Kruskal-Wallis nonparametric oneway ANOVA, or the student's *t* test. Differences between means at a level of *p* 0.05 were considered to be significant.

RESULTS

Up-regulation of TRPV1 in colonic afferent neurons during colitis

We examined the expression of TRPV1 in FB-labeled colonic afferent neurons in DRG from control and TNBS-treated animals. The L1 and S1 DRGs were chosen because our previous studies demonstrated that these segments contained neurons project specifically to the distal colon (Qiao and Grider, 2007). Immunohistochemistry with a specific antibody against TRPV1 (Figure 1A and 1D, green cells) showed that colitis at both 3 and 7 days post TNBS treatment increased the number of colonic afferent neurons (Figure 1B and 1E, blue cells) that expressed TRPV1 (Figure 1C and 1F, cells indicated by yellow arrows) by approximate 1.5–2 folds in the L1 (Figure 1G) and S1 (Figure 1H) DRGs at both time points. We found that in addition to colonic afferent neurons TRPV1 expression was also increased in DRG neurons that were not labeled by FB (summary data was not shown since they were not the

focus of this study). These neurons could be colonic afferent neurons that failed to uptake FB or neurons that projected to a different organ other than the distal colon. To confirm our immunohistochemistry results, we performed western blot (Figure 1I). We found that TNBS 3 or 7-day colitis increased the protein levels of TRPV1 in the L1/S1 DRGs (Figure 1I). To enrich samples, we pooled the L1 and S1 DRG together for western blot.

At both 3 and 7 days of colitis, we did not find changes in the TRPV1 mRNA levels in the labeled colonic afferent neurons (Figure 2A). These neurons were microdissected out from T13-L2 and L6-S1 DRG to increase the sample pools of the labeled neurons because a single segment of DRG did not provide enough samples for subsequent analysis. To examine whether there is a segment-dependent change in the TRPV1 transcriptional level in the DRG, we performed real-time PCR of each L1 or S1 DRG and found that there was no change in these segments at both 3 days and 7 days of colitis (Figure 2B–C).

TRPV1 protein up-regulation in the DRG was regulated by NGF-mediated PI3K/Akt pathway

It was reported that TRPV1 sensitization in sensory neurons was evoked by NGF (Zhu and Oxford, 2007; Zhuang et al., 2004), suggesting that TRPV1 nociceptive neurons are responsive to NGF. After NGF treatment of DRG explant culture, we found that NGF evoked a time-dependent increase in the protein expression level of TRPV1 (Figure 3A). NGF-increased TRPV1 protein up-regulation occurred at 4 h after addition of NGF (Figure 3B). However, NGF did not change the level of TRPV1 mRNA in the cultured DRG explants at time points of 45 min, 2 h, 4 h, or 8 h of treatment (Figure 3C). It has been reported by us and others that NGF up-regulates brain-derived neurotrophic factor (BDNF) mRNA and protein in DRG neurons (Matsuoka et al., 2007; Michael et al., 1997; Yu et al., 2012a). Thus we analyzed the same samples for BDNF mRNA as positive controls showing that NGF-facilitated a time-dependent increase in BDNF transcripts in the cultured DRG explants (Figure 3D). Interestingly, we found that in the same study for calcitonin gene-related peptide (CGRP), NGF failed to up-regulate CGRP mRNA levels within the examined time frame (45 min to 8 h, data not shown since there was no change). This was consistent to previous findings in isolated DRG neuron culture that NGF did not increase CGRP mRNA at 2 or 6 h of treatment (Xu and Hall, 2007).

Similar to other cell systems, NGF treatment increased the activity (phosphorylation level) of Akt in the DRG (Figure 3E). We then found that inhibition of the Akt kinase, PI3K, with LY294002 reduced NGF-facilitated TRPV1 protein expression (Figure 3F). We then examined whether the TRPV1 protein up-regulation in vivo by colitis was also regulated by the endogenous PI3K/Akt pathway. We found that TRPV1 protein up-regulation in the DRG during TNBS colitis was not affected by vehicle treatment, but was reduced by PI3K inhibitor LY294002 (Figure 4).

Since the above data suggests an existence of a NGF-Akt-TRPV1 axis in the DRG, we then examined the co-distribution of these molecules in DRG neurons during colitis. We found that a subpopulation of TrkA DRG neurons (Figure 5A, green cells) also expressed the active forms (phosphorylation) of Akt (Figure 5B, red cells). We also found that Akt phosphorylation during colitis (Figure 5D, red cells) was also expressed in TRPV1-positive DRG neurons (Figure 5E, green cells). We analyzed the trend of changes in the co-labeled

cells in the S1 DRG and found that the number of cells co-expressing TrkA and p-Akt (Figure 5G), or TRPV1 and p-Akt (Figure 5H) was increased in TNBS-treated animals when compared to vehicle treatment.

Up-regulation of protein synthesis in the DRG during colitis

The fact that both NGF and colitis increased the expression levels of TRPV1 protein but not mRNA in the DRG which involved activation of the PI3K/Akt pathway suggests that TRPV1 up-regulation could be regulated at the stage of mRNA translation. Thus we examined the key molecule in the protein synthesis pathway. We focused on 4E-BP1 because its phosphorylation by the Akt/mTOR cascade releases its inhibition on translation initiation factor eIF4E thereby promoting protein translation (Hinnebusch, 2012). In DRG explants, treatment with NGF for 25 min increased the phosphorylation level of 4E-BP1 (Figure 6A, 6B), which was inhibited by pre-treatment with LY294002 (Figure 6A, 6C). In colitis, the phosphorylation level of 4E-BP1 was also increased in the DRG (L1 and S1 combined) at 7 days post colitis induction, which was also inhibited by suppression of the endogenous PI3K activity with LY294002 (Figure 6D, 6E).

Regulation of colitis-induced hyperalgesic responses by TRPV1 and the PI3K/Akt pathway

The level of TRPV1 expression is closely related to painful sensation. A number of studies demonstrate that TRPV1 antagonisms either by specific TRPV1 inhibitors or genetic deletion of TRPV1 gene (TRPV1^{-/-}) have blocked colitis-induced visceral inflammatory and postinflammatory pain (Lapointe et al., 2015; Miranda et al., 2007; Vermeulen et al., 2013). Here we inhibited TRPV1 by prolonged RTX treatment either intraperitoneally (i.p) or intracolonicly (i.c.) and also revealed the same role of TRPV1 in TNBS colitis associated visceral hypersensitivity (Figure 7A–B). Specifically, we found that in animals with colitis the threshold force that caused abdominal withdraw was significantly lower when compared to control in the test of von Frey filaments stimulation of abdomen (Figure 7A). The same sets of animals were then tested with CRD (Figure 7B). The AWR scores were recorded according to previously described criteria (Al-Chaer et al., 2000). The gradually increased pressure was introduced as 20, 40, and 60 mmHg (Schwartz and Gebhart, 2014). In control animals, there was an increased trend in the baseline sensitivity in response to an increased pressure (Figure 7B). We were more interested in the comparison of control and colitic animals showing that the colitic animals demonstrated enhanced sensory hypersensitivity especially when the pressure increased above noxious stimuli at 40 mmHg (Figure 7B). In both tests of animal hyperalgesic responses to von Frey filaments stimulation and CRD, RTX pre-treatment reversed colitis-induced visceral hypersensitivity (Figure 7A–B). We then examined the level of TRPV1 in the DRG following RTX pre-treatment. We found that RTX pre-treatment by either i.p. or i.c. blocked TNBS-induced TRPV1 up-regulation in L1 and S1 DRG (pooled samples for western blot) (Figure 7C–D).

Since the TRPV1 up-regulation was regulated by the PI3K/Akt pathway, we tested whether inhibition of the PI3K/Akt had a role in colitis-induced hyperalgesic responses (Figure 8). We found that the animals that received both TNBS and LY294002 treatment had a higher threshold force that caused abdominal withdraw when compared to TNBS treatment alone in response to von Frey filaments stimulation (Figure 8). The same sets of animals were then

tested with CRD (Figure 8). Treatment of colitis animals with LY294002 decreased the AWR scores when compared to TNBS treatment alone tested under the pressure of 40 and 60 mmHg (Figure 8).

Akt phosphorylation was also increased in specifically labeled colonic afferent neurons during colitis

Our previous studies with western blot showed that the level of Akt phosphorylation was increased in the L1/S1 DRG at 7 days of colitis (Xia et al., 2016). We then asked whether the increased Akt phosphorylation occurred in specifically labeled colonic afferent neurons. Using immunohistochemistry, we assessed the expression of phospho-Akt (Figure 9, red cells) in colonic afferent neurons (Figure 9, blue cells) before and post TNBS treatment for 7 days and found that the percentage of colonic afferent neurons expressing phospho-Akt was significantly increased in colitis when compared to control (Figure 9G, 9H). Both L1 (Figure 9G) and S1 (Figure 8H) DRGs showed increases in the level of phospho-Akt during colitis.

DISCUSSION

The present study combines *in vivo* and *in vitro* approaches and determines that sensory hypersensitivity is associated with TRPV1 protein, but not mRNA, up-regulation in the DRG. We sensitized the DRG neurons by NGF treatment in DRG culture as well as *in vivo* by colonic inflammation to induce colonic hypersensitivity and achieved consistent results that the PI3K/Akt pathway mediated TRPV1 protein up-regulation in the DRG through up-regulating the mRNA translational process. At 3 or 7 days after colitis induction, the TRPV1 protein levels were increased in specifically labeled colonic afferent neurons (Figure 1). NGF treatment also increased TRPV1 protein levels in the DRG culture (Figure 3). The TRPV1 mRNA levels were not changed by either NGF or colitis (Figures 2 & 3). Both NGF- and colitis-induced TRPV1 protein up-regulation in the DRG were mediated by the PI3K/Akt-induced protein synthesis pathway (Figures 3, 4 & 6). Examination of the hyperalgesic responses of the animals shows that TRPV1 and PI3K/Akt activities were strongly associated with colitis-induced colonic hypersensitivity. Desensitization of TRPV1 by pre-treatment with RTX prevented the development of colonic hypersensitivity induced by colitis (Figure 7). Inhibition of the PI3K/Akt activity also reduced the hyperalgesic responses of animals to colitis (Figure 8). These results imply that sensory hypersensitivity regulated by TRPV1 is governed by PI3K/Akt-mediated protein synthesis which may underlie colitis-induced visceral pain.

TRPV1 is a member of the TRPV (vanilloid) group of ion channels that are highly sensitive to capsaicin and acts as capsaicin receptor. TRPV1 immunoreactivity is expressed in small to medium sized nociceptors, presumably the unmyelinated nociceptive C-fiber afferents (Pezet et al., 2005; Shi et al., 2009). TRPV1 receptor antagonists are often used for prevention of chronic pain, e.g., RTX, a potent analog of capsaicin, is suggested for its therapeutic intervention of visceral disorders (Chen et al., 2005; Cruz et al., 1997; Payne et al., 2005). In terms of colitis, antagonism of the TRPV1 normalizes TNBS colitis-induced pelvic afferent C-fiber sensitization in response to CRD (De Schepper et al., 2008) and reduces chemical colitis- or stress-facilitated visceromotor response to CRD (Miranda et al.,

2007; van den Wijngaard et al., 2009; Vermeulen et al., 2013; Wiskur et al., 2010). Prolonged RTX treatment shown in the current study also reduced TNBS-induced visceral hyperalgesia to CRD and von Frey filament stimulation of the abdomen (Figure 7). At 3 or 7 days post TNBS treatment, TRPV1 immunoreactivity in the TL and LS DRGs is increased but not in the cell bodies of pelvic afferent NF200 positive neurons (De Schepper et al., 2008; Miranda et al., 2007). Consistent to these immunostaining and behavioral results, the present study, using western blot, reveals the same result that TRPV1 protein expression is up-regulated in the L1 and S1 DRGs at 3 and 7 days of TNBS colitis (Figure 1). In TNBS-induced colitis and colonic hypersensitivity, we also show that TRPV1 protein up-regulation occurs in the specifically labeled colonic afferent neurons (Figure 1) and TRPV1 desensitization reversed colitis-induced visceral pain (Figure 7). This confirms a similar observation that TRPV1 protein levels are increased in the TL and LS DRG and are expressed by specifically labeled colonic afferent neurons in a rat model of neonatal acetic acid-induced chronic colonic hypersensitivity (Winston et al., 2007). Our results also show that colitis-induced TRPV1 fold increase in the DRG detected by western blot is higher than that detected by immunohistochemistry (Figure 1). One of the explanations for the discrepancy may be that TRPV1 expression in the nerve fibers or other structures other than neuronal cell bodies could be picked up by western blot but not by cell counting. During colitis, there is also a higher number of non-labeled DRG neurons that express TRPV1 when compared to control (Figure 1, cells indicated by green arrows). We do not know whether these neurons are colonic afferent neurons that fail to uptake FB or neurons that innervate a different organ. In spite of the advantage of the neuronal tracer dye back-labeling that it can identify specific colonic afferent neurons, the limitation of this technique is that it can only reveal a subset of colonic afferent neurons and provide sampling but not all of the colonic afferent populations. This is because of that FB is only injected to a segment of the distal colon (approximately 2 cm long and 4 cm away from the anus), and that FB is only injected to several areas by a Hamilton syringe which may not deliver FB to the proximity of every nerve terminals of the colonic afferent neurons. Therefore, this method identifies an adequate number of colonic afferent neurons as study samples for characterization of changes in these neurons, but it won't include the entire population of colonic afferent neurons. The results that TRPV1 expression is increased in the labeled colonic afferent neurons during colitis (Figure 1) indicate that colitis can sensitize colonic afferent neurons but it does not preclude that colitis does not sensitize other neurons.

Previous studies show that TRPV1 activity is regulated by NGF (Wang et al., 2014; Zhu and Oxford, 2007). The present study shows that NGF treatment of DRG explants increases TRPV1 protein production (Figure 3). The PI3K/Akt pathway is one of the major pathways downstream of NGF and participates in sensory hypersensitivity in animal models such as bladder hyperactivity and somatic pain (Arms and Vizzard, 2011; Choi et al., 2010; Kay et al., 2013; Pezet et al., 2008; Sun et al., 2006; Xu et al., 2007; Xu et al., 2011). In these studies, inhibition of PI3K and Akt with specific inhibitors significantly attenuates visceral and peripheral inflammatory or nerve injury pain. Akt activation is found to be up-regulated in the DRG following peripheral nerve injury (Shi et al., 2009; Xu et al., 2007), colonic inflammation (Xia et al., 2016), or upon peripheral capsaicin stimulation (Pezet et al., 2005; Zhuang et al., 2004). This study also shows that the Akt activity (phosphorylation) is

increased in specifically labeled colonic afferent neurons during colitis (Figure 9) and is co-expressed with TRPV1 and NGF high affinity receptor TrkA (Figure 5). In the DRG, Akt is required for the protein up-regulation of Nav1.7 and Nav1.8 channels (Liang et al., 2013), and also mediates NGF-induced neuronal sensitization in response to capsaicin (Wang et al., 2014; Zhu and Oxford, 2007). Here we show that Akt activity is also involved in colitis-induced TRPV1 protein production (Figures 3 & 4). PI3K/Akt-mediated NGF-induced TRPV1 protein up-regulation may underlie the mechanism of NGF-potentiated TRPV1 activity (Wang et al., 2014; Zhu and Oxford, 2007; Zhuang et al., 2004). Another TRP channel potentiated by NGF in DRG neurons is the TRPA1 (Malin et al., 2011). In TNBS-induced acute inflammation (3 days) and visceral hypersensitivity, blockade of both TRPV1 and TRPA1 synergistically enhances the reduction of visceromotor responses when compared to inhibition of a single channel alone (Vermeulen et al., 2013), suggesting that TRPA1 could be as important as TRPV1 in NGF-potentiated sensory neuron activation and colitis-induced visceral pain. The regulation and enhancement of TRPA1 by NGF could be an interesting topic for future studies.

In NGF- and colitis-induced TRPV1 up-regulation in the DRG, the TRPV1 transcription level remains the same, suggesting a possibility of an up-regulation in the TRPV1 mRNA translational process. Similarly, NGF treatment of DRG neurons fails to increase the mRNA level of CGRP within 6 hours of treatment examined by others (Xu and Hall, 2007) and within 8 hours of treatment examined by us (data is not shown since there is no change). NGF-induced CGRP protein up-regulation occurs after 12 hours of treatment (Yu et al., 2012b), which is prior to NGF-induced CGRP mRNA up-regulation after 24 hours of treatment (Xu and Hall, 2007). This suggests that NGF-induced CGRP up-regulation may share the same mechanism as NGF-induced TRPV1 up-regulation, which involves an enhanced process of protein synthesis. The well-known downstream signaling pathways of PI3K/Akt that are involved in protein synthesis are the mammalian target of rapamycin (mTOR)-mediated phosphorylation and activation of p70S6 kinase (p70^{S6K}) and 4E-BP1 (Gingras et al., 1999). mTOR is expressed in DRG, primary sensory axons, and dorsal horn neurons (Xu et al., 2010). Its role as a potential pharmacological target in the management of chronic pain is recently reviewed (Lisi et al., 2015). The present study demonstrates that the phosphorylation of 4E-BP1 is also up-regulated, dependent on the PI3K/Akt pathway, in the DRG by TNBS colitis and NGF (Figure 6), further confirms an important role of protein synthesis in the regulation of sensory activity. A recent study in IBS patient biopsy as well as in TNBS colitis (30 days)-induced visceral hypersensitivity demonstrates that TRPV1 up-regulation at the protein level in the colon and in the DRG are mediated by decreases in microRNA miR-199, which modulates TRPV1 protein synthesis (Shen et al., 2015). It is reported that miR-199 can negatively regulate the Akt/mTOR signaling pathway in cancer cells (Shen et al., 2015); this may also possibly apply to the DRG neurons where the decreases in miR-199 lead to up-regulation of the Akt/mTOR pathway thereby promoting 4E-BP1 phosphorylation and TRPV1 protein synthesis. These studies do not exclude the role of NGF in promoting gene transcription in the DRG neurons. In fact, NGF-induced gene transcription occurs at a much later time (8 hours for BDNF, Figure 3), and 24 hours for CGRP (Xu and Hall, 2007). This may pose a more economic pathway that NGF utilizes

the existing mRNA to generate functional products (the proteins) before it initiates the gene transcription process in response to injury (e.g. colitis in the present study).

Sensory hypersensitivity following visceral insult is a highly complex entity that can occur due to hyperexcitability of the primary sensory afferents in the DRG. Aberrant level of neurochemical compounds, especially ion channels, within the DRG neurons are critical components in modulating neuronal excitability. TRPV1 is a nonselective cation channel which provides sensation of scalding heat and pain. TRPV1 activity can be regulated by phosphorylation (activation) and de-phosphorylation (deactivation) and enhanced by elevated production and trafficking to plasma membrane. The PI3K/Akt pathway is implicated to regulate neuronal excitability and is found to be a critical pathway in regulating colitis-induced visceral hypersensitivity. The underlying molecular mechanism is largely unknown. The present study demonstrates that activation of the PI3K/Akt pathway in colonic afferent neurons regulates visceral hypersensitivity through regulating TRPV1 protein synthesis, independent of mRNA transcription, providing a novel mechanisms underlying sensory sensitization.

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Highlights

- TRPV1 protein, not mRNA, up-regulation in DRG is associated with colonic hypersensitivity.
- The PI3K/Akt pathway mediates colitis- and NGF-induced TRPV1 protein up-regulation.
- PI3K/Akt activation up-regulates the protein synthesis pathway contributing to TRPV1 protein up-regulation in vitro during NGF treatment and in vivo during colitis.

Figure 1. Up-regulation of TRPV1 protein levels in colonic afferent neurons during colitis
 The expression profile of TRPV1 (A and D, green cells indicated by yellow and white arrows) was examined in the FB-labeled colonic afferent neurons (B and E, blue cells indicated by white and yellow arrows) before (A–C) and during colitis (D–F). The percentage of colonic afferent neurons that expressed TRPV1 was calculated and shown in histograms G and H for L1 and S1 DRG, respectively. Western blot (I) analysis also showed that colitis increased the level of TRPV1 protein production in the DRG (L1 and S1 combined). Three or four animals of each group were analyzed. Nonparametric oneway

ANOVA was used for analysis. *, $p < 0.05$ vs control. Calibration bar = 80 μm . The sample microphotographs were from the L1 DRG.

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Figure 2. TRPV1 mRNA production was not increased in the colonic afferent neurons during colitis

Real-time PCR analysis showed that TRPV1 mRNA levels were not changed at either 3 days or 7 days post colitis induction in colonic afferent neurons (A), L1 whole DRG (B), and S1 whole DRG (C). Three or four animals of each group were analyzed. Nonparametric one-way ANOVA was used for analysis.

Figure 3. NGF treatment increased TRPV1 protein expression, but not mRNA, in the DRG explants, which was mediated by the PI3K/Akt pathway

Pair-matched DRG explants were incubated in the absence or present of NGF for designated time points to examine the level of TRPV1 protein (A), TRPV1 mRNA (C), BDNF mRNA (D), or the level of phospho-Akt (E). Treatment with NGF increased the expression level of TRPV1 protein (B: n=3; *, p<0.05 vs control (Con)), BDNF mRNA (D: n=4, *, p<0.05 vs control (Con)), as well as the activity of Akt (E: n=5; *, p<0.05 vs control). Treatment with NGF did not increase the level of TRPV1 mRNA (C: n=4). For inhibition test, pair-matched DRG explants were pre-incubated with LY294002 or vehicle, respectively, for 30 min, and

then were treated by NGF for 8 h (F). Inhibition of the PI3K/Akt pathway reduced NGF-elicited TRPV1 protein expression (F: n=4; *, $p<0.05$ vs NGF treatment alone). Nonparametric one-way ANOVA was used for analysis of results shown in A-D; student's t test was used for analysis of results shown in E-F.

Figure 4. Colitis-increased TRPV1 protein expression was mediated by the PI3K/Akt pathway Western blot (A) showed that colitis increased the expression level of TRPV1 in the DRG (sample are L1 and S1 combined). Inhibition of the PI3K activity with LY294002 (LY) blocked the up-regulation of TRPV1 caused by colitis (A and B). *, $p < 0.05$ vs vehicle control; #, $p < 0.05$ vs TNBS colitis. Data were from three animals for each group.

Figure 5. Co-localization of TrkA with phospho-Akt, and phospho-Akt with TRPV1 in DRG at 7 days of colitis

Double staining of the DRG sections from rats with 7 days of colitis revealed co-localization of TrkA with phospho-Akt (A-C, arrows), and co-localization of phospho-Akt with TRPV1 (D-F, arrows). Representative sections were S1 DRG from colitic animals. The number of cells that co-expressed two molecules of interest in each S1 section was counted and analyzed which was shown in G-H (n=3, *, p<0.05 vs control, student's t test was used for statistical analysis). Calibration bar = 20 μ m.

Figure 6. Up-regulation of 4E-BP1 phosphorylation by NGF and colitis

Treatment of DRG explants with NGF (30 min) increased the phosphorylation level of 4E-BP1 when compared to the non-treated contralateral control (A, B: $n=4$, *, $p < 0.05$ vs control). NGF-increased 4E-BP1 phosphorylation was inhibited by pre-treatment with LY294002 (A, C: $n=3$; #, $p < 0.05$ vs NGF+vehicle). TNBS colitis also increased the phosphorylation level of 4E-BP1 in the DRG when compared to vehicle control, which was inhibited by LY294002 treatment (D, E: $n=3$). Samples are L1 and S1 combined for western blot for in vivo studies.

Figure 7. Desensitization of TRPV1 reduced colitis-induced visceral hypersensitivity

Visceral nociception in response to von Frey filaments (A) and CRD (B) was tested in control, colitis, colitis plus RTX (i.p.), and colitis plus RTX (i.c.) treatment. Colitis enhanced visceral sensitivity detected by both methods. Desensitization of TRPV1 inhibited colitis-initiated visceral pain (A, B: n=4–6 for each group. *, p<0.05 vs control; #, p<0.05 vs TNBS colitis). The same animal was tested for both assessments. Von Frey test was conducted prior to CRD in the same animal. The effects of RTX on TRPV1 expression in the DRG were examined by western blot (C, D: n=3, *, p<0.05 vs control; #, p<0.05 vs TNBS colitis).

Figure 8. The PI3K/Akt pathway participated in colitis-induced visceral hypersensitivity
Visceral nociception in response to von Frey filaments (A) and CRD (B) was tested in control, colitis and colitis plus LY294002 treatment. Colitis enhanced visceral sensitivity detected by both methods. Suppression of the PI3K/Akt pathway inhibited colitis-initiated visceral pain. *, $p < 0.05$ vs control; #, $p < 0.05$ vs TNBS colitis. Six animals per group were tested. The same animal was tested for both assessments. Von Frey test was conducted prior to CRD in the same animal.

Figure 9. Colitis increased the immunoreactivity of phospho (p)-Akt in colonic afferent neurons
 The expression profile of p-Akt (A and D, red cells indicated by yellow and blue arrows) was examined in the FB-labeled colonic afferent neurons (B and E, blue cells indicated by white and yellow arrows) before (A–C) and during colitis (D–F). The merged photograph (C and F) showed co-localization of p-Akt and FB-labeled neurons in DRG (purple cells indicated by yellow arrows). The sample microphotographs were from L1 DRG. Histograms G and H showed the percentage of colonic afferent neurons expressing p-Akt in L1 (G) and

S1 (H) DRGs. *, $p < 0.05$. Five animals of each group were analyzed. Calibration bar = 80 μm .

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