INVOLVEMENTS OF TRP CHANNELS AND OXIDATIVE STRESS IN PAIN

EDITED BY: Jose A. Pariente, Cristina Carrasco, Mustafa Naziroglu and Laszlo Pecze

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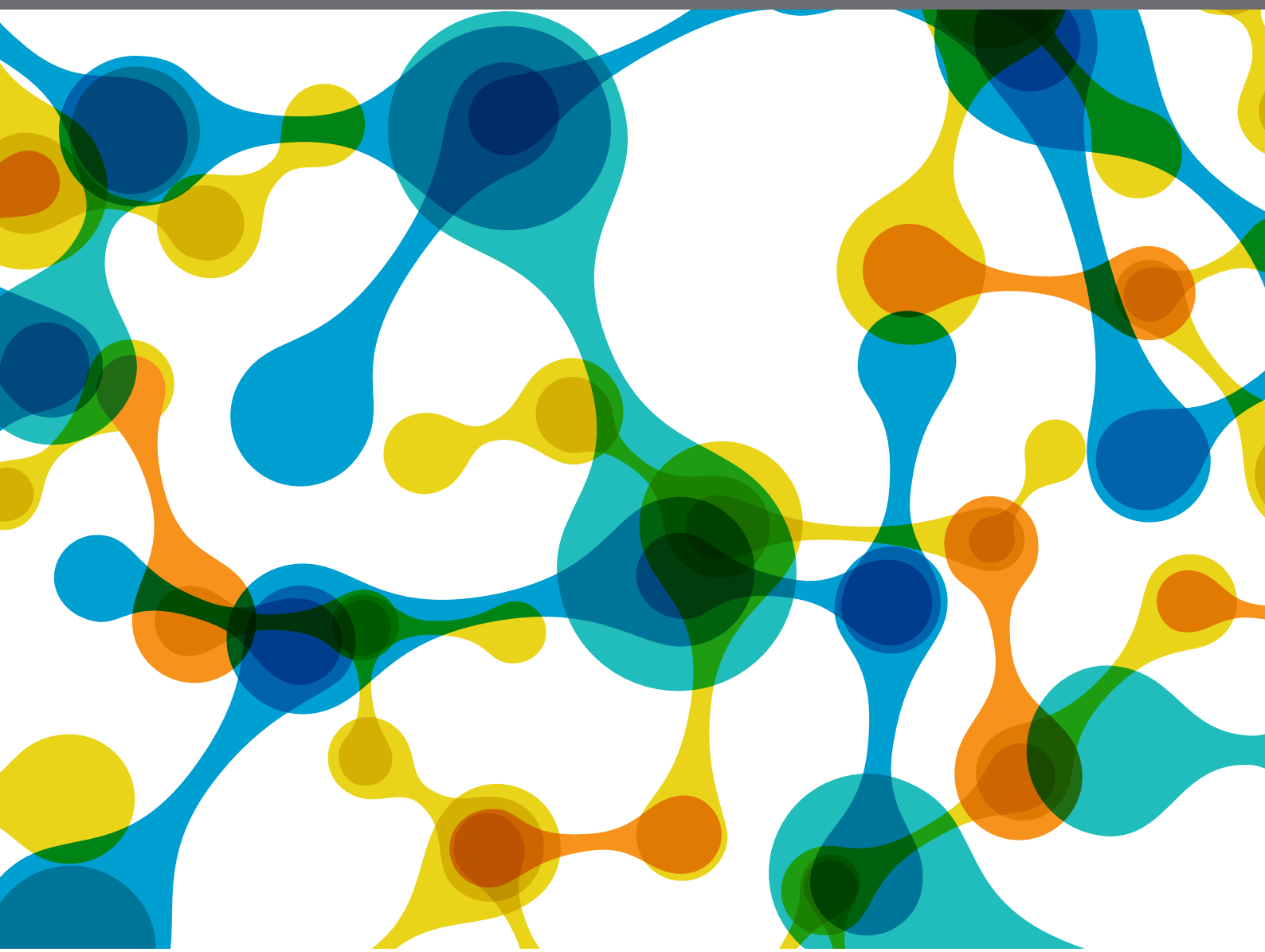

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INVOLVEMENTS OF TRP CHANNELS AND OXIDATIVE STRESS IN PAIN

Topic Editors:
Jose A. Pariente, University of Extremadura, Spain
Cristina Carrasco, University of Extremadura, Spain
Mustafa Naziroglu, Suleyman Demirel University, Turkey
Laszlo Pecze, University of Fribourg, Switzerland

Undoubtedly, pain conditions the quality of life of millions of people worldwide suffering a wide range of diseases. Major research efforts are being made by the international scientific community to determine the mechanisms underlying nociception. Growing evidence points out a complex network including oxidative and nitrosative stress, inflammatory response and cation signaling. In this sense, transient receptor potential (TRP) channels have attracted researchers' attention. Expression levels are very different in tissues and cells mediating a myriad of processes in our organism. At the neurological level, it has been observed that the expression levels of four TRP channels (TRPA1, TRPM2, TRPV1, and TRPV4) are high in neurons related to nociception, including dorsal root ganglion and trigeminal ganglia neurons.

For this reason, this research field promises to shed light on this intricated matrix linking oxidative stress, calcium signaling (via TRP channels), and inflammatory signals in different pain modalities, including neuropathic pain and chemotherapy-induced peripheral pain. In such a way, all this intense research activity will enable us to design individual and rational treatment strategies for pain relief, such as the use of molecular neurosurgery.

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EDITORIAL
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Editorial: Involvements of TRP Channels and Oxidative Stress in Pain

Cristina Carrasco[1], *Mustafa Naziroglu*[2], *Laszlo Pecze*[3] *and José A. Pariente*[1]*

[1] Department of Physiology, Faculty of Sciences, University of Extremadura, Badajoz, Spain, [2] Neuroscience Research Center, Suleyman Demirel University, Isparta, Turkey, [3] Department of Medicine, Faculty of Sciences, University of Fribourg, Fribourg, Switzerland

Keywords: TRP channels, oxidative stress, pain, nervous system, nociception

Editorial on the Research Topic

Involvements of TRP Channels and Oxidative Stress in Pain

Defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, pain impairs the quality of life of millions of people worldwide suffering from a wide range of diseases. Thus, efficient pain relief is a socioeconomic priority at the present time. Significant research efforts are being made by the international scientific community to determine the mechanisms underlying nociception, that is, the transmission and integration of noxious stimuli by our nervous system. Growing evidence points to a complex network including oxidative and nitrosative stress, inflammatory response and cation signaling. In this sense, transient receptor potential (TRP) channels have attracted researchers' attention. Discovered in the photoreceptor cells of Drosophila flies, TRP superfamily is presented in different species and grouped into seven subfamilies: TRPA, TRPC, TRPM, TRPML, TRPP, TRPV, and TPRN. The activation of these channels depends on a number of different physical or chemical stimuli. In mammals, 28 isoforms have been identified. Expression levels are very different in tissues and cells, mediating a myriad of processes in our organism, such as sensory physiology. Last years, it has been observed that the expression levels of four TRP channels (TRPA1, TRPM2, TRPV1, and TRPV4) are high in neurons related to nociception, including dorsal root ganglia (DRG) and trigeminal ganglia neurons. In the next decades, all this intense research on the involvements of TRP channels and oxidative stress in pain could provide essential information for the design of individual and rational treatment strategies for pain relief.

As an introduction to this interesting topic, Jardín et al. propose an update review focused on the function of TRPs in the transduction of noxious sensation, especially TRPV1 and TRPA1, and the current knowledge about differential expression and sensitivity in mammals. Moreover, as many researchers have pointed out, there is a functional link between these two channels. It has been observed that some TRPV1-positive neurons co-express the TRPA1 channel; in addition, its activities are closely modulated by TRPV1 channel. In this sense, the research published by Masuoka et al. in this special issue describes that the activities of TRPV1 channel are also modulated by the presence of TRPA1 channel in primary sensory neurons. Thus, this kind of bidirectional link between specific TRP channels activities is another interesting issue which should be further investigated in the future. Finally, we must not forget that research in other species can shed light on intramolecular mechanisms underlying the functioning of TRP channels. For that reason, Kühn et al. offer us a fascinating vision of the TRPM2 channel in both humans and sea anemone *Nematostella vectensis*. As authors reflect, this particular chanzyme co-activated by intracelullar ADP-ribose and Ca^{2+} has evolved from one gene in a strikingly divergent manner but also has gained analogous functional properties in both species.

Edited by:
Ali Mobasheri,
University of Surrey, United Kingdom

Reviewed by:
Ana Gomis,
Consejo Superior de Investigaciones Científicas (CSIC), Spain

***Correspondence:**
José A. Pariente
pariente@unex.es

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Turning to mammals, a growing number of evidences suggest that oxidative stress and TRP channels are involved in noxious sensation. Firstly, different types of pain must be distinguished, such as neuropathic pain (NP). As discussed by Carrasco et al., the incidence of this condition may increase in next years and become to a worldwide public health problem. Current knowledge points out mitochondrial dysfunction induced by nitro-oxidative stress, inflammatory signals, and the overload in intracellular calcium ion as possible underlying mechanisms. Among other chronic diseases, NP is present in cancer patients following treatment with chemotherapeutic agents, mainly oxaliplatin, what is known as chemotherapy-induced peripheral pain (CIPP). Naziroglu and Braidy summarize the scientific evidence related to five temperature-regulated TRP channels (TRPA1, TRPM8, TRPV1, TRPV2, and TRPV4) as novel targets for treating this hardly bearable condition that may persist from months to years following cessation of treatment. Focusing on CIPP caused by oxalipatin, this special issue also contains an experimental research performed by Miyake et al. This interesting study clarifies how this noxious chemotherapeutic agent can act differently on TRPA1 channel, depending on the dose. In addition, results showed that ROS-mediated TRPA1 activation may be a common mechanism in the CIPP caused by low and high oxaliplatin concentrations. Likewise, Uslusoy et al. have observed that increased mitochondrial ROS levels, as well as excessive Ca^{2+} entry and apoptosis, are involved in NP caused by sciatic nerve injury (SNI). As authors demonstrated, this condition can be experimentally alleviated in rats by *Hypericum perforatum* treatment. Thus, the vegetal extract seems to act through inhibition of TRPM2 and TRPV1 channels in sciatic nerve and DRG neurons of SNI-induced rats. Furthermore, the Research Topic also collects experimental works about other pain modalities. The study performed by Sandoval et al. showed the interconnection between Cdk5 activation and ROS production by NOX1 and NOX2/NADPH oxidase complexes during inflammatory pain. Moreover, oxidative stress has been observed to be involved in urinary bladder disorders, particularly, mediating the activation of TRPA1. Although this channel seems to be responsible for urinary bladder abnormalities and hyperalgesia in acute cystitis, its implication in chronic bladder inflammation is less clear. In their work, Oyama et al. provide evidence supporting that, as suspected, TRPA1 might not play a major role in the physiopathology of long-lasting cystitis.

In summary, this research field promises to shed light on this intricated matrix linking oxidative stress, calcium signaling (via TRP channels) and inflammatory signals in different pain modalities. In such a way, all this intense research activity will enable us to design individual and rational treatment strategies for pain relief. In fact, molecular neurosurgery appears as one the future medical possibilities. As explained by Pecze et al., some molecular agents can be used as nano-surgery scalpels, which selectively would remove neurons responsible for noxious sensation. Thus, resiniferatoxin has recently entered to phase II clinical trial, demonstrating its safety and efficay in the removal of specific TRPV1+ inflammatory pain-sensing neurons. This new medical concept could revolutionize not only the treatment of morphine-insensitive pain conditions but also the cancer research.

In this research topic issue, 61 authors contributed 10 articles (6 original research and 4 reviews) from different countries in the world (Spain, Turkey, Chile, USA, Australia, Japan, Germany, Switzerland, and Hungary). This multidisciplinary approach (Physiology, Neuroscience, Geroscience, Pharmacology, Chemistry, Medicine, and Biophysics) gives the special issue to a comprehensive analytical insight, discussing current understanding and offering new experimental data about the involvements of TRP channels and oxidative stress in pain. We hope this compilation provides a knowledge base for future researchers of this promising research area.

AUTHOR CONTRIBUTIONS

CC wrote the editorial. MN, LP, and JP critically revised the work and approved its version to be submitted.

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REVIEW
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TRPs in Pain Sensation

Isaac Jardín[1], José J. López[1], Raquel Diez[1], José Sánchez-Collado[1], Carlos Cantonero[1], Letizia Albarrán[1], Geoffrey E. Woodard[2], Pedro C. Redondo[1], Ginés M. Salido[1], Tarik Smani[3] and Juan A. Rosado[1]*

[1] Cell Physiology Research Group, Department of Physiology, University of Extremadura, Cáceres, Spain, [2] Department of Surgery, Uniformed Services University of the Health Sciences, Bethesda, MD, United States, [3] Department of Medical Physiology and Biophysics, Institute of Biomedicine of Sevilla, University of Seville, Sevilla, Spain

According to the International Association for the Study of Pain (IASP) pain is characterized as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage". The TRP super-family, compressing up to 28 isoforms in mammals, mediates a myriad of physiological and pathophysiological processes, pain among them. TRP channel might be constituted by similar or different TRP subunits, which will result in the formation of homomeric or heteromeric channels with distinct properties and functions. In this review we will discuss about the function of TRPs in pain, focusing on TRP channles that participate in the transduction of noxious sensation, especially TRPV1 and TRPA1, their expression in nociceptors and their sensitivity to a large number of physical and chemical stimuli.

Keywords: calcium entry, TRPs, TRPA1, TRPV1, noxious sensation

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Edited by:
Laszlo Pecze,
University of Fribourg, Switzerland

Reviewed by:
Carlos Villalobos,
Consejo Superior de Investigaciones Científicas (CSIC), Spain
Javier González-Gallego,
Universidad de León, Spain

****Correspondence:***
José J. López
jjlopez@unex.es

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INTRODUCTION

Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) is a key factor for the regulation of a large variety of cellular functions, ranging from short-term processes, such as muscle contraction, exocytosis, or platelet aggregation, to long-term events, including cell proliferation or apoptosis (Berridge et al., 2000). Physiological agonists modulate $[Ca^{2+}]_c$ through the regulation of a number of Ca^{2+} transport mechanisms, based on the activation of more or less Ca^{2+} selective channels and transporters. Agonist-induced Ca^{2+} mobilization consist, among others, of (1) the release of Ca^{2+} from agonist-sensitive Ca^{2+} stores, mostly the endoplasmic reticulum (ER) and acidic organelles (Lopez et al., 2005; Galione, 2006; Aulestia et al., 2011), (2) extracellular Ca^{2+} entry through plasma membrane permeable channels (Salido et al., 2009a), (3) cytosolic Ca^{2+} clearance either by Ca^{2+} uptake into intracellular stores (Lipskaia et al., 2014) or Ca^{2+} extrusion across the plasma membrane (Redondo et al., 2005), and (4) Ca^{2+} buffering with the participation of the mitochondria (Montero et al., 2001). While Ca^{2+} release from the finite intracellular Ca^{2+} compartments has been reported to regulate different cellular events, Ca^{2+} entry from the extracellular medium is required for the replenishment of the internal stores and also for full activation of different cellular functions. Ca^{2+} entry might occur through a variety of mechanisms, which might be grouped in voltage-operated and receptor-operated Ca^{2+} influx processes. In turn, according to the activation route, receptor-operated Ca^{2+} entry might be classified into receptor-mediated, second messenger-operated and store-operated Ca^{2+} entry mechanisms. The simplest mechanism is receptor-mediated Ca^{2+} influx, which occurs through channels allosterically regulated by agonist binding. Second messenger-operated Ca^{2+} entry requires the generation of a second messenger that directly gates the channel. On the other hand, store-operated Ca^{2+} entry (SOCE) is regulated by the filling state of the intracellular Ca^{2+} stores, mainly the ER (Putney, 1986), but also acidic organelles (Zbidi et al., 2011). According to this, a reduction in

the intraluminal Ca^{2+} concentration results in the opening of channels in the plasma membrane (Putney, 1986).

Among the wide variety of Ca^{2+}-permeable channels identified, TRP channels play a relevant functional role in mammalian cells. TRP channels were identified in a *Drosophila* mutant with visual defects, where a mutation in a channel permeable to Na^+ and Ca^{2+} leads to transient, rather than sustained, receptor potential in the photoreceptors (Minke, 1977). Since the identification of the mammalian homologs of the *Drosophila* TRPs in 1995 (Wes et al., 1995; Zhu et al., 1995) these channels have been proposed as candidates to conduct both second messenger- as well as store-operated Ca^{2+} entry.

TRP channels are a group of ion channels located in the plasma membrane as well as in the membrane of a number of intracellular organelles, where they participate in the homeostasis of intracellular Ca^{2+}, as well as other ions, such as Mg^{2+} (Fleig and Penner, 2004; Ambudkar et al., 2007; Salido et al., 2009b). Since TRP proteins were first described a number of isoforms have been identified, which are grouped into seven subfamilies: TRPC, TRPV, TRPM, TRPP, TRPML, TRPA, and TRPN (the latter only expressed in fish, flies, and worms) and each subfamily includes one or more members (Montell et al., 2002; Li et al., 2011).

The structure of TRP channels comprises six membrane-spanning helices with a pore-forming loop between the last two transmembrane segments. The N- and C-terminal segments are located in the cytosol and vary in the number of amino acids and the functional motifs among the different subfamilies. Thus, the N termini of TRPC, TRPA, TRPV, and TRPN subfamilies contain between 4 and 30 tandem copies of ankyrin repeat domains, involved in protein-protein interaction (Latorre et al., 2009). Furthermore, the cytoplasmic N and/or C-termini of TRPC, TRPM, TRPP, and TRPV channels have been reported to contain coiled coil domains, which play an important role in the assembly of homomeric and heteromeric complexes (Lepage and Boulay, 2007; Schindl and Romanin, 2007) as well as in the interaction with the ER Ca^{2+} sensor STIM1 (Lee et al., 2014). TRPC, TRPV, and TRPM subfamilies also contain a conserved TRP box, a short hydrophobic region located just C-terminal of the putative last transmembrane segment (Nilius et al., 2006). Certain TRP members are regulated by cytosolic Ca^{2+} through the interaction with C-terminal located EF-hand motifs, including TRPA1, TRPML1, and TRPP2 (Tsiokas, 2009), or calmodulin and IP3 receptor-binding regions, this is the case of TRPC, TRPM, and TRPV members (Tang et al., 2001; Dionisio et al., 2011). Finally, other more restricted motifs have been reported in different TRP members, including the tubulin-binding domain reported in TRPV1 (Sardar et al., 2012), the kinase domain of TRPM6 and TRPM7 (Schlingmann and Gudermann, 2005), the conserved proline-rich region, downstream of the EWKFAR motif, responsible for the interaction with Homer proteins and immunophilins (Yuan et al., 2003; Sinkins et al., 2004; Jardin et al., 2013; Lopez et al., 2013; Dionisio et al., 2015) or the voltage sensing domain reported in TRPV1, TRPV3, TRPM8, and TRPM4 (Nilius et al., 2003, 2005), among others.

TRP channels are activated and modulated by a wide variety of chemical and physical stimuli including receptor occupation via activation of phospholipase C, which, in turn, leads to the hydrolysis of phosphatidylinositol 4,5-bisphospate (PIP_2) and the generation of lipid messengers, biosynthesis of IP_3, and subsequent Ca^{2+} release from the intracellular stores, the activation of serine/threonine or tyrosine kinases or ligand binding, including exogenous ligands, such as capsaicin or allyl isothiocyanate, and endogenous molecules, including eicosanoids, diacylglycerol, phosphoinositides, purine nucleotides, or inorganic ions, such as Ca^{2+} and Mg^{2+} (Harteneck et al., 2011; Vetter and Lewis, 2011).

The sensitivity of TRP channels to a number of physical and chemical stimuli allows these channels to be essential components of different sensory processes, such as vision, hearing, taste, tactile and thermal sensation, redox status, or pain (Voets et al., 2005; Woodard et al., 2007; Wetsel, 2011; Feng, 2014; Ogawa et al., 2016).

TRP CHANNELS AND NOCICEPTION

Pain and Nociception

Pain is a subjective unpleasant sensory experience that might be associated to real or potential damage. Noxious stimuli are detected by pain receptors or nociceptors, nerve endings that specifically respond to damaging stimuli and transmit the information to the spinal cord, through which the message is transmitted to higher nerve centers, including the brain stem reticular formation, thalamus, somatosensory cortex, and limbic system (Osterweis et al., 1987). Nociception, therefore, is the process of transmission of noxious signals by nociceptors in the primary afferent nerve fibers (Dai, 2016). Noxious stimuli are classified into chemical, mechanical and thermal. The transduction of nociception includes several chemical compounds that might be released by damaged tissue, such as K^+, histamine and serotonin, or generated by enzymes activated by tissue damage, including prostaglandins, leukotrienes, or bradikinin (Schaible et al., 2011; Viguier et al., 2013).

A major function of the nociceptors is to detect potentially damaging stimuli with a threshold that allows perform activities without pain but sensitive enough to warn of the risk of damage (Patapoutian et al., 2009). The detection of noxious stimuli by nociceptors involves the expression of nociceptive ion channels, which basically define the functional properties of nociceptors. The largest group of nociceptive ion channels is the TRP channel family (Clapham, 2003; Patapoutian et al., 2009), especially TRPV1 and TRPA1 members. Activation of nociceptive TRP ion channels in sensitive (i.e., dorsal root ganglion, DRG) neurons leads to the influx of Na^+ and Ca^{2+} across the plasma membrane resulting in membrane depolarization that, in turn, might trigger voltage-gated ion channel-dependent action potentials (Gees et al., 2010) that transmit the information to the spinal cord and the higher nerve centers as described above.

Nociceptive TRP Ion Channels

TRPV1

TRPV1 is one of the six members of the TRPV subfamily and is involved in the detection of noxious sensation (Caterina and Julius, 2001). TRPV1 has been found to be highly expressed in

the plasma membrane of nociceptive DRG neurons (Caterina and Julius, 2001). Furthermore, functional expression of this channel has also been reported to be expressed in the ER of DRG neurons, where it is involved in Ca^{2+} efflux from the ER upon stimulation with vanilloids; although its sensitivity to agonists is smaller when located in the ER membrane probably due to a mechanism mediated by calmodulin, which might be important for neuronal biology (Gallego-Sandin et al., 2009). The structure of TRPV1 follows the pattern of the TRP channels, with six transmembrane spanning domains, six ankyrin repeats in the N-terminus and a large C-terminal region (Cao et al., 2013; **Figure 1**). Three splice variants of TRPV1 have been described: VR.5'sv, TRPV1b, and TRPV1var. VR.5'sv (vanilloid receptor 5' splice variant) shows a shorter N-terminal region due to both an alternative initiation of translation and the lack of transcription of an exon resulting in loss of 60 amino acids in the N-terminus (Schumacher et al., 2000) and do not respond to capsaicin (Eilers et al., 2007). TRPV1b shows a modification in the N-terminal region encoded by exon 7 that leads to loss of 10 amino acids (Wang et al., 2004) and, as well as the VR.5'sv variant, has been propose to function as a dominant-negative channel subunit (Pecze et al., 2008; Schumacher and Eilers, 2010).

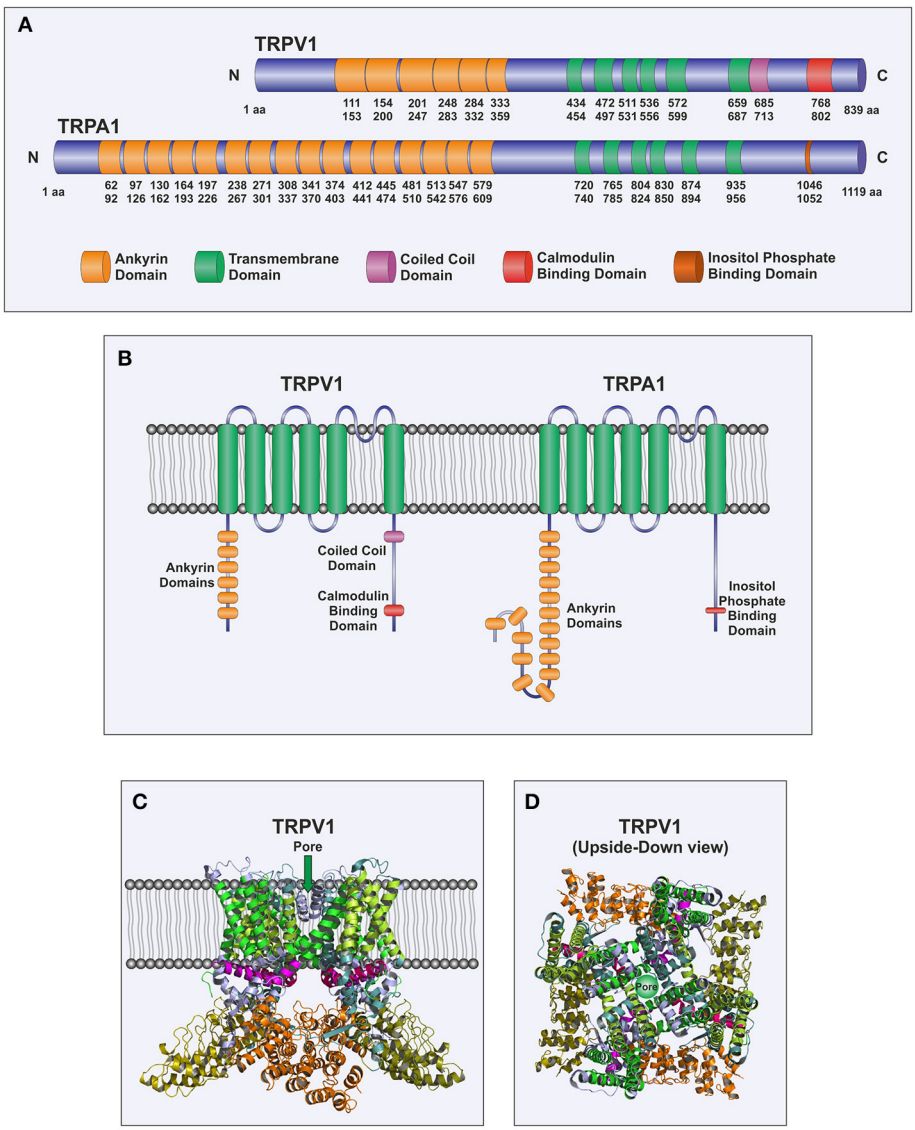

FIGURE 1 | Molecular structure of TRPA1 and TRPV1. **(A)** Scheme of TRPV1 and TRPA1 channels depicting individual domains. Numbers correspond to amino acids positions of human TRPV1 and TRPA1, respectively. **(B)** Cartoon representing TRPV1 and TRPA1 monomers morphology within a bilayer membrane. The channel spans the membrane up to six times with the pore located between transmembrane domains (TM) 5 and 6, and both N-terminal and C-terminal domains situated in the cytosol. **(C,D)** Cartoons depicting the tetrameric assembly of TRPV1 subunits based on the X-ray crystal structure of *Rattus norvegicus* as described in Cao et al. (2013). As mentioned above, the ion permeation pathway is formed by TM5 and TM6, while the remaining TM domains 1–4, surround the pore. **(C)** Represents a frontal view of the channel while **(D)** sketches an upside-down perspective.

TRPV1var is generated by a failure to splice out intron 5, thus leading to translation of a portion of the N-terminal region that lacks the transmembrane spanning domains and the C-terminal intracellular region (Tian et al., 2006). It has been reported that TRPV1var, when coexpressed with the full-length TRPV1 subunits, might modulate its responses, for instance, it has been shown to increase the response of TRPV1 to resiniferatoxin (Tian et al., 2006).

TRPV1 is a polymodal channel sensitive to different physical and chemical stimuli, including heat (see above), pH under 5.9 (Tominaga et al., 1998), and mechanical stimuli (Walker et al., 2003). In addition, TRPV1 is activated by a variety of ligands (**Table 1**) including vanilloids, such as capsaicinoids (the most representative is capsaicin, the major pungent constituent of *Capsicum* fruit; Caterina et al., 1997) and resiniferanoids (Szallasi and Blumberg, 1989), α, β-unsaturated dialdehydes isolated from a variety of plants, fungi, algae, sponges, arthropods, and molluscs (Jonassohn et al., 1995), cannabinoids from *Cannabis sativa* (Bisogno et al., 2001), ginsenosides found in the ginseng *Panax ginseng* (Jung et al., 2001), a number of animal-derived toxins, such as VaTx1, VaTx2, and VaTx3 found in the venom of the tarantula *Psalmopoeus cambridgei* activates TRPV1 channels (Siemens et al., 2006) while other toxins, including agatoxin 489 and agatoxin 505, from the spider *Agelenopsis aperta*, and the analgesic polypeptide HC1, from the see anemona *Heteractis crispa*, elicits TRPV1 inhibition (Kitaguchi and Swartz, 2005; Andreev et al., 2008). Furthermore, a number of endogenous molecules, known as endovanilloids, including leukotriene B4 and 12-S-HPETE and anandamide (a cannabinoid receptor agonist) have been found to be potent activators of TRPV1 channels (Di Marzo et al., 2002; Hermann et al., 2003). On the other hand, alkaloids, such as nicotine (from *Nicotiana tabacum*) or yohimbine (from the bark of the tree *Pausinystalia yohimbe*), phenols like grifolin, neogrifolin, and albaconol (present in the

TABLE 1 | Agonists and antagonists of TRPV1 and TRPA1 channels.

Channel	Agonist	Potency (EC_{50})	Antagonist	Potency (IC_{50})	References
TRPV1	Capsaicin	0.04–1 μM	Agatoxin 489	0.3 μM	Jung et al., 2001; Behrendt et al., 2004; Varga et al., 2005; Rami et al., 2006; Harteneck et al., 2011; Planells-Cases et al., 2011; Vetter and Lewis, 2011; Xia et al., 2011
	Eugenol	1 mM	Agatoxin 505	0.3 μM	
	Resiniferatoxin	39 nM	APHC1	54 nM	
	Polygodial	5 μM	Capsazepine	420 nM	
	Cinnamodial	0.6 μM	Nicotine	1 mM	
	Isovelleral	100 nM	Yohimbine	25 μM	
	Cannabidiol	3 μM	Acetylsalicylic acid	1 μM	
	Ginsenoside Rc	?	Grifolin	26 μM	
	VaTx1	12 μM	Neogrifolin	7 μM	
	VaTx2	3 μM	Albaconol	17 μM	
	VaTx3	0.3 μM	BCTC	35 nM	
	Leukotriene B4	30 μM	AMG-517	32 nM	
	12-S-HPETE	10 μM	SB366791	651 nM	
	Anandamide	30 μM			
TRPA1	Allyl isothiocyanate	1–6.5 μM	Camphor	0.6 mM	Karashima et al., 2007; Trevisani et al., 2007; Cruz-Orengo et al., 2008; Eid et al., 2008; Sculptoreanu et al., 2010; Harteneck et al., 2011; Vetter and Lewis, 2011; Trevisan et al., 2014; Wei et al., 2009; McGaraughty et al., 2010; Brenneis et al., 2011; Sisignano et al., 2012
	Cinnamaldehide	60μM	HC-030031	6.2 μM	
	Methyl salicylate	600 μM	A-967079	67 nM	
	Allicin	7.5 μM	Chembridge-5861528	?	
	Ajoene	0.5 μM			
	Diallyl trisufphide	0.5 μM			
	Hydroxy-α-sanshool	69 μM			
	Acrolein	5 μM			
	Crotonaldehyde	16 μM			
	Δ9 tetra-hydrocannabinol	12 μM			
	Cannabinol	20 μM			
	Hydrogen peroxide	?			
	Nitrooleic acid	?			
	4-hydroxy-2-nonenal	27 μM			
	15-deoxy- Δ12,14-PGJ2	?			
	5,6-EET	?			
	8,9-EET	?			
		?			

mushroom *Albatrellus confluens*) or acetylsalicylic acid have been found to inhibit the activity of TRPV1 channels, for a review see Vetter and Lewis (2011). Endogenous modulators of TRPV1 activity include noradrenaline, which is able to attenuate capsaicin-activated response by ∼60%, a mechanism mediated by activation of α2 adrenergic receptors that has been reported to underlie the inhibition of the incoming noxious stimuli at the dorsal horn of the spinal cord (Chakraborty et al., 2017).

TRPA1

TRPA1, also known as P120 and ANKTM1, is the sole member of the TRPA subfamily. It was first described in human fibroblasts where its expression is lost after oncogenic transformation (Jaquemar et al., 1999). TRPA1 has been found to be expressed in peptidergic nociceptors, as well as in a number of non-neuronal cells, including keratinocytes (Atoyan et al., 2009), megakaryocytes (Albarran et al., 2013) or enterochromaffin cells (Nozawa et al., 2009), and tissues (for a review see Benemei et al., 2014).

In addition to TRPV1, TRPA1 is specialized in the transduction of noxious stimuli in mammals. In fact, a certain degree of interaction between both channels has been reported. TRPV1 is expressed in most TRPA1-expressing neurons and about 30% of TRPV1-expressing sensory neurons also exhibit TRPA1 expression (Story et al., 2003). Furthermore, TRPV1 has been reported to influence several features of the TRPA1 channel, such as voltage–current relationships and open probability (Staruschenko et al., 2010). Further pieces of evidence for the functional interaction between both channels comes from studies reporting that the biophysical properties of TRPA1 are different when TRPA1 is expressed alone or coexpressed with TRPV1 and that the TRPV1 and TRPA1 agonists, capsaicin and mustard oil, are able to induce heterologously desensitization of TRPA1 and TRPV1 via calcineurin-dependent and independent pathways, respectively (Ruparel et al., 2008).

The structure of TRPA1 shows the features of the TRP family and consists of six membrane-spanning domains and a presumed pore-forming region between the fifth and sixth transmembrane domains. Its N- and C-terminal segments are predicted to be located in the cytoplasm (**Figure 1**). In addition, an unusual and characteristic feature of TRPA1 is the presence of a very long N-terminus, which contains at least 16 predicted ankyrin repeat domains (Story et al., 2003; Paulsen et al., 2015). It is the only mammalian TRP channel with such high number of ankyrin repeats, which might provide the protein a certain degree of elasticity, as well as, the ability to interact with other proteins, especially those of the cytoskeleton (Corey et al., 2004; Sotomayor et al., 2005).

TRPA1 is a polymodal ion channel that can be activated by a number of physical and chemical stimuli. Among the physical stimuli, TRPA1 is sensitive to temperature. The 10 thermo-TRP channels identified to date, including TRPV1-4, TRPM2, TRPM4, TRPM5, TRPM8, TRPC5, and TRPA1, are activated by different temperature ranges. The mammalian TRPs activated by heat are TRPV2 (activated at temperatures over 52°C), TRPV1 (sensitive to temperatures over 42°C), TRPV4 (activated by temperatures between 27 and 42°C), TRPV3 (by temperature over 33°C), TRPM2 (sensitive to temperatures between 35 and 42°C), TRPM4 and TRPM5 (sensitive to temperatures between 15 and 35°C). On the other hand, TRPC5 activity is potentiated at temperatures below 30°C, TRPM8 is sensitive to temperatures below 25°C and TRPA1 is activated at temperatures below 17°C (Caterina et al., 1999; Dhaka et al., 2006; Vriens et al., 2014). As TRPV1 has been associated to painful heat, TRPA1 has been reported to be associated to noxious cold sensation (Patapoutian et al., 2003). The thermal sensitivity of TRPA1 is conserved throughout evolution, although the range of temperatures that activates the channel differs among the distinct vertebrates, thus, in reptiles and amphibians TRPA1 is sensitive to heat and, in certain snakes, TRPA1 provides sufficient thermal sensitivity for infrared detection (Poletini et al., 2015; Kang, 2016).

In addition, TRPA1 can be activated by a number of chemical stimuli (**Table 1**), including exogenous compounds, such as isothiocyanates, cinnamaldehyde, and methyl salicylate (the pungent compounds associated to burning sensation present in mustard oil, wasabi, horseradish, cinnamon and wintergreen oil; Bandell et al., 2004), allicin, ajoene, and diallyl sulfides (organosulfur compounds present in garlic; Bautista et al., 2006), acrolein, and crotonaldehyde (present in cigarette smoke; Andre et al., 2008), cannabinoids, such as Δ^9 tetra-hydrocannabinoland cannabinol (Jordt et al., 2004), alkylamides, including hydroxy-α-sanshool (one of the compounds of the Szechuan pepper; Riera et al., 2009; Vetter and Lewis, 2011), or endogenous compounds such as hydrogen peroxide (Trevisan et al., 2014), nitro-oleic acid, a byproduct of nitric oxide (Sculptoreanu et al., 2010), 4-hydroxy-2-nonenal (Trevisani et al., 2007), the cyclopentenone prostaglandin D2 metabolite 15-deoxy- $\Delta^{12,14}$-prostaglandin J_2 (Cruz-Orengo et al., 2008), and different epoxyeicosatrienoic acids (EET), including 5,6-EET (Sisignano et al., 2012) and 8,9-EET (Brenneis et al., 2011). By contrast, a number of TRPA1 antagonists have been identified, including the synthetic HC-030031, its derivative chembridge-5861528 or A-967079, among others (**Table 1**). Most exogenous compounds activate TRPA1 channels by covalent modification of cysteines and lysines in the N-terminus (Hinman et al., 2006; Macpherson et al., 2007; Nilius et al., 2011), although it remains to be determined the mechanism of activation of the channel by certain endogenous compounds.

In addition to TRPV1 and TRPA1, other TRP members have been associated to noxious sensation, including TRPM3, expressed in a number of small-diameter sensory neurons from dorsal root and trigeminal ganglia where it is involved in the nocifensive response to heat (Vriens et al., 2011), TRPV4, presented as an osmo-transducer in primary afferent nociceptive nerve fibers (Alessandri-Haber et al., 2003), TRPC1 and TRPC6, which cooperate with TRPV4 in the mediation of hyperalgesia to mechanical and hypotonic stimuli induced by inflammatory mediators (Alessandri-Haber et al., 2009), TRPV3, a channel sensitive to farnesyl pyrophosphate that is involved in the sensitivity to noxious heat (Bang et al., 2010) TRPM8, involved in cold hyperalgesia and tactile allodynia (Salat and Filipek, 2015), TRPC3, associated to the mediation of store-

and receptor-operated Ca^{2+} entry in DRG neurons (Alkhani et al., 2014), TRPC4, which is required for the detection or transmission of colonic visceral pain sensation, and TRPC5, which, together with TRPC4, is relevant for pain hypersensitivity and neuropathic pain (Westlund et al., 2014; Wei et al., 2015); however, the involvement of these channels in pain detection or transmission has been less characterized than that of TRPV1 or TRPA1.

NOCICEPTIVE TRP CHANNELS AND PAIN PATHOLOGIES

Nociceptive TRP channels have been found to be involved in a number of pain modalities, including inflammatory pain, neuropathic pain, visceral pain, and pain associated to certain pathological conditions, including cancer or migraine (Mickle et al., 2016).

The involvement of TRPV1 in inflammatory pain is the most prominent among the TRP channels. TRPV1 antagonists have been shown to be efficient attenuating thermal hyperalgesia induced under inflammatory conditions and increasing the noxious heat threshold (Tekus et al., 2010) and similar results have been obtained in TRPV1 lacking mice models (Davis et al., 2000). Further evidence supporting the role of TRPV1 in inflammatory pain comes from studies reporting that TRPV1 is essential for the analgesia induced by electroacupuncture in a mouse model of inflammatory pain (Liao et al., 2017). In addition to TRPV1, TRPA1 has been presented as a candidate to mediate inflammatory mechanical hyperalgesia as well as cold hyperalgesia under inflammatory conditions (Eid et al., 2008; Da Costa et al., 2010). Furthermore, TRPA1 has been reported to modulate inflammation and pruritogen responses in allergic contact dermatitis. TRPA1 is involved in skin edema, leukocyte infiltration and antihistamine-resistant scratching in mice treated with oxazolone (Liu et al., 2013).

Neuropathic pain occurs as a result of nerve injury. The role of TRPV1 has been demonstrated in neuropathic pain associated to diabetes or the administration of chemotherapeutics (Bourinet et al., 2014). A more recent study has revealed a high coexpression between TRPV1 and different sensitizing agents, such as PKCε, during the development of neuropathic pain (Malek et al., 2015) and blockade of this mechanism by quercetin has been found to attenuate paclitaxel-induced neuropathic pain (Gao et al., 2016). TRPA1 has also been proposed to mediate mechanical hyperalgesia and allodynia during neuropathic pain in diabetic patients or derived from the administration of chemotherapeutics, probably mediated by the synthesis of reactive oxygen and nitrogen species (Kim and Hwang, 2013; Huang et al., 2017), which are well-known TRPA1 activators (Trevisan et al., 2016). TRPA1 and TRPV1 have also been involved in the development of migraine, which can be activated by a number of TRPA1 agonists (Benemei et al., 2014) and might be attenuated by repeated desensitizing administration of capsaicin to the nasal mucosa (Fusco et al., 2003). Furthermore, ethanol, a well-known trigger of migraine, has been reported to induce TRPV1 activation (Nicoletti et al., 2008). Activation of TRP channels, such as TRPV1 and TRPA1, has been reported to induce the trigeminal calcitonin gene-related peptide pathway, which mediate neurogenic inflammation, thus leading to the migraine attacks (Benemei et al., 2013).

Visceral pain occurs in internal organs and its transduction involves different TRP family members, including TRPV1, TRPA1, and TRPM8. Silencing the expression of TRPV1 by RNAi has been reported to attenuate visceral pain *in vivo* (Christoph et al., 2006). Consistent with this, a more recent study has revealed that decreased expression of miR-199 in irritable bowel syndrome, which results in enhanced expression of TRPV1, leads to increased visceral hypersensitivity (Zhou et al., 2016). On the other hand, the luminal gasotransmitter hydrogen disulphide has been reported to induce colonic pain and hyperalgesia via activation of $Ca_v3.2$ and TRPA1 channels (Tsubota-Matsunami et al., 2012). Furthermore, the TRPA1 agonist ASP7663 has been reported to prevent constipation (a gastrointestinal motility disorder) when administered orally, and induce analgesic abdominal effects when it is intravenously administered (Kojima et al., 2014). Finally, TRPM8 has been found to play a relevant role in overactive bladder and painful bladder syndrome and it has been reported that administration of the TRPM8 channel blocker AMTB is able attenuate this syndrome in rats (Lashinger et al., 2008).

Chronic pain is also a multidimensional complication of cancer or its treatment. The role of TRPV1 in bone cancer pain has been widely investigated. TRPV1 has been found to be associated to bone cancer pain, as demonstrated by pharmacological inactivation of TRPV1 as well as disruption of the TRPV1 gene (Ghilardi et al., 2005). Furthermore, the TRPV1 antagonist SB366791 has been reported to potentiate the analgesic effect of intraperitoneal administration of morphine in a mouse model of bone cancer pain. The expression of TRPV1, as well as the TRPV1-dependent currents, have been found to be enhanced upon the development of bone cancer in DRG neurons. In these cells, capsaicin-mediated currents were potentiated by administration of lysophosphatidic acid through a mechanism dependent on PKCε but independent on PKA and the small GTPase Rho (Pan et al., 2010). Two more recent studes have revealed that the up-regulated expression and function of TRPV1 in bone cancer pain might be attributed to the the presence of tumor tissue-derived endogenous formaldehyde, which enhances TRPV1 expression via mitogen-activated protein kinase and PI3K, but independently on PKC (Han et al., 2012), as well as the regulatory effects of insulin-like growth factor-1 (Li et al., 2014). Finally, JAK/PI3K-dependent TRPV1 up-regulation has been reported to be involved in peripheral sensitization and bone cancer-induced pain evoked by interleukin-6 (Fang et al., 2015). TRPV1 and TRPA1 have also been found to be involved in neuropathic pain due to the administration of chemotherapeutics, including oxaliplatin (Park et al., 2015), 5-fluorouracil (Yamaguchi et al., 2016), or docetaxel (Huang et al., 2017).

Currently, there is a body of studies and clinical trials identifying new antagonists of the nociceptive TRPs and characterizing their effects in the *in situ* attenuation of pain transduction at the nociceptors.

AUTHOR CONTRIBUTIONS

JR, IJ, and JL drafted the manuscript. IJ performed the figure. RD, JS, CC, LA, and TS performed the bibliographic revision. GW, GS, and PR revised the manuscript and performed the final check.

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TRPA1 Channels Modify TRPV1-Mediated Current Responses in Dorsal Root Ganglion Neurons

*Takayoshi Masuoka *, Makiko Kudo, Yuka Yamashita, Junko Yoshida, Noriko Imaizumi, Ikunobu Muramatsu, Matomo Nishio and Takaharu Ishibashi*

Department of Pharmacology, School of Medicine, Kanazawa Medical University, Uchinada, Japan

The transient receptor potential vanilloid 1 (TRPV1) channel is highly expressed in a subset of sensory neurons in the dorsal root ganglia (DRG) and trigeminal ganglia of experimental animals, responsible for nociception. Many researches have revealed that some TRPV1-positive neurons co-express the transient receptor potential ankyrin 1 (TRPA1) channel whose activities are closely modulated by TRPV1 channel. However, it is less investigated whether the activities of TRPV1 channel are modulated by the presence of TRPA1 channel in primary sensory neurons. This study clarified the difference in electrophysiological responses induced by TRPV1 channel activation between TRPA1-positive and TRPA1-negative DRG. TRPV1 and TRPA1 channel activations were evoked by capsaicin (1 μM), a TRPV1 agonist, and allyl isothiocyanate (AITC; 500 μM), a TRPA1 agonist, respectively. Capsaicin perfusion for 15 s caused a large inward current without a desensitization phase at a membrane potential of −70 mV in AITC-insensitive DRG (current density; 29.6 ± 5.6 pA/pF, time constant of decay; 12.8 ± 1.8 s). The capsaicin-induced currents in AITC-sensitive DRG had a small current density (12.7 ± 2.9 pA/pF) with a large time constant of decay (24.3 ± 5.4 s). In calcium imaging with Fura-2, the peak response by capsaicin was small and duration reaching the peak response was long in AITC-sensitive neurons. These electrophysiological differences were completely eliminated by HC-030031, a TRPA1 antagonist, in an extracellular solution or 10 mM EGTA, a Ca^{2+} chelator, in an internal solution. Capsaicin perfusion for 120 s desensitized the inward currents after a transient peak. The decay during capsaicin perfusion was notably slow in AITC-sensitive DRG; ratio of capsaicin-induced current 60 s after the treatment per the peak current in AITC-sensitive neurons (78 ± 9%) was larger than that in AITC-insensitive neurons (48 ± 5%). The capsaicin-induced current in the desensitization phase was attenuated by HC-030031 in AITC-insensitive DRG. These results indicate that (1) TRPV1-mediated currents in TRPA1-positive neurons characterize small current densities with slow decay, which is caused by TRPA1 channel activities and intracellular Ca^{2+} mobilization and (2) desensitization of TRPV1-mediated current in TRPA1-positive neurons is apparently slow, due to appending TRPA1-mediated current.

Keywords: TRPA1, TRPV1, dorsal root ganglion, intracellular calcium ion, membrane current

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Edited by:
Laszlo Pecze,
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Reviewed by:
Mustafa Naziroglu,
Süleyman Demirel University, Turkey
Hai Huang,
Tulane University, USA
Csaba Vizler,
Biological Research Centre (MTA), Hungary

****Correspondence:***
Takayoshi Masuoka
masuoka@kanazawa-med.ac.jp

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INTROUCTION

The transient receptor potential vanilloid 1 (TRPV1) channel is a polymodal sensor, which is sensitive to noxious heat, acidic pH, and irritant vanilloids, and is highly expressed in a subset of sensory neurons in the dorsal root ganglia (DRG) and trigeminal ganglia, predominantly contributing to nociception. The activation of TRPV1 channels, Ca^{2+}-permeable cation channels, induces a large inward current, thereby elevating intracellular Ca^{2+} concentration because of the influx of extracellular Ca^{2+} in primary sensory neurons (Caterina et al., 1997). The depolarization of sensory neurons induced by the TRPV1-mediated current was shown to be transmitted as nociceptive information to the central nervous system (Caterina et al., 2000). The TRPV1-mediated current responses were found to be regulated by various receptors, such as bradykinin receptors (Tang et al., 2004), purinergic receptors (Stanchev et al., 2009), and glutamate receptors (Masuoka et al., 2015; Szteyn et al., 2015), thereby modulating nociception. In contrast, elevated intracellular Ca^{2+} concentration induced by TRPV1 channel activation affects the functions of nociceptors and ion channels, modulating nociception in primary sensory neurons. The Ca^{2+} influx feeds back on the TRPV1 channels, inhibiting their gating by binding to the intracellular Ca^{2+} sensor, calmodulin (Rosenbaum and Simon, 2007; Lau et al., 2012). The Ca^{2+} influx by TRPV1 directly activates anoctamin 1 chloride channels, potentiating TRPV1-mediated pain in DRG neurons (Takayama et al., 2015). TRPV1 channel activation with capsaicin inhibits the mechanosensitive Piezo1 and Piezo2 channels by depleting phosphatidylinositol 4,5-bisphosphate and its precursor phosphatidylinositol 4-phosphate from the plasma membrane through Ca^{2+}-induced phospholipase Cδ activation in DRG neurons (Borbiro et al., 2015).

Some TRPV1-positive DRG neurons co-express the transient receptor potential ankyrin 1 (TRPA1) channel. Recent studies have revealed the functional and mechanical interactions between TRPA1 and TRPV1 channels, although the physiological significance of TRPA1 channels has been poorly understood in TRPV1-expressing sensory neurons. The first report showing the TRPA1-TRPV1 interaction described that the pharmacological desensitization of TRPA1-mediated responses is more pronounced in sensory neurons that lack TRPV1 than in neurons that express TRPV1 (Akopian et al., 2007). Thereafter, the probability of TRPA1 being open at negative holding potentials is reduced by TRPV1 channels because of the complex formation of TRPV1 and TRPA1 channels (Staruschenko et al., 2010). Tmem100 protein was recently reported to decrease the interaction between TRPV1 and TRPA1 channels in DRG neurons, potentiating TRPA1 channel properties (Weng et al., 2015). Therefore, TRPA1 channel-mediated responses are largely modulated by TRPV1 channels in primary sensory neurons. However, studies have scarcely investigated whether TRPV1 channel activities are modulated in the presence of TRPA1 channels. Here, we examined the differences in the kinetics of current responses induced by TRPV1 channel activation in TRPA1-positive and TRPA1-negative DRG neurons to elucidate the modulating effect of TRPA1 channels on TRPV1 channel activities.

MATERIALS AND METHODS

Animals

All mice were purchased from SLC (Shizuoka, Japan). The mice were housed in clear acrylic cages in a temperature-controlled room (25 ± 1°C) with a 12-h light/dark cycle (lights on from 07:00 to 19:00). All animal procedures were approved by the Ethics Committee of Kanazawa Medical University. The mice were humanely treated, according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Guiding Principles for the Care and Use of Laboratory Animals set by the Japanese Pharmacological Society.

Preparation of Primary Cultures

Culture preparation was conducted as previously described (Masuoka et al., 2015, 2016). The C57BL/6J male and female mice (6- to 14-day-old) were anesthetized by the inhalation of isoflurane (Escain®; Mylan Inc., Cecil Township, PA, USA). DRG were rapidly dissected in ice-cold Ca^{2+}/Mg^{2+}-free artificial cerebrospinal fluid (Ca^{2+}/Mg^{2+}-free ACSF; 143.9 mM NaCl, 3.35 mM KCl, 21 mM $NaHCO_3$, 9.9 mM glucose, 0.6 mM NaH_2PO_4) gassed with a mixture of 95% O_2 and 5% CO_2 (pH 7.4). Neurons were dissociated following treatment with 0.1% type II collagenase (240–265 U/mg; Worthington Biochemical Co., Lakewood, NJ, USA), 0.1% trypsin (Gibco, San Diego, CA, USA), and 0.01% DNase I (Sigma, St. Louis, MO, USA) in Ca^{2+}/Mg^{2+}-free ACSF and shaken (35 cycle/min) in a water bath at 37°C for 30 min. Cells were gently triturated in Dulbecco's modified Eagle medium (Sigma) containing 10% horse serum (Gibco), 5% fetal calf serum (Gibco), and 1% penicillin–streptomycin (Wako, Osaka, Japan). Dispersed cells were passed through a 100-μm cell strainer (BD Biosciences, San Jose, CA, USA), and the filtered cells were seeded on glass coverslips (13 mm in diameter) coated with poly-L-lysine (Matsunami Glass Ind., Osaka, Japan).

Whole-Cell Patch Clamp Recording

Cultured neurons were plated onto coverslips, transferred to the recording chamber, and superfused with ACSF (138.6 mM NaCl, 3.35 mM KCl, 21 mM $NaHCO_3$, 9.9 mM glucose, 0.6 mM NaH_2PO_4, 2.5 mM $CaCl_2$, and 1 mM $MgCl_2$). Neurons were visually identified using a 60 × microscope objective (DIAPHOT300; Nikon, Tokyo, Japan). Whole-cell recording were performed from small and medium size of neurons (<25 μm diameter). Pipettes for whole-cell recordings were made from borosilicate glass capillaries (1.5-mm outer diameter; World Precision Instruments Inc., Sarasota, FL, USA). Patch pipettes (4–6 MΩ) were filled with an internal solution containing 120 mM KCH_3SO_3, 5 mM KCl, 0.1 mM K-ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 5 mM Na-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 3 mM Mg-adenosine triphosphate, and 0.4 mM Na-guanosine triphosphate (pH 7.4). Series resistance was 8–20 MΩ, which was

monitored throughout the recording. Membrane currents were recorded in a whole-cell configuration using an Axopatch-1D amplifier and pCLAMP 10 software (Axon Instruments, Foster City, CA, USA), digitized, and stored on a computer disk for offline analysis. Current responses mediated by TRPV1 and TRPA1 were induced through 0.03–1 μM capsaicin (a TRPV1 agonist) perfusion for 15 or 120 s and 500 μM AITC (a TRPA1 agonist) perfusion for 30 s, respectively. Capsaicin responses were recorded >5 min after establishing whole-cell configuration. AITC-induced responses were observed >5 min after capsaicin perfusion. The extracellular solution was perfused at 2 mL/min. To clarify contribution of TRPA1 channels in capsaicin-induced current, HC-030031 (a TRPA1 antagonist) was dissolved in ACSF with or without capsaicin and was perfused. Temperature in recording chamber was maintained at 30.0 ± 1.0°C with in-line solution heaters (SF-28; Warner Instruments, Hamden, CT, USA) and temperature controller (TC-324C; Warner Instruments).

Calcium Imaging

Changes in intracellular calcium were measured with a fluorescent calcium indicator, as described previously (Masuoka et al., 2015, 2016). For microscopic fluorometric measurement, cultured DRG neuronal cells were washed twice with ACSF and incubated for 45 min in the CO_2 incubator (37 ± 2°C) in a solution of 3 μM of Fura-2-acetoxymethyl ester (Fura-2 AM; Dojindo Laboratories, Kumamoto, Japan) and 0.005% Cremophor EL (Sigma). After incubation, cells were washed in ACSF for 30 min and culture dishes were placed on the stage of an inverted microscope (ECLIPSE TE 300, Nikon, Tokyo, Japan) equipped with a 20 × S-fluor objective. Fluorescence images were recorded and analyzed using a video image analysis system (ARGUS/HiSCA, Hamamatsu Photonics, Hamamatsu, Japan). Experimental agents were dissolved in ACSF and delivered by continuous perfusion in the recording chamber (2 mL/min) with a peristaltic pump. Capsaicin (1 mμM), AITC (500 μM) and KCl (50 mM) were respectively perfused for 15, 30, and 30 s in this order. Image pairs were captured at 5 s (beginning 5 min) or 10 s intervals. Fura-2 fluorescence was recorded at an emission wavelength of 510 nm by exciting Fura-2 at 340 and 380 nm. The 340–380 nm fluorescence ratio (F340/F380) was used as a parameter of intracellular calcium concentration.

Drugs

Capsaicin and allyl isothiocyanate (AITC) were obtained from Sigma-Aldrich (St. Louis, MO, USA); 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamide (HC-030031) was obtained from Abcam (Cambridge, United Kingdom). The stock solution of capsaicin and AITC were dissolved in ethanol at the concentration of 10 mM and 2 M, respectively. HC-030031 was dissolved in DMSO at 100 mM. The stock solutions were diluted with ACSF just before the experiment. The working solutions were made not exceeding 0.03% ethanol, because 0.1–3% ethanol facilitates TRPV1 channels activity induced by capsaicin (Trevisani et al., 2002).

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM), and "n" represents the number of cells examined. Data were analyzed using the SigmaPlot 13.0 software (Systat Software Inc., San Jose, CA, USA). Data were assessed using the unpaired *t*-test. A P value of < 0.05 was considered significant.

RESULTS

We clarified the difference in kinetics of current responses to TRPV1 channel activation between TRPA1-negative and TRPA1-positive DRG neurons using whole-cell recording. TRPV1 channel activation was evoked by the perfusion of 1 μM capsaicin, a TRPV1 agonist. To identify TRPA1-positive and TRPA1-negative neurons, AITC (a TRPA1 agonist) were perfused after recording of capsaicin responses in this study. Some studies clarified that high concentration (1 mM and over) of AITC activates porcine, mouse and human TRPV1 as well as TRPA1 (Ohta et al., 2007; Everaerts et al., 2011; Gees et al., 2013). Therefore, TRPA1-negative and TRPA1-positive DRG neurons were distinguished by responsiveness to perfusion of 500 μM AITC for 30 s, >5 min after capsaicin perfusion. In this condition, peak current were observed at 15–25 s after starting AITC perfusion (**Figure 1B**), which were completely blocked by 10 μM HC-030031, a TRPA1 antagonist. A few recording showed very small currents (<100 pA) without peak response before washout of AITC; they were probably TRPV1-mediated current by AITC. In this study, we excluded these data to accurately identify TRPA-positive and TRPA1-negative neurons. Capsaicin perfusion for 15 s caused a large inward current without a desensitization phase at a membrane potential of −70 mV (**Figures 1A,B**). The capsaicin-induced currents in AITC-sensitive DRG neurons had a small current density (**Figure 1C**) with a large time constant of decay (**Figure 1D**). Membrane capacitance in recording neurons demonstrated no significant difference between the two groups (**Figure 1E**). The capsaicin-induced current density increased in a concentration-dependent manner (**Figure 1F**). The maximum response was significantly smaller in AITC-sensitive DRG neurons (14.9 ± 4.7 pA/pF) than in AITC-insensitive ones (43.8 ± 4.3 pA/pF); furthermore, EC_{50} showed no discernible difference between the two groups (0.088 ± 0.008 and 0.104 ± 0.024 μM, respectively). Fluorescence calcium imaging is useful to analyze TRPV1 channels activities, because TRPV1 channels are high-conductance Ca^{2+}-permeable TRP channels. Intracellular calcium mobilizations by capsaicin were examined with Fura-2 (**Figure 2**). Perfusion of 1 μM capsaicin increased the intracellular calcium concentration in a subset of DRG neurons. Elevations of intracellular calcium concentration in AITC-sensitive neurons were significantly smaller than that in AITC-insensitive neurons (**Figures 2B,C**). In addition, duration reaching peak responses after starting the perfusion was significantly longer in AITC-sensitive neurons (**Figures 2B,D**). These results confirmed that small current densities and slow decay of capsaicin-induced current in AITC-sensitive neurons. We examined the possible contributions of TRPA1

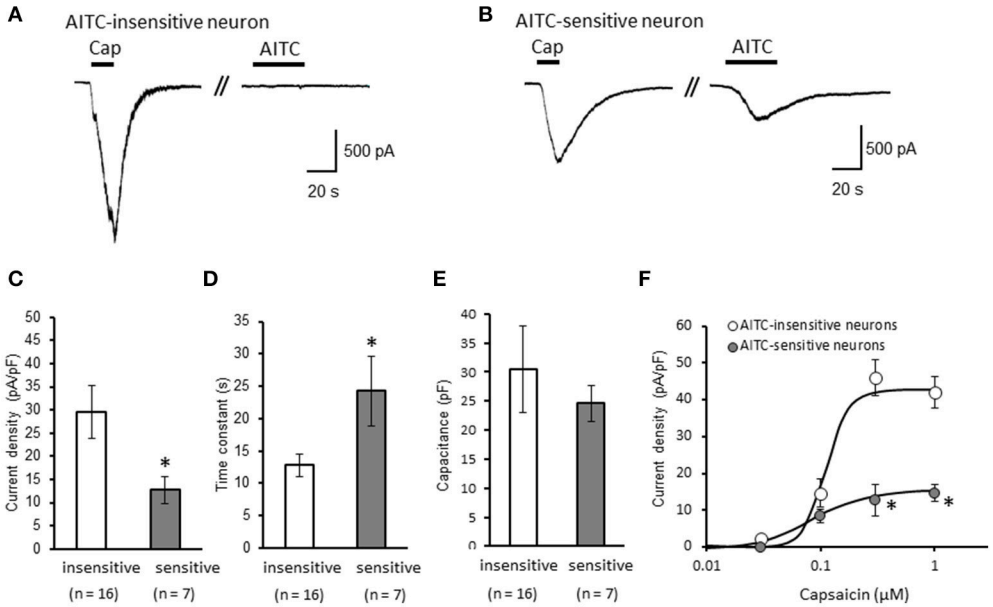

FIGURE 1 | Differences in capsaicin-induced current between AITC-insensitive and AITC-sensitive DRG neurons. (A,B) Representative traces of current response induced by 1 μM capsaicin (cap) perfusion for 15 s and 500 μM AITC perfusion for 30 s in AITC-insensitive **(A)** and AITC-sensitive **(B)** DRG neurons. The currents were recorded with a potassium methanesulfonate internal solution at membrane potentials of −70 mV. **(C–E)** The differences in the capsaicin-induced current density **(C)**, time constant of decay **(D)**, and membrane capacitance **(E)** between AITC-insensitive DRG neurons (open columns) and AITC-sensitive DRG neurons (gray columns) are shown. **(F)** Concentration–response curves to capsaicin in AITC-insensitive and AITC-sensitive DRG neurons. Each column and vertical bar represent the mean ± standard error of the mean (SEM). *$P < 0.05$ by unpaired *t*-test.

channels, intracellular Ca^{2+} concentration, and potassium channels to the difference in kinetics of TRPV1-mediated current between AITC-sensitive DRG neurons and AITC-insensitive ones (**Figures 1C,D**). Capsaicin responses under presence of HC-030031 were examined more than 5 min after perfusion of HC-030031. The differences in current density and time constant of decay were completely eliminated in the presence of 5 μM HC-030031, a TRPA1 antagonist, in the extracellular solution (**Figures 3A,B**). To explore the contributions of intracellular Ca^{2+} to the different kinetics of capsaicin-induced currents between AITC-insensitive and AITC-sensitive DRG neurons, we recorded the TRPV1-mediated current induced by capsaicin with intracellular solutions containing 10 mM EGTA, a Ca^{2+} chelator. The differences in the current density and time constant of decay (**Figures 1C,D**) completely disappeared when a high EGTA concentration was added in the internal solution (**Figures 3C,D**). In contrast, although potassium in the internal solution was replaced by Cs^{+} to block the potassium channels, TRPV1-mediated currents in AITC-sensitive DRG neurons were significantly smaller with a larger time constant of decay than those in AITC-insensitive DRG neurons (**Figures 3E,F**). Presence of 10 μM capsazepine, a TRPV1 antagonist, strongly inhibited capsaicin-induced current in both AITC-insensitive and AITC-sensitive neurons, which has no difference between two groups (**Figure 3G**). These results suggest that the different kinetics of capsaicin-induced current between AITC-insensitive and AITC-sensitive DRG neurons are caused by TRPA1 channels activities and the dynamics of intracellular Ca^{2+} concentration.

To elucidate the presence of TRPA1 current in the basal condition in AITC-sensitive neurons, the effect of HC-030031 was examined before the application of capsaicin and AITC. The perfusion of HC-030031 (5 μM) for 60 s slightly but significantly attenuated the basal inward current in capsaicin- and AITC-sensitive neurons (-17.3 ± 4.4 pA, $n = 5$, $P < 0.05$), although it had no effect in capsaicin-sensitive and AITC-insensitive neurons (2.0 ± 2.7 pA, $n = 13$). This implied that a tonic TRPA1 current exists in AITC-sensitive neurons in the absence of exogenous TRPV1 and TRPA1 agonists.

Next, we examined the difference in the desensitization of current induced by TRPV1 channel activation between AITC-sensitive and AITC-insensitive DRG neurons. When 1 μM capsaicin was perfused for 120 s (**Figure 4A**), the transient peak current was observed 15–30 s after the perfusion in all neurons, which then gradually desensitized the inward currents. As the decay of the capsaicin-induced current was difficult to fit with a logarithmic curve in many AITC-sensitive DRG neurons, we calculated the ratio of current 60 s after treatment of capsaicin per peak current as an alternative measure. The ratio of the current was significantly larger in AITC-sensitive DRG neurons than in AITC-insensitive DRG neurons, indicating that the decay of the capsaicin-induced current in the desensitization phase was significantly slower in TRPA1-positive DRG neurons than in TRPA1-negative DRG neurons (**Figure 4B**).The effects of HC-030031 were subsequently examined. HC-030031 perfusion significantly inhibited capsaicin-induced currents in AITC-sensitive DRG neurons, which was absent in AITC-insensitive

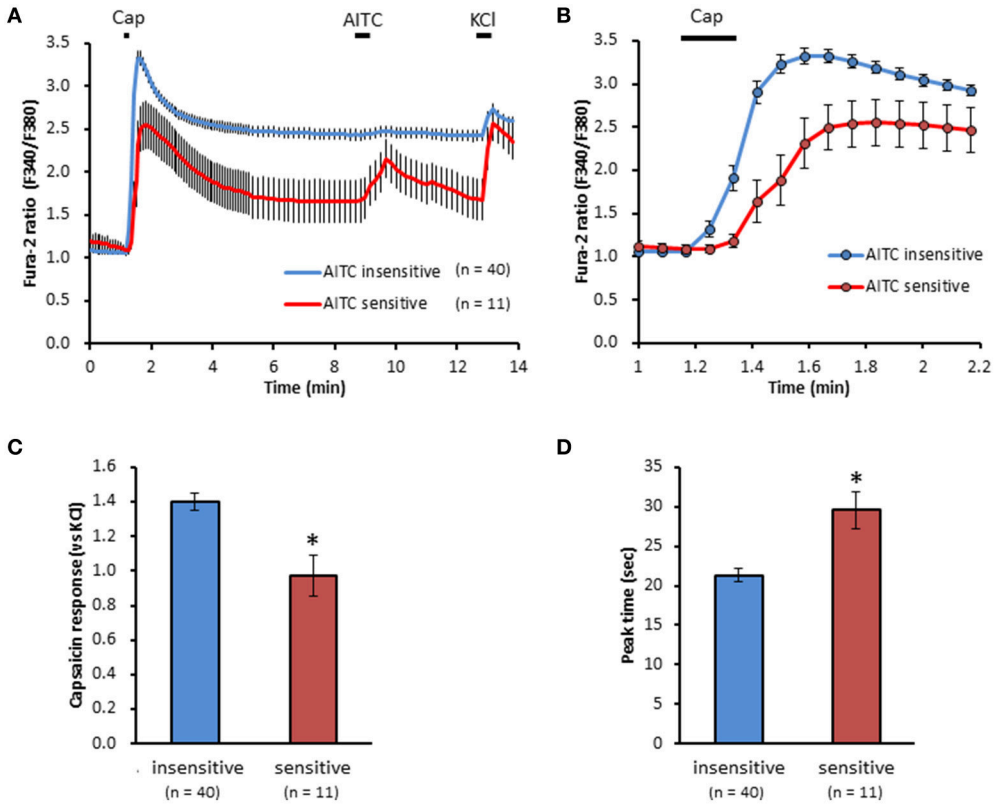

FIGURE 2 | Differences in capsaicin-induced intracellular calcium elevation between AITC-insensitive and AITC-sensitive DRG neurons. (A) Time course of F340/F380 ratio before and after the perfusion of capsaicin (cap, 1 μM) AITC (500 μM) and KCl (50 mM) using Fura-2 AM dye. Horizontal bars represent periods of capsaicin, AITC and KCl perfusion. **(B)** Changes in F340/F380 ratio with a widespread expression of x axis between time 1 and 2.2 min of **(A)**. The difference in peak response **(C)** and time reaching peak response **(D)** on capsaicin-induced elevation of intracellular calcium in DRG neurons. Data presented as mean ± SEM. *$P < 0.05$ against AITC-sensitive group.

DRG neurons (**Figure 5A**). The inhibitory effect of HC-030031 was significantly larger in AITC-sensitive DRG neurons than in AITC-insensitive DRG ones (**Figure 5B**). Therefore, TRPA1-mediated current is contained in the capsaicin-induced current in the desensitization phase in AITC-sensitive DRG neurons. To clarify the difference in the desensitization of the TRPV1-mediated current by repeated stimulation between AITC-sensitive and AITC-insensitive DRG neurons, 1 μM capsaicin perfusion for 15 s was repeated five times. Repeated short-term capsaicin perfusion gradually desensitized capsaicin-induced current responses (**Figure 6A**), and no difference was found between the two groups (**Figure 6B**).

DISCUSSION

We clarified that the kinetics of TRPV1-mediated current induced by capsaicin differed depending on the coexistence of TRPA1 channels in DRG neurons. First, the current densities were significantly smaller in AITC-sensitive DRG neurons than in AITC-insensitive DRG neurons. The difference disappeared in the presence of a high EGTA concentration in internal solutions and in the presence of HC-030031 in extracellular solutions. Therefore, TRPA1 channels suppress TRPV1 channel activity, possibly by regulating the basal intracellular Ca^{2+} concentration. TRPA1 channels are activated by exogenous irritants, including mustard oil, allicin, acrolein (Bautista et al., 2006), and alkaline pH (Fujita et al., 2008), and by endogenous substances, including hydrogen peroxide, nitric oxide, hydrogen sulfide, oxidized lipids, and general long-chain polyunsatulated fatty acids (Andersson et al., 2008, 2012; Cavanaugh et al., 2008; Takahashi et al., 2008; Motter and Ahern, 2012). Basal inward currents in AITC-sensitive neurons were slightly attenuated by a TRPA1 antagonist, which implies that a tonic TRPA1 current exists in DRG neurons. Therefore, the spontaneous TRPA1 channel activity possibly induced by endogenous substances inhibits proximate TRPV1 channels mediated by Ca^{2+} elevation. Intracellular calcium concentrations regulate the activities of protein kinase A, protein kinase C, Ca^{2+}/Calmodulin-dependent protein kinase II, and calcineurin that regulate TRPV1 channels activities through phosphorylation or dephosphorylation of several amino acid residues in TRPV1 channels, such as Ser502, Thr704, and Ser800 (Rosenbaum and Simon, 2007). The dephosphorylation of TRPV1 by calcineuline that activated by weak intracellular calcium elevation desensitizes TRPV1 channels activities by vanilloid stimulation, capsaicin (Jung et al., 2004). The balance of phosphorylation and dephosphorylation

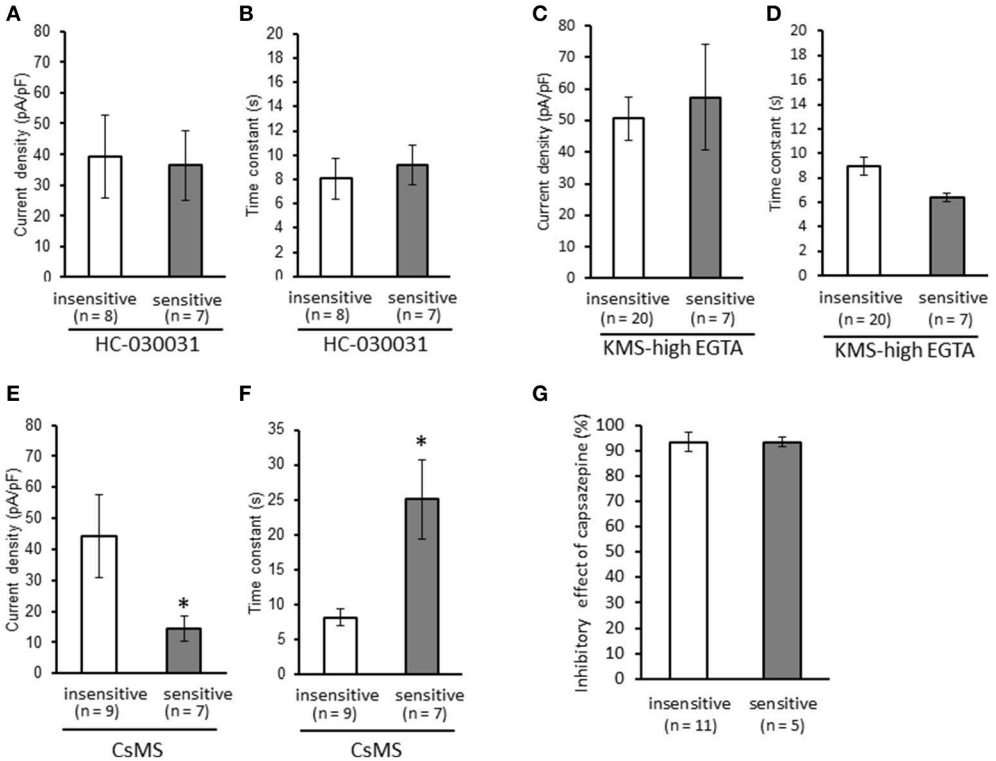

FIGURE 3 | Contributions of TRPA1 channels, intracellular Ca^{2+} concentration, and potassium channels to the differences in kinetics of capsaicin-induced currents between AITC-insensitive and AITC-sensitive DRG neurons. Capsaicin-induced currents were recorded **(A,B)** in the presence of HC-030031 (5 μM, a TRPA1 antagonist), **(C,D)** with intracellular solutions containing a high EGTA concentration (10 mM, a Ca^{2+} chelator), and **(E,F)** with intracellular solutions containing cesium methanesulfonate (CsMS) instead of potassium methanesulfonate (KMS) to block potassium channels. The differences in the current density **(A,C,E)** and time constant of decay **(B,D,F)** are shown in each recording condition. **(G)** Effect of capsazepine on capsaicin-induced current in AITC-sensitive and AITC-insensitive neurons. Each column and vertical bar represent the mean ± SEM. *$P < 0.05$ by unpaired *t*-test.

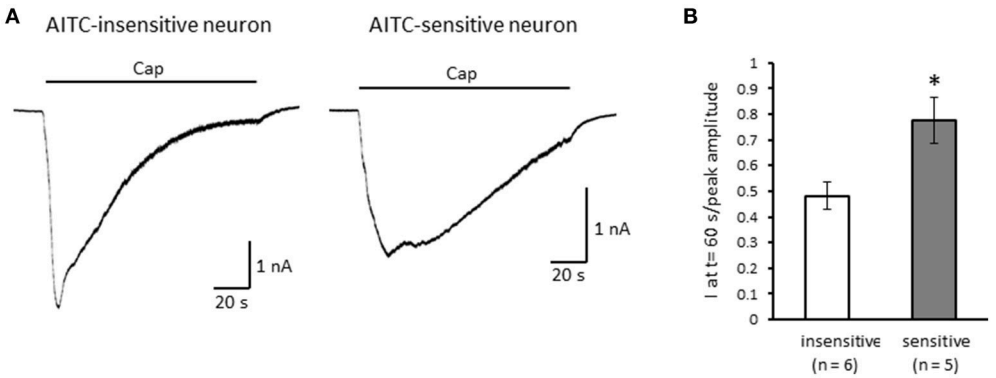

FIGURE 4 | The differences in desensitization of currents induced by persistent perfusion of capsaicin. (A) Representative traces of current response induced by 1 μM capsaicin (cap) perfusion for 120 s in AITC-insensitive (left) and AITC-sensitive (right) DRG neurons. **(B)** The difference in the decay of capsaicin-induced current 60 s after initiating the perfusion between AITC-insensitive DRG neurons (open column) and AITC-sensitive DRG neurons (gray column). Each column and vertical bar represent the mean ± SEM. *$P < 0.05$ by unpaired *t*-test.

of TRPV1 under presence of TRPA1 possibly contributes to the mechanism of small current densities of TRPV1 channels. Second, the latencies of decay in capsaicin-induced currents were significantly longer in AITC-sensitive DRG neurons than in AITC-insensitive DRG neurons, which was blocked by an internal solution containing a high EGTA concentration and in the presence of HC-030031 in an extracellular solution. In addition, desensitization during persistent capsaicin perfusion

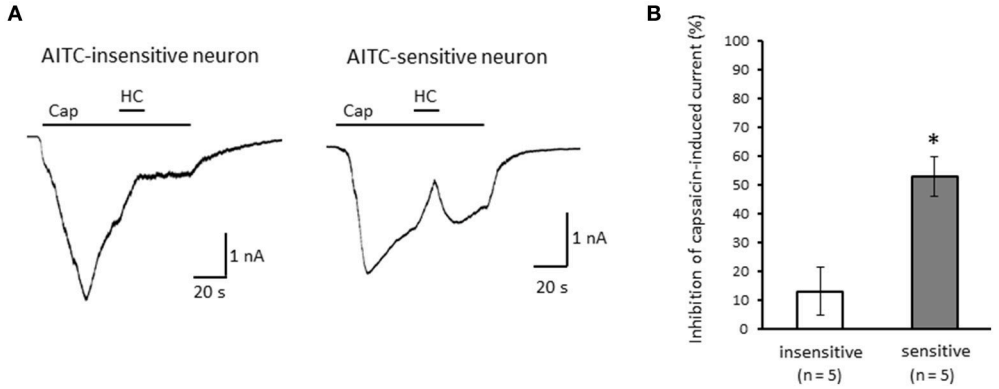

FIGURE 5 | Effects of HC-030031 on capsaicin-induced current in the desensitization phase. (A) Representative traces of capsaicin (cap)-induced current during 2 μM HC-030031 (HC) perfusion in AITC-insensitive (left) and AITC-sensitive (right) DRG neurons. **(B)** Inhibitory effects of HC-030031 on capsaicin-induced current in AITC-insensitive DRG neurons (open column) and in AITC-sensitive DRG neurons (gray column). Each column and vertical bar represent the mean ± SEM. $^*P < 0.05$ by unpaired *t*-test.

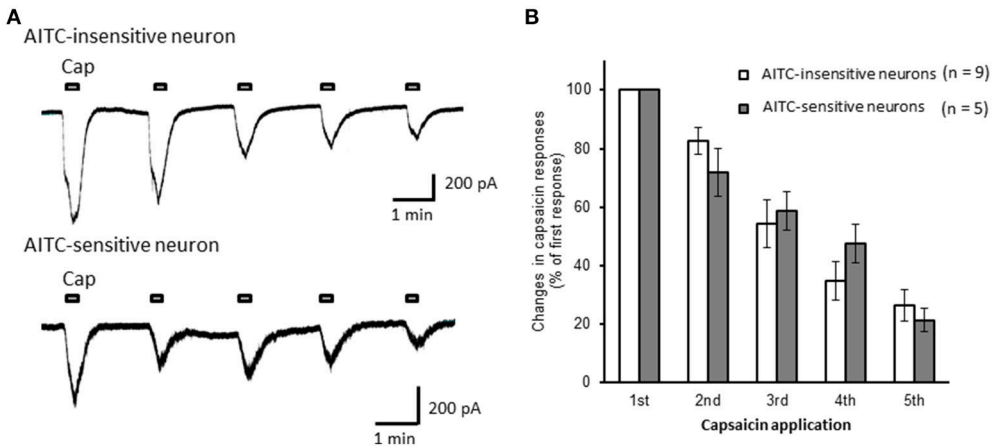

FIGURE 6 | The desensitization of capsaicin-induced currents by repeated application in AITC-insensitive and AITC-sensitive DRG neurons. (A) Representative traces of current induced by repeated capsaicin (cap) perfusion in AITC-insensitive (upper) and AITC-sensitive (lower) DRG neurons. **(B)** Comparison of desensitization of capsaicin-induced current between AITC-insensitive DRG neurons (open columns) and AITC-sensitive DRG neurons (gray columns). Each column and vertical bar represent the mean ± SEM.

was notably slower in AITC-sensitive DRG neurons than in AITC-insensitive DRG neurons. The capsaicin-induced current in the desensitization phase was attenuated by HC-030031 in AITC-sensitive DRG neurons. Therefore, it seems that TRPA1-mediated current are evoked after activation of TRPV1 channels by capsaicin. TRPA1 channels are activated by intracellular Ca^{2+} binding to the EF-hand domain (Zurborg et al., 2007; Paulsen et al., 2015). TRPA1 channels are directly activated by endogenous substance produced by intracellular calcium elevation. For instance, nitric oxide is synthesized by nitric oxide synthase (NOS) whose activation is essential for increase in intracellular Ca^{2+} bringing about calmodulin binding (Förstermann and Sessa, 2012), leading to activates TRPA1 channels through nitrosylation of Cys421, Cys641, and Cys665 (Takahashi et al., 2008). Actually, the neuronal NOS predominantly expresses in small and medium size of DRG neurons (Terenghi et al., 1993; Kolesár et al., 2016). Considering the abovementioned reports, TRPV1 channels may directly and/or indirectly activate TRPA1 channels by elevating intracellular Ca^{2+} concentration in DRG neurons, which seems to underlie slow decay of capsaicin-induced current after the brief perfusion and slow desensitization of capsaicin-induced current during the long-term perfusion in AITC–sensitive neurons.

Small peak currents induced by TRPV1 channel activation in TRPA1-positive DRG neurons might implicate low excitability for noxious stimuli in sensory neurons that co-express TRPV1 and TRPA1 channels under normal conditions. In contrast, the slow desensitization of TRPV1-mediated current in TRPA1-positive DRG neurons might enable nociceptive stimuli to persistently excite primary sensory neurons. The tumor necrosis

factor-alpha released from mast cells, lymphocytes, and skin keratinocytes during inflammation increases the cotrafficking of TRPA1/TRPV1 in trigeminal ganglion, which is involved in hypersensation in an inflammatory disorder (Meng et al., 2016). A previous study revealed that long-term treatment with glutamate, an endogenous pain modulator and inducer of inflammation, drastically increased TRPV1-mediated currents induced by capsaicin in TRPA1-expressing DRG neurons (Masuoka et al., 2016). The facilitation of TRPV1-mediated currents in TRPA1-positive DRG neurons produced heat hyperalgesia in mice (Masuoka et al., 2016). Therefore, TRPV1-mediated electrophysiological responses in TRPA1-expressing sensory neurons might be related to the molecular basis of nociception in chronic abnormal pain induced by inflammation. Recent studies clarified that sensory neurons that express TRPA1 regulate inflammation and pruritogen responses (Wilson et al., 2011; Liu et al., 2013; Cevikbas et al., 2014). For instance, TRPA1 is required for Mas-related G protein-coupled receptor-mediated signaling, which is activated by mast cell mediators and promotes histamine-independent itch (Wilson et al., 2011). Interleukin-31 (IL-31), T helper cell type 2- derived cytokine, activates a small subpopulation of primary sensory neurons expressing TRPV1, and TRPA1 through IL-31 receptor, and produced inflammatory and lymphoma-associated itch (Cevikbas et al., 2014). Therefore, TRPV1 channel activation in DRG neurons that co-express TRPA1 channels might contribute to the itch response. Our findings might help in understanding the characteristics and molecular mechanisms of itch often accompanied by pain.

CONCLUSION

TRPV1 mediated-currents in TRPA1-positive neurons are characterized small densities with slow decay, which is caused by TRPA1 channels activation and intracellular calcium mobilization. In addition, TRPV1-mediated current in TRPA1-possitive neurons slowly desensitize, due to appending TRPA1-mediated current.

AUTHOR CONTRIBUTIONS

TM designed and performed the research, analyzed the data, and wrote the paper. MK and YY performed research and analyzed data. JY, NI, IM, MN, and TI wrote and revised the paper.

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REVIEW
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Different Principles of ADP-Ribose-Mediated Activation and Opposite Roles of the NUDT9 Homology Domain in the TRPM2 Orthologs of Man and Sea Anemone

Frank Kühn, Cornelia Kühn and Andreas Lückhoff*

Medical Faculty, Institute of Physiology, RWTH Aachen University, Aachen, Germany

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Edited by:
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****Correspondence:***
Frank Kühn
fkuehn@ukaachen.de

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A decisive element in the human cation channel TRPM2 is a region in its cytosolic C-terminus named NUDT9H because of its homology to the NUDT9 enzyme, a pyrophosphatase degrading ADP-ribose (ADPR). In *h*TRPM2, however, the NUDT9H domain has lost its enzymatic activity but serves as a binding domain for ADPR. As consequence of binding, gating of the channel is initiated. Since ADPR is produced after oxidative DNA damage, *h*TRPM2 mediates Ca^{2+} influx in response to oxidative stress which may lead to cell death. In the genome of the sea anemone *Nematostella vectensis (nv)*, a preferred model organism for the evolution of key bilaterian features, a TRPM2 ortholog has been identified that contains a NUDT9H domain as well. Heterologous expression of *nv*TRPM2 in HEK-293 cells reveals a cation channel with many close similarities to the human counterpart. Most notably, *nv*TRPM2 is activated by ADPR, and Ca^{2+} is a co-agonist. However, the intramolecular mechanisms of ADPR gating as well as the role of NUDT9H are strikingly different in the two species. Whereas already subtle changes of NUDT9H abolish ADPR gating in *h*TRPM2, the region can be completely removed from *nv*TRPM2 without loss of responses to ADPR. An alternative ADPR binding site seems to be present but has not yet been characterized. The ADP-ribose pyrophosphatase (ADPRase) function of *nv*NUDT9H has been preserved but can be abolished by numerous genetic manipulations. All these manipulations create channels that are sensitive to hydrogen peroxide which fails to induce channel activity in wild-type *nv*TRPM2. Therefore, the function of NUDT9H in *nv*TRPM2 is the degradation of ADPR, thereby reducing agonist concentration in the presence of oxidative stress. Thus, the two TRPM2 orthologs have evolved divergently but nevertheless gained analogous functional properties, i.e., gating by ADPR with Ca^{2+} as co-factor. Opposite roles are played by the respective NUDT9H domains, either binding of ADPR and mediating channel activity, or controlling the availability of ADPR at the binding site located in a different domain.

Keywords: *Nematostella vectensis*, ADP-ribose, calcium, oxidative stress, 2-APB

HUMAN TRPM2: HISTORY AND HALLMARKS OF AN EXCEPTIONAL CATION CHANNEL

The scientific community was very much excited when in 2001, Perraud et al. reported that the human Ca^{2+}-permeable cation channel LTRPC2 was activated by intracellular ADP-ribose. Two characteristics of this channel, renamed in the meantime to TRPM2, were particularly fascinating. The first one is its activation by ADPR, a metabolite which had not been on the list of potential stimuli of ion channels at this time, although it was known to induce the fertilization current in oocytes of the sea squirt *Ciona intestinalis* (Wilding et al., 1998). From then on it was quickly realized that ADPR, produced in response to oxidative stress and as consequence of DNA damage, mediates Ca^{2+} influx through TRPM2 channels which may eventually lead to apoptosis or other forms of cell death. Second, TRPM2 contains a homology region within the cytosolic C-terminus that strongly resembles the human NUDT9 pyrophosphatase as well as homologous bacterial enzymes of the NUDIX-family (Bessman et al., 1996; Perraud et al., 2003). Hence, TRPM2 might be considered a "chanzyme," a channel protein that additionally displays enzymatic activity intimately linked to channel function.

It has been well established that ADPR is the principal activator of TRPM2. Few related substances have been reported to share its agonistic properties (Grubisha et al., 2006; Tóth et al., 2014, 2015; Fliegert et al., 2017). However, a major role as an essential co-factor is played by Ca^{2+} (McHugh et al., 2003; Starkus et al., 2007; Csanády and Töröcsik, 2009). For an effective stimulation by ADPR, Ca^{2+} must be presented either on the extracellular or the intracellular side of the plasma membrane and its action is likely to take place within the pore region (as discussed later). In particular, intracellular Ca^{2+} strongly modulates the sensitivity of TRPM2 to ADPR, to an extent that in neutrophil granulocytes activation of TRPM2 occurs without an apparent increase in the intracellular concentration of ADPR as soon as intracellular Ca^{2+} is elevated (Heiner et al., 2006). Since basal levels of ADPR are sufficient to enable Ca^{2+}-directed TRPM2 gating, ADPR renders TRPM2 a Ca^{2+}-activated cation channel that is indispensible for some but not all responses of neutrophils during antibacterial defense. Especially chemotaxis seems to be critically dependent on the preceding stimulation of TRPM2 and is significantly impaired in TRPM2 knock-out mice (Sumoza-Toledo et al., 2011).

Neutrophil granulocytes are among the few cells that are not equipped with poly(ADP-ribose)-Polymerases (PARPs; Sanghavi et al., 1998). Along with poly(ADP-ribose) glycohydrolases (PARGs), these are key enzymes involved in the formation of ADPR after oxidative damage to the DNA (e.g., reviewed by Yamamoto and Shimizu, 2016). Therefore, in many other cell types including neurons, ADPR-induced Ca^{2+} influx through TRPM2 is a decisive element in the process that terminates in apoptosis after initiation by oxidative stress. Experimentally, oxidative stress is frequently induced by extracellular application of hydrogen peroxide (H_2O_2) to the cells. Indeed, in cell models with overexpression of TRPM2, H_2O_2 is a well-established stimulus of Ca^{2+} influx (Hara et al., 2002; Wehage et al., 2002). Another extracellularly applicable stimulus of TRPM2 (as opposed to the strictly intracellular application of ADPR) is N-Methyl-N′-nitro-N-nitrosoguanidine (MNNG) that, like H_2O_2, is an activator of PARP-1 (Buelow et al., 2008; Chiu et al., 2011). It is believed that the action of H_2O_2 is an indirect one, depending on the intracellular accumulation of ADPR (Perraud et al., 2005). Consequently, current induction in response to H_2O_2 takes some time, in contrast to the fast onset after stimulation of TRPM2 with high concentrations of intracellular ADPR during patch-clamp experiments. The co-operation of ADPR and Ca^{2+}, along with the positive feed-back constituted by Ca^{2+} entry through already activated TRPM2 channels (McHugh et al., 2003; Heiner et al., 2006; Csanády and Töröcsik, 2009; Tóth and Csanády, 2010), explains why any effect of H_2O_2 is strongly dependent on Ca^{2+}, even more strictly than under experimental conditions when ADPR in excess is directly applied to the channel. Therefore, an elevated intracellular Ca^{2+} concentration of 1 μM is routinely used in our lab in patch-clamp experiments when TRPM2 or TRPM2 variants are tested for sensitivity toward H_2O_2.

Mammalian TRPM2 channels are moreover sensitive to temperature (Togashi et al., 2006; Kashio et al., 2012) and are apparently involved in temperature sensing (Song et al., 2016; Tan and McNaughton, 2016), but this is beyond the scope of this review, as is its role as a channel in membranes of intracellular organelles (Lange et al., 2009).

TRPM2 so far is the only ion channel that is directly activated by ADPR. This should not be confused with other regulatory functions of ADPR, notably ADP ribosylation, which takes place, e.g., in the purinergic P2X7 receptor and leads to channel activation (Adriouch et al., 2008). Among the four known "chanzymes," the most prominent member is still the cystic fibrosis transmembrane conductance regulator (CFTR) channel (Ramjeesingh et al., 1999), whereas the other three ones all belong to the melastatin-subfamily of TRP channels (Perraud et al., 2001; Runnels et al., 2001; Schlingmann et al., 2002). Until recently, it was believed that the catalytic activity of the respective enzyme domain contributes decisively to their gating process. However, now it seems clear that for TRPM6 and TRPM7, the enzyme domain is not really essential for gating but rather performs a regulatory function (Matsushita et al., 2005; Thébault et al., 2008; Cai et al., 2017). In the case of CFTR and also of human TRPM2, multiple lines of evidence suggest that not catalysis but binding of the substrate represents the critical step for channel activation (Tóth et al., 2014; Mihályi et al., 2016).

The principal structure of the NUDT9 homology domain of TRPM2 which is very similar to the almost identical NUDT9 enzymes of man and sea anemone is outlined in **Figure 1**. The more C-terminally localized catalytic center is formed by a strongly conserved amino acid sequence, the so-called NUDIX-box (Bessman et al., 1996). It has been experimentally demonstrated that the two successive amino acid residues glutamate-phenylalanine of this region are especially important for the activity of the human enzyme (Perraud et al., 2003). If these residues are mutated to isoleucine-leucine, the activity is reduced to about 1% (Shen et al., 2003). Exactly this substitution is present in the NUDT9H domain of human TRPM2 which strongly suggests that the enzymatic activity has been largely

FIGURE 1 | Multiple Sequence alignment of the NUDT9-related channel domains of *h*TRPM2 and *nv*TRPM2 and the NUDT9 enzymes of man and sea anemone. Alignment was performed (using the tool at www.uniprot.org/align/) with amino acid sequences of the human NUDT9 enzyme (aa 59–350) the NUDT9 homology (NUDT9H) domains of human TRPM2 (aa 1236–1503) and sea anemone TRPM2 (aa 1,271–1,551) as well as of the putative NUDT9 enzyme of the sea anemone (aa 1–281). The NUDIX sequence motif containing the catalytic active site (bold) is given in red. The functionally important deletion downstream of the NUDIX box which is exclusively present in *h*TRPM2 is indicated with a green dashed line. Individual residues found to be directly involved in the binding of ADP-ribose in *h*TRPM2 (Yu et al., 2017) are in gray. Most of these residues are conserved in the corresponding region of *nv*TRPM2 and the NUDT9 enzymes. Symbols (*, :, .) denote the degree of conservation observed in each column as specified on the website indicated above.

abolished while NUDT9 has undergone the adaption to a channel domain of TRPM2. Importantly, the reciprocal mutation of the critical sequence of TRPM2 back to that of the NUDT9 enzyme abolishes any channel function (Kühn and Lückhoff, 2004; Perraud et al., 2005; Du et al., 2009) suggesting that ADPR hydrolysis and channel function are incompatible with each other. Moreover, channel activation can be readily achieved with the non-hydrolyzable ADPR analog alpha, beta-methylene ADP-ribose (AMPCPR) (Tóth et al., 2014). Taken together, there is ample and strong evidence for the notion that catalytic activity is not necessary and even detrimental for the activation of TRPM2.

The currently favored view is that the NUDT9H region of TRPM2 ensures the specific binding of the channel agonist ADPR. Several studies have clearly demonstrated that already subtle changes within the structure of the NUDT9H domain may lead to a complete loss of channel function (e.g., Kühn and Lückhoff, 2004; Perraud et al., 2005). Obviously, binding

and subsequent channel gating have very restricted structural requirements that can easily be disturbed.

STUDIES ON ORTHOLOGOUS CHANNELS FROM DISTANTLY RELATED SPECIES TO GET MECHANISTIC INSIGHT

While it is generally accepted and well documented that gating of TRPM2 requires binding of ADPR to the NUDT9H domain, the subsequent steps that ultimately lead to pore opening are far from being understood. Methodological approaches that would provide a straightforward interpretation are not obvious. In this situation, a strategy may be helpful that has been successfully employed for several other ion channels: the structural and functional comparison of species variants. Prominent examples are the capsaicin receptor TRPV1 (Jordt and Julius, 2002), the menthol receptor TRPM8 (Chuang et al., 2004) and the chemoreceptor TRPA1 (Laursen et al., 2015).

In this review, we will summarize the findings and perspectives gained from studies on the TRPM2 ortholog of the sea anemone *Nematostella vectensis*.

Of course, the question arises why choosing the sea anemone as species variant. There are several good reasons for this choice. First, the evolution especially of the TRPM channel subfamily seems to have taken a very interesting course. In basal metazoans and even in unicellular protists, there is only one representative of the TRPM subfamily and this is clearly classified as TRPM2-like (Mederos y Schnitzler et al., 2008). This and other indications allow the conclusion that in the beginning of the metazoan evolution, a TRPM2-like channel stepped on stage which probably represents the evolutionary ancestor of all modern TRPM channels. This scenario implicates that the archetypal TRPM channel should have had structural and functional features that are at least partially present in all modern TRPM subtypes, including TRPM2. *Nematostella vectensis* today represents a preferred model organism for the study of the evolution of some archetypal metazoan blueprints such as the immune system and the nervous system. Especially for comparative studies on TRPM2, it is noteworthy that the natural habitats of *Nematostella vectensis* are salt marshes along the coasts of the northern Atlantic Ocean. Here, animals are commonly exposed to UV radiation and diverse chemicals, all of which can exert oxidative stress (Tarrant et al., 2014). Because the mammalian TRPM2 ortholog represents a central player in the process of oxidative-stress mediated apoptosis, the suitability of *Nematostella vectensis* as a simplistic model appears evident.

The comparative studies on *h*TRPM2 and *nv*TRPM2 reveal that both of these far distantly related channel orthologs are activated by ADPR. However, and unexpectedly, this is achieved by vastly different mechanisms and parts of the channel protein.

TOPICAL AND DETAILED STRUCTURE OF *nv*TRPM2

The genome of the starlet sea anemone *Nematostella vectensis* was sequenced and assembled by whole genome shotgun by Putnam et al. (2007). A search of Nudix-linked TRPM proteins in genomic sequence databases by Mederos y Schnitzler et al. (2008) revealed that they are invariably present in chordates, molluscs, echinodermates and also in basal metazoans like cnidarians and even in unicellular protists. As the complete expressed sequence tag (start codon to stop codon open reading frame) of the sea anemone TRPM2-like channel was published in the joint genome institute database (jgi.Nemve1.248535|estExt_fgenesh1_pg.C_6220005), it was possible to make this gene available for functional expression in heterologous expression systems by commercial gene synthesis (Kühn et al., 2015).

The sea anemone TRPM2 open reading frame (ORF) contains 1551 amino acid residues (aa) and on closer inspection represents the only full-length TRPM gene product of *Nematostella vectensis* (Mederos y Schnitzler et al., 2008; Peng et al., 2015). The sea anemone TRPM2 open reading frame displays a total sequence identity of 31% to the corresponding sequence of human TRPM2 (1503 aa). The similarity is greatest in the N-terminal region upstream of the putative transmembrane segments (36% identity) and in the NUDT9H domain (39% identity), whereas the regions containing the transmembrane segments (25% identity) and the connecting linker to the NUDT9H domain (27% identity) are less conserved. Furthermore, the NUDT9H domain of *nv*TRPM2 (aa 1271–1,551) shows 49% sequence identity to the corresponding sequence of the *h*NUDT9 enzyme (aa 59–350) which is notably higher than between the *h*NUDT9 enzyme and NUDT9H (aa 1,236–1,503) of *h*TRPM2 (34%; Kühn et al., 2015; see also **Figure 1**). Compared to the *h*NUDT9-enzyme, in both *nv*TRPM2 and *h*TRPM2, the putative ADPR binding domain of the NUDT9H domain is well conserved, including the critical residue N1326 of *h*TRPM2 (Kühn and Lückhoff, 2004; Kühn et al., 2016). However, the active site of the *h*NUDT9 enzyme containing the NUDIX box signature $GX_5EX_7REUXEEXGU$ (Bessman et al., 1996) is slightly different in NUDT9H of *nv*TRPM2 and markedly different in NUDT9H of *h*TRPM2 (**Figure 1**). This fact strongly suggests that the NUDT9H domain of *nv*TRPM2, in contrast to the *h*TRPM2 counterpart, is very likely to have a largely intact catalytic function.

A short amino acid motif within the proximal part of the predicted pore loop contributes significantly to the Ca^{2+} permeation of enzyme-linked TRPM channels (Mederos y Schnitzler et al., 2008). In the non-selective group, among them *h*TRPM2, this motif consists of the amino acid triplet glutamine-isoleucine-proline (QIP), whereas in the more Ca^{2+}-selective members, as for example TRPM7, this motif is changed to glutamate-valine-tryptophane (EVY). In general, the TRPM2-like channels of diverse organisms ranging from choanoflagellates to primitive chordates and also *nv*TRPM2 contain the motif glutamate-leucine-phenylalanine (ELF) which indicates the signature of a more Ca^{2+}-permeable channel (Mederos y Schnitzler et al., 2008).

As a striking difference to the primary structure of *h*TRPM2, the *nv*TRPM2 channel exhibits a much longer S1-S2 linker region with numerous glutamate and lysine residues. Notably, this region shows significant similarity to the corresponding

region of the *h*TRPM3 channel which strengthens the hypothesis that a TRPM2-like channel represents a common ancestor of the contemporary TRPM-subfamily (Mederos y Schnitzler et al., 2008; Kühn et al., 2015).

FUNCTIONAL EXPRESSION OF *nv*TRPM2 IN HUMAN CELLS REVEALS CATION CURRENTS INDUCED BY ADPR AND BY Ca^{2+}

From the overall high topological similarity between *nv*TRPM2 and mammalian TRPs, we were confident in the beginning of our studies that the sea anemone ortholog could be functionally overexpressed with standard methods in mammalian cells, although until then only few examples existed where a successful heterologous expression of such far distantly related ion channels had been achieved and this was in oocytes of *Xenopus laevis* (e.g., Jegla et al., 2012; Assmann et al., 2014; Baker et al., 2015). The standard procedure of commercially available gene synthesis was used and the codon usage was adapted to the human expression system (Ikemura, 1985). This manipulation is frequently a prerequisite for the successful heterologous expression of proteins from distantly related species. The successful expression of all TRPM2 channels (human or *Nematostella* orthologs) in HEK-293 cells was verified by cell surface biotinylation assay and Western-blot-analysis with variants containing hemagglutinin (HA) tags downstream from the respective open reading frame (Kühn et al., 2016). This procedure was chosen to minimize the danger of artifacts due to species-specific antibodies. Wild-type and mutant *nv*TRPM2 channels were expressed in the plasma membrane with no obvious difference to the human ortholog.

For functional analysis, mostly the variants without HA tags were studied using the standard whole-cell patch-clamp technique. The non-electrophysiologists among the readers should understand that with this technique, the cytosol of the cells is replaced with the solution in the pipette within seconds by diffusion. Thus, the intracellular concentrations of the standard stimulus, ADPR, as well as the intracellular concentration of Ca^{2+} is completely controlled by the composition of the pipette fluid. For some selected *nv*TRPM2 variants, the biophysical properties were explored with single-channel analysis in inside-out patches (Kühn et al., 2016).

The electrophysiological studies demonstrate that *nv*TRPM2 is expressed in HEK-293 cells as fully functional cation channel activated by ADPR and by its co-agonist Ca^{2+}. Thus, the principal activators of *h*TRPM2 are effective in the ortholog of a distantly related species as well. In addition to many common features of ADPR-induced currents, however, there were several properties unique for *nv*TRPM2. These include in the first line the concentration-effect-relation as well as the on and off kinetics.

In human TRPM2, stimulation with ADPR results in a current that reaches its maximum within several tens of seconds. A run-down takes place over several minutes and is usually incomplete within the time frame of the experiments (**Figure 2A**). The amplitudes and kinetics depend significantly on Ca^{2+} which must be present on at least one side of the plasma membrane for the induction of any current (Perraud et al., 2001; McHugh et al., 2003; Starkus et al., 2007; Csanády and Törocsik, 2009; Kühn et al., 2010). The elevation of the Ca^{2+} concentration either on the extracellular or the intracellular side fail to stimulate TRPM2 channels in the absence of ADPR. On the other hand, removal of Ca^{2+} from the extracellular side promptly abolishes ADPR-induced currents when Ca^{2+} is absent in the cytosol. These two findings establish the role of ADPR and Ca^{2+} as essential co-agonists. In the presence of 1 μM Ca^{2+} in the cytosol, half-maximal current amplitudes are reached with ADPR concentrations of about 100 μM. The ADPR concentration needs to be increased to 500 μM when Ca^{2+} is removed from the pipette fluid.

In characteristic distinction to *h*TRPM2, the *Nematostella* ortholog *nv*TRPM2 displays much faster developing currents of large amplitude after stimulation with ADPR; however, the currents return to baseline within a few seconds (**Figure 2B**). These responses are induced by already moderate concentrations of ADPR (25–50 μM) in the absence of intracellular Ca^{2+} ($\leq$10 nM); ADPR concentrations as low as 10 μM were sufficient with 1 μM Ca^{2+}. No currents were observed when Ca^{2+} was missing on both sides of the plasma membrane (Kühn et al., 2015). Therefore, Ca^{2+} is an essential co-factor as in *h*TRPM2. However, one order of magnitude less ADPR is required for *nv*TRPM2 than for *h*TRPM2.

With respect to many biophysical properties, *nv*TRPM2 and *h*TRPM2 appear closely similar. Single channel open times are extremely long in inside-out patches with ADPR on the cytosolic side of the plasma membrane, frequently reaching several hundreds of milliseconds. Likewise, there is almost no discrimination between monovalent and divalent cations, as shown by the reversal potential close to 0 mV in asymmetric solutions (Kühn et al., 2015).

The on-kinetics, which is markedly accelerated by Ca^{2+} in the case of *h*TRPM2, was not modified in *nv*TRPM2 by increasing the concentrations of ADPR and Ca^{2+}. This may have not been expected anyway because they are extremely fast for a ligand-gated channel already at standard conditions. Interestingly, the on-kinetics remained fast when the pore signature glutamate-leucine-phenylalanine (ELF) of *nv*TRPM2 was changed to glutamine-leucine-proline (QLP) which is characteristic for TRP channels with little Ca^{2+} permeability (Mederos y Schnitzler et al., 2008). It appears that although Ca^{2+} in the pore is essential, it is not required in high amounts or concentrations; a graded modulation of ADPR-induced currents by intracellular Ca^{2+} cannot be demonstrated experimentally.

Beyond the effects of Ca^{2+} on the on-kinetics of ADPR-dependent currents, Ca^{2+} has a strong impact on the off-kinetics. When extracellular Ca^{2+} is removed, ADPR induces currents that are sustained over extended periods of time. Already this finding suggests that Ca^{2+} exerts an action on the pore to induce a rapid current decline of the current.

In any case, Ca^{2+} entry profoundly affects the kinetics of ADPR-induced currents, whereas intracellular Ca^{2+} facilitates the principal activation in most experimental conditions.

Further evidence for this interpretation is discussed later in context of the effects of 2-APB. We therefore propose that the

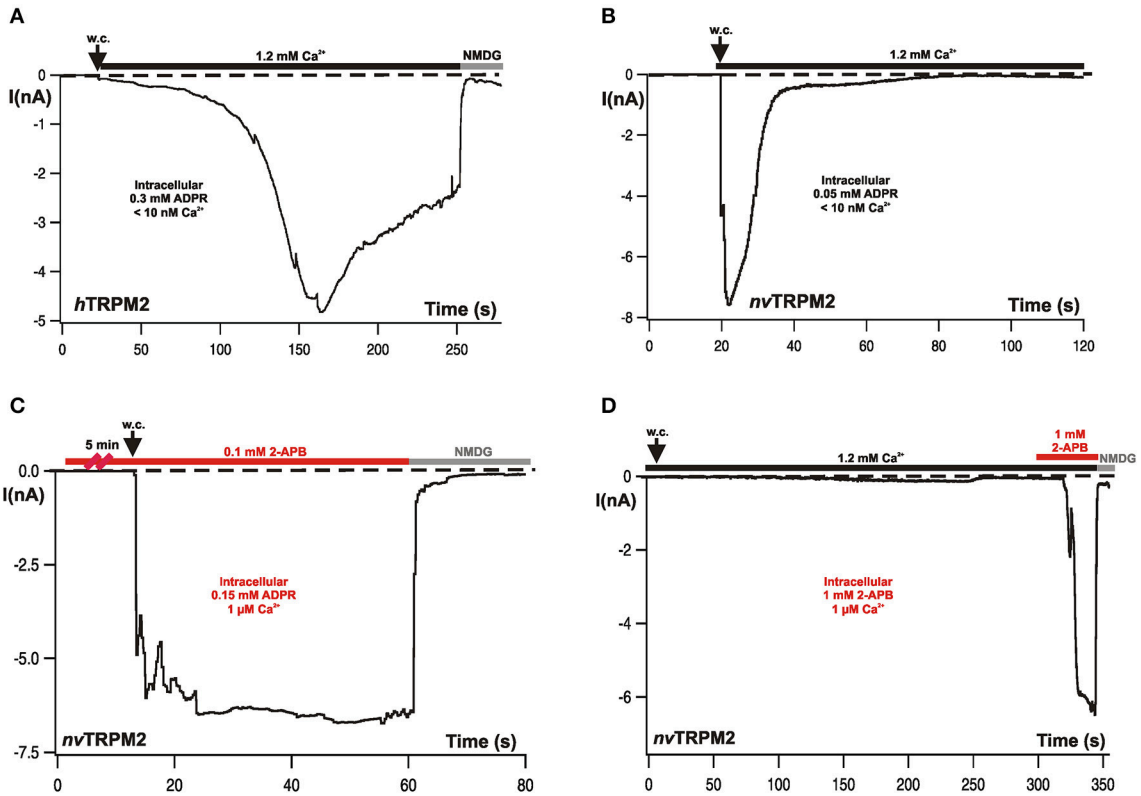

FIGURE 2 | Typical whole-cell patch-clamp recordings from *h*TRPM2 and from *nv*TRPM2 channels in HEK-293 cells. **(A)** Characteristic currents of *h*TRPM2 during stimulation with ADPR infused through the patch-pipette. Intracellular (pipette) concentrations of ADPR and Ca^{2+} are indicated. Both activation and inactivation kinetics are comparatively slow. At the end of the experiment, the currents were fully blocked by a substitution of extracellular monovalent cations with NMDG. **(B)** Currents of *nv*TRPM2 in response to ADPR showing the characteristic fast kinetics of activation and inactivation. **(C)** Similar experimental conditions as shown before but the cells had been pre-incubated for at least 5 min in bath solution containing 2-APB (0.1 mM). The rapid inactivation is completely suppressed. **(D)** No stimulation of *nv*TRPM2 with 2-APB (1 mM) instead of ADPR in the pipette solution. Only when applied from the extracellular side of the plasma membrane high concentrations of 2-APB (≥1 mM) induces a strong and fast current activation after a characteristic delay, while inactivation does not occur. The standard Ca^{2+} concentration in the bath solution for the measurements is 1.2 mM Ca^{2+}. Figures are slightly modified from Kühn et al. (2015, 2016, 2017).

current decline should be referred to as inactivation because it relates to a pore-dependent mechanism. The term desensitization should, in our opinion, not be used because it may be understood to describe a process that affects binding of ADPR for which no experimental indication exists. Unfortunately, it is not easily possible to remove the stimulus ADPR during one experiment and repeat its application several times. In inside-out patches, this would be feasible; however, for some reasons that are not understood, single channel activity in response to ADPR persists much longer than whole-cell currents.

We have not performed a determination of the Ca^{2+} permeability deduced from reversal potentials in non-physiological high Ca^{2+} concentrations because this approach is unlikely to yield a true estimation of the contribution of Ca^{2+} to the total current under physiological ion conditions (Dzeja et al., 1999). However, studies on the QLP variant demonstrate that indeed Ca^{2+} access to the pore is improved by the ELF motif which, interestingly, preferentially concerns permission of activation by intracellular Ca^{2+}, in co-operation with ADPR.

Taken together, *nv*TRPM is rapidly activated by ADPR and Ca^{2+} as co-agonists, with considerably higher sensitivity and faster kinetics than *h*TRPM2. A fast inactivation takes place through the action of Ca^{2+} entering the pore.

2-APB AS A Ca^{2+}-DEPENDENT GATING MODIFIER OF TRPM2 CHANNELS

A general problem in the investigation of TRP channels, especially of the TRPM subfamily, is the lack of specific inhibitors. 2-Aminoethyl-diphenylborinate (2-APB) is one of the better candidates since its effects on TRPM channels are at least rapidly and completely reversible. On the other hand, the compound is by no means channel-specific and its effect can be inhibitory as well as activating. This depends on its concentration and the channel type. Even on one particular channel, it may exert both these opposite effects in a concentration dependent manner (e.g., Li et al., 2006; Jansen et al., 2016). Likewise, the human TRPM2 ortholog was exclusively inhibited already by moderate concentrations of 2-APB (0.1 mM), whereas the *nv*TRPM2 ortholog shows different and complex responses in the presence of 2-APB. In no case, an inhibition was observed;

2-APB left the activation by ADPR completely intact. However, the fast inactivation that is a characteristic feature of *nv*TRPM2 is completely abolished by the compound (**Figure 2C**) such that sustained currents are induced by ADPR in the presence of 2-APB (0.1–0.5 mM). At higher concentrations (1 mM), 2-APB activated large currents by itself (**Figure 2D**); again, these currents did not inactivate over time (Kühn et al., 2017).

Both these effects were strictly dependent on an extracellular application and were completely absent when 2-APB was present only in the pipette (intracellular) solution (**Figure 2D**; as also demonstrated for *h*TRPM2; Togashi et al., 2008; Kühn et al., 2017). This finding suggests that 2-APB acts on the channel pore; this view is supported by experiments on *nv*TRPM2 variants in which genetic manipulations have been performed in the pore region and which show altered responses to 2-APB, in comparison with wild-type *nv*TRPM2 (Kühn et al., 2017). We are convinced that it is a safe assumption that 2-APB indeed is a modifier of the pore properties and that it can therefore be used as a tool to explore these properties further, in particular with respect to Ca^{2+}-mediated effects on the pore. The potential of 2-APB in this respect has not yet been fully exploited but already the initial results reveal surprising insight, as well as they give rise to further questions and to hypotheses that should be tested in the near future.

There are several key findings for 2-APB on *nv*TRPM2 that in combination result in a straightforward interpretation of its modes of action, although still some detailed questions remain open.

First, these are the peculiar on and off kinetics of 2-APB when used as a channel stimulus, i.e., in high concentrations. There is a lag time of several tens of seconds before any effect can be observed but afterwards, the development of currents occurs very rapidly within seconds. Wash-out of 2-APB, on the other hand, leads to an immediate cessation of the currents, much faster than their onset (Kühn et al., 2017). Thus, access to the pore is restricted as long as the channels are in a closed state but becomes fast as soon they are opened by 2-APB. As a result, an almost all-or-nothing kind of response to 2-APB is observed with an extremely steep concentration-response relation. While there is no apparent activation by 0.5 mM 2-APB, a full activation takes place if that concentration is doubled. Removal of 2-APB then lets the stimulus quickly leave the pore and the currents recede (Kühn et al., 2017).

The second key finding is the strict requirement on Ca^{2+} for the channel stimulation by 2-APB. Ca^{2+} must be present on both sides of the plasma membrane, in contrast to experiments with ADPR as stimulus of *nv*TRPM2 when either extracellular or intracellular Ca^{2+} was sufficient. It is tempting to speculate but not yet proven that this relates to multiple binding sites for Ca^{2+}, as has been proposed for the pore of *h*TRPM2 (Csanády and Töröcsik, 2009). However, some clarification is gained by experiments with the QLP-variant of *nv*TRPM2. This mutation is supposed to change the pore signature to that of a less Ca^{2+} selective channel (Mederos y Schnitzler et al., 2008).

Indeed, this mutation seems to impede the access of Ca^{2+} to the pore, and not only from the extracellular but from the intracellular side as well. Removal of extracellular Ca^{2+}, which has no dramatic effect on the stimulation of wild-type *nv*TRPM2, abolishes ADPR responses of *nv*TRPM2-QLP completely when the standard intracellular solution is used (with a Ca^{2+} concentration of 1 μM). Currents can be restored when intracellular Ca^{2+} is increased to 100 μM (a certainly non-physiological concentration). Likewise, the QLP variant was not stimulated by 2-APB when the extracellular Ca^{2+} concentration was normal. But with 10 mM Ca^{2+}, again non-physiologically high, currents reappeared. The inactivation of ADPR-induced currents in the QLP variant was normal, suggesting that not so much Ca^{2+} is required for the inactivation as for the co-agonism with 2-APB. On the other hand, as co-agonist with ADPR, intracellular Ca^{2+} is more effective than extracellular one. These findings again may point to multiple Ca^{2+} binding sites within the pore with different functions, as more extensively discussed on *h*TRPM2 (Csanády and Töröcsik, 2009; Tóth and Csanády, 2012).

It should be kept in mind in this context that Ca^{2+} not only accesses the pore but that it permeates it. The latter, however, takes place only after opening of the channel. Access, on the other hand, is decisive prior to the channel's full activation and may occur in its closed state or when only few channel openings happen that do not produce a noticeable current but allow Ca^{2+} to reach its target within the pore. Again, Ca^{2+} mediates a self-enhancing process as a co-agonist for ADPR and for 2-APB because it leads to pore opening and at the same time its access is favored by pore opening. Moreover, whenever differences are observed between extracellular and intracellular Ca^{2+}, it is difficult to decide whether these reflects steric reasons within the pore's architecture or merely a matter of the required Ca^{2+} concentration because the intracellular Ca^{2+} is low and cannot reasonably be increased too much.

The notion that Ca^{2+} within the pore is a prerequisite for channel activation not only by ADPR but by 2-APB as well is elegantly underlined by experiments on the QLP variant where a low concentration of ADPR is present in the pipette. A relatively small current is induced and inactivation takes place. Then, addition of 2-APB evokes currents with two remarkable properties. They are larger in amplitude than the previous ADPR-dependent ones, and they occur with a shorter delay than typical for 2-APB effects in the absence of ADPR. Our interpretation is that some Ca^{2+} has remained at the putative activating site in the pore and that the positive feedback of 2-APB and Ca^{2+} can now progress earlier.

An extremely interesting process in *nv*TRPM2 is the fast current inactivation which discriminates it from its human ortholog. In *h*TRPM2, the current decline after stimulation with ADPR is remarkably slow, such that the activation is frequently perceived as permanent. However, also in *h*TRPM2, inactivation may be important (Starkus et al., 2007), although at a different timescale. In *nv*TRPM2, inactivation takes place within fractions of a minute. As molecular mechanisms for this phenomenon, the experiments with 2-APB and on the pore mutant QLP provide strong evidence that inactivation represents processes within the pore and should therefore, as noticed before, referred to as inactivation, rather than desensitization. It is also clear that it is extracellular Ca^{2+} passing through the pore that mediates

inactivation. Less clear is how this is prevented by 2-APB. The compound could interfere with the binding of Ca^{2+} to a (yet undefined) site specific for inactivation. Alternatively, it may be hypothesized that some Ca^{2+}-induced pore collapse takes place as basis for inactivation. Such a mechanism has been proposed for *h*TRPM2 (Tóth and Csanády, 2012). As soon as 2-APB is present in the pore, collapse may be prevented without direct interference with Ca^{2+}. In any case, the effects of 2-APB are immediately reversible after wash-out.

As a side-note with the potential of an experimental pitfall, we would like to add that 2-APB may interfere with the large cation NMDG in a manner that is difficult to interpret biologically but may lead to incorrect conclusions in some experiments. When extracellular Na^+ is substituted by NMDG and Ca^{2+} is present as sole permeable cation at a concentration of 10 mM, ADPR induces Ca^{2+} influx but 2-APB does not. However, this is not due to an inhibition of Ca^{2+} permeation by 2-APB because isosmotic substitution of NMDG with sucrose restitutes Ca^{2+} currents (Kühn et al., 2017). Corresponding observations were made with 2-APB and the TRPV6 channel (Kovacs et al., 2012). Thus, it appears that NMDG blocks pore entry of 2-APB.

It is hoped that further comparison between *nv*TRPM2 and *h*TRPM2 and the study of pore chimeras will produce insight on the structural requirements that govern inactivation.

RESPONSES TO H_2O_2 DEMONSTRATE THE FUNCTIONAL ROLE OF THE NUDT9H DOMAIN

A key feature of all TRPM2 channel orthologs studied previously (which were all mammalian representatives without exception) is their activation in response to oxidative stress (Hara et al., 2002; Fonfria et al., 2004) that is experimentally simulated by the extracellular application of H_2O_2 (Wehage et al., 2002). Currently the most accepted hypothesis is that H_2O_2 activates the channel indirectly through an accumulation of intracellular ADPR (Perraud et al., 2005). This view is supported by inside-out patch-clamp experiments in which H_2O_2 apparently had no direct effects on human TRPM2 (Tóth and Csanády, 2010). In extension of this view, a recent study reported that H_2O_2 sensitizes *h*TRPM2 to the activation by physiological body temperatures; the sensitization is achieved by the oxidation of a methionine residue localized in the N-terminus of the channel (Kashio et al., 2012). This mechanism, under some experimental conditions and probably *in vivo*, may contribute to channel activation in response to oxidative challenges.

Since not only this specific methionine residue is also conserved in *nv*TRPM2 but also *nv*TRPM2 is more sensitive to ADPR than *h*TRPM2, it was expected to confirm H_2O_2 responses as well, and probably stronger and faster ones because accumulated ADPR should activate *nv*TRPM2 more easily than *h*TRPM2. The opposite findings were obtained. H_2O_2 completely failed to induce any currents. This could not be helped by increasing the concentration or the time of incubation of H_2O_2; *nv*TRPM2 presented itself as a channel highly sensitive to ADPR but entirely insensitive to H_2O_2 (Kühn et al., 2015).

Genetic manipulations of the NUDT9H domain in *h*TRPM2 have revealed that its function is easily disturbed by subtle changes. There are quite a few point mutations that render channels completely insensitive to ADPR. Several short sequences were deleted or substituted with the same result (Hara et al., 2002; Kühn and Lückhoff, 2004; Perraud et al., 2005). When analogous changes were introduced in *nv*TRPM2, again surprising findings were obtained. In no case, any change in the response to ADPR could be demonstrated. However, these manipulations produced channels that were now readily activated by H_2O_2. It is not worthwhile to summarize here the specific alterations of NUDT9H that were studied because it turned out that none of them contributes to the understanding of *nv*TRPM2 channel function. Instead, they gave rise to a radically different perspective on the role that NUDT9H plays in *nv*TRPM2, additionally guided by elegant experiments from Perraud et al. (2005) who co-expressed TRPM2 channels along with a cytosolic variant of the ADPR-degrading human NUDT9 enzyme. This co-expression suppressed the H_2O_2-induced activation of human TRPM2 which is accomplished by intracellularly accumulating ADPR. Therefore, we speculated that the NUDT9H domain of *nv*TRPM2 did not mediate the activation by ADPR at all; instead, it prevented the activation by H_2O_2 by degrading ADPR in the vicinity of the channel pore. This latter role would fit very well to the two critical residues (EF instead of IL) in the enzymatic domain; furthermore, all changes that created H_2O_2 sensitivity could then be interpreted as loss of ADPR degradation.

As a definite experimental test of the hypothesis, a *nv*TRPM2 channel variant was constructed in which the entire NUDT9H domain had been deleted (*nv*TRPM2-ΔNUD). The absence of large parts of the C-terminus may lead to unpredictable structural changes of the protein, possibly resulting in misfolding and aberrant surface expression. Accordingly, it was mandatory to verify the correct surface expression of this variant. It is later discussed that incidentally, these expression studies revealed unforeseen insight into the function of the human NUDT9H domain. Not unforeseen, however, but rather hoped for as confirmation of the tested hypothesis, were the results on *nv*TRPM2-ΔNUD. To begin with, the surface expression was almost normal which is prerequisite for further functional studies. These studies then revealed that sizeable currents were induced by ADPR, such that the absence of the NUDT9H domain did by no means preclude channel activation by ADPR. In further confirmation of the hypothesis, H_2O_2 proved as an effective current activator on *nv*TRPM2-ΔNUD, in line with a missing ADPR degradation in the absence of a NUDT9H domain. As a more direct proof for the catalytic activity of the NUDT9H domain of *nv*TRPM2 and for its role in preventing channel activation by H_2O_2, calcium imaging experiments were performed on HEK-293 cells in which *nv*TRPM2-ΔNUD was co-expressed together with one of the following NUDT9 variants (see **Figure 1**): an essential part of the human NUDT9 enzyme (aa 77–350), the isolated NUDT9H domain of *nv*TRPM2 (aa 1,289–1,551), or the isolated NUDT9H domain of *h*TRPM2 (aa 1,253–1,503). Stimulation with H_2O_2 resulted in Ca^{2+} influx through *nv*TRPM2-ΔNUD when the enzymatic inactive

NUDT9H domain of *h*TRPM2 was co-expressed. In contrast, co-expression of the human NUDT9 enzyme as well as of the NUDT9H domain of *nv*TRPM2 drastically suppressed the H_2O_2 responses of *nv*TRPM2-ΔNUD (Kühn et al., 2016). Therefore, the evidence is compelling that the ADPRase activity of the NUDT9H domain in *nv*TRPM2 is of decisive functional relevance, as is in the opposite way the loss of ADPRase activity in *h*TRPM2, which has already been demonstrated with the analogous experiments by Perraud et al. (2005).

Thus, the original approach of studying species variants, to elucidate the apparently unique NUDT9H-directed activation of TRPM2 by ADPR, led to the realization that there exist two completely different mechanisms for ADPR-dependent channel gating (Kühn et al., 2016), one present in mammals and one in cnidarians.

The cartoon in **Figure 3** is intended to illustrate the results on H_2O_2 stimulation of heterologously expressed wild-type *h*TRPM2, wild-type *nv*TRPM2, and of *nv*TRPM2 variants in which different parts of the NUDT9H domain have been modified or deleted.

Several questions immediately arise. The first one is how wild-type *nv*TRPM2 can show as extremely sensitive to ADPR in patch-clamp experiments (Kühn et al., 2015) when the NUDT9H domain degrades all ADPR in the vicinity of the channel. Possibly, its ADPRase activity is overpowered by the inexhaustible ADPR supply of the patch pipette. This interpretation is in line with the experimental findings of Perraud et al. (2005) where the ADPRase activity of the co-expressed human NUDT9 enzyme lost its relevance when increased concentrations of ADPR were used in the patch-clamp pipette.

As second question for which no easy answer is available at present, we have to ask how ADPR accomplishes gating in the absence of NUDT9H and whether there is an additional binding site for ADPR. It is plausible that such a binding site should be in the N-terminus as only longer intracellular region of *nv*TRPM2-ΔNUD.

*nv*TRPM2—A PROTOTYPE FOR A NOVEL MECHANISMS OF ADPR-DIRECTED CHANNEL ACTIVATION

In principle, ADPR-dependent channel activation would not necessarily require a binding site. Alternatively, mechanisms like ADP-ribosylation should be discussed. PARPs, however, would be no good candidates which could achieve such a modification of an ion channel because they are all transferases and transfer the ADPR-moiety from the cofactor NAD^+ to the protein (Barkauskaite et al., 2015); free ADPR is not a suitable substrate for PARP enzymes. Moreover, ADPR is able to induce channel gating in cell-free patches. Unless there would be membrane-associated enzymes that accomplished ADP-ribosylation of *nv*TRPM2, which seems somewhat remote in our opinion, the finding strongly contradicts such a mechanism (Kühn et al., 2016).

Potential binding sites might either exhibit known motifs for ADPR binding such as the Nudix box, or represent a new type of interaction. They are not expected to have ADPRase activity because the non-hydrolyzable ADPR analog AMPCPR is fully accepted as an activator (own unpublished results).

While Nudix box motifs cannot be found in the N-terminus or in other cytosolic parts of *nv*TRPM2, future search of binding sites may be guided by studies on a protein module ubiquitous in eukaryotes, bacteria, and archaea (Chakravarthy et al., 2005). The module was originally characterized in the histone variant macroH2A (Chakravarthy et al., 2005) and is well known for its capability to bind ADPR (Karras et al., 2005).

POSSIBLE ROLE OF *nv*TRPM2 *IN VIVO*

Considering the unique functional properties demonstrated by the *nv*TRPM2 channel in HEK-293 cells, the question arises how a combination of an ADPR-sensitive channel and a catalytic active ADPRase function might work *in vivo* in the sea anemone. Concededly, it is not the primary goal of our present research or of this review to describe the physiological role that TRPM2 plays in *Nematostella vectensis*. Moreover, fundamental information is lacking that would put any speculation on such a role on a more solid basis. In particular, data on the spatial and temporal expression of TRPM2 in *Nematostella vectensis* are still missing. Nevertheless, some thoughts on this topic may be outlined here.

During evolution, the sea anemone has separated from man some 800 million years ago. Notwithstanding, there is a striking degree of conservation concerning the gene families in the genome of *Nematostella* and vertebrates, as revealed by expressed sequence tag (EST) and genome analyses. This indicates that many ancestral traits have been preserved in *Nematostella* (Genikhovich and Technau, 2009). For this very reason, *Nematostella vectensis* currently represents a model organism in which fundamental biological processes are intensely studied, such as axial patterning, plasticity of the nervous system or stress responses (Layden et al., 2016).

Certainly, oxidative stress plays an important role in sea anemones in their natural habitat (Goldstone, 2008; Reitzel et al., 2008; Tarrant et al., 2014). However, at present there is no information about the intracellular regulation of the ADPR concentration. In particular, it has not been proven that intracellular ADPR is mobilized by oxidative stress, as in mammalian cells, although PARPs and PARGs as well as the NUDT9 enzyme are represented in the genome of *Nematostella vectensis* (as derived from the Joint genome institute database). Thus, at least the signaling cascade that leads to the activation of a Ca^{2+}-permeable, depolarizing cation channel as consequence to DNA damage, seems to be fully constituted in *Nematostella*.

Speculations on how this cascade proceeds are nourished by the peculiar kinetics of *nv*TRPM2. In studies on human TRPM2, extracellular application of H_2O_2 as an experimental paradigm of oxidative stress leads to an extended channel activation, resulting invariably in a permanent and massive elevation of the intracellular Ca^{2+} concentration (**Figure 4A**). This kind of response fits well to other observations in which H_2O_2 induces apoptosis in a TRPM2-dependent manner (Miller and Zhang, 2011; Naziroglu, 2011; Takahashi et al., 2011). In contrast, the consequences of *nv*TRPM2 activation *in vivo* are anticipated to

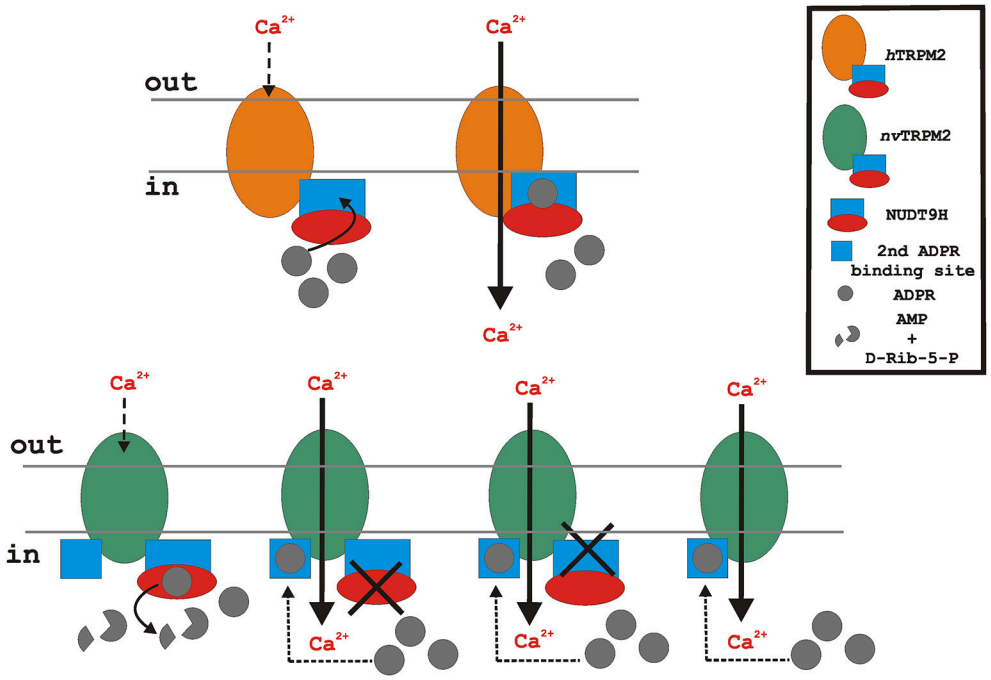

FIGURE 3 | Putative functional role of the endogenous NUDT9H domain of the TRPM2 orthologs during oxidative stress (stimulation with H_2O_2). Oxidative stress leads to intracellular accumulation of ADPR. In the case of *h*TRPM2 (upper), ADPR binds to the cytosolic NUDT9H domain (lacking significant ADPRase activity) and initiates channel activation. In contrast, *nv*TRPM2 (lower, left) exhibits full catalytic activity. Therefore, ADPR is degraded and the cytosolic concentration of ADPR remains too low to initiate channel activation. However, when the enzymatic function of the NUDT9H domain of *nv*TRPM2 is disrupted due to point mutations, interfering either with binding or with cleavage of ADPR, or due to the deletion of the entire NUDT9H, the accumulated intracellular ADPR enables channel gating via a second interaction site. The cartoon reflects the experimental situation of *nv*TRPM2 over-expression in mammalian cells (HEK-293) with no external ADPR added to the cytosol. Note that for the proper function of *nv*TRPM2 in their native environment, a cellular mechanism is required that controls the catalytic activity of the NUDT9H domain and thereby enables ADPR-dependent gating of *nv*TRPM2.

be far less drastic than cell death because channel activation is short and followed by immediate inactivation. This is already evident in patch-clamp experiments. An approach that is closer to a physiological situation are calcium imaging experiments because the cytosol is left intact and can be controlled and regulated by the cells. However, an ADPR-mediated stimulation of *nv*TRPM2 is not feasible in such experiments because ADPR cannot be applied intracellularly and because application of H_2O_2 is without effect on wild-type *nv*TRPM2 (**Figure 4B**). As discussed, this lack of *nv*TRPM2 response, which occurs in spite of the high ADPR sensitivity, is due to the degradation of ADPR by the catalytic active NUDT9H domain. When the enzymatic activity is abrogated by genetic manipulations, e.g., by deletion of the entire NUDT9H domain, H_2O_2 becomes effective and evokes increases in $[Ca^{2+}]_i$ by Ca^{2+} entry through the *nv*TRPM2 variants. As expected, these Ca^{2+} responses are characterized by a lag time, by a rapid increase of $[Ca^{2+}]_i$ after the lag time, and by a fast decline. Moreover, oscillations of $[Ca^{2+}]_i$ are consistently found that display as sharp peaks of $[Ca^{2+}]_i$, fast returns to baseline, and extended periods at baseline level prior to the next sharp peak (**Figure 4C**). In many experiments, such oscillations were observed already in the absence of H_2O_2. Therefore, the basal ADPR concentration in the chosen cell model for heterologous overexpression (HEK-293) is sufficient for *nv*TRPM2 stimulation, provided the ADPR degradation by the NUDT9H domain is prevented.

Without doubt, $[Ca^{2+}]_i$ oscillations play a pivotal role in many important physiological processes (e.g., circadian rhythm, fertilization) and oscillatory Ca^{2+} signaling associated with endogenously expressed TRPM channels has been described in *Caenorhabditis elegans* (Xing and Strange, 2010); hence, the finding may well be meaningful for the physiological role of TRPM2 in the sea anemone. Unfortunately, there are still a lot of fundamental questions about the oscillations.

Primarily, it is unclear how degradation of ADPR by the NUDT9H domain in *nv*TRPM2 should be prevented in *Nematostella in vivo*. One way how this might happen was demonstrated by Carloto et al. (2006) in studies with the human NUDT9 enzyme. In the presence of H_2O_2, the preferred divalent cation for the ADPRase activity becomes Mn^{2+} rather than Mg^{2+} which then can no longer act as cofactor; the result is an increased K_m for ADPR. Accordingly, treatment with H_2O_2 virtually abolishes the enzymatic activity with Mg^{2+} as cofactor (Carloto et al., 2006).

Mechanistically, it is not easy to understand how the $[Ca^{2+}]_i$ oscillations are accomplished. Channel inactivation is certainly a key element because 2-APB (1 mM) induces no oscillations but instead causes a permanent increase in $[Ca^{2+}]_i$ (**Figure 4D**).

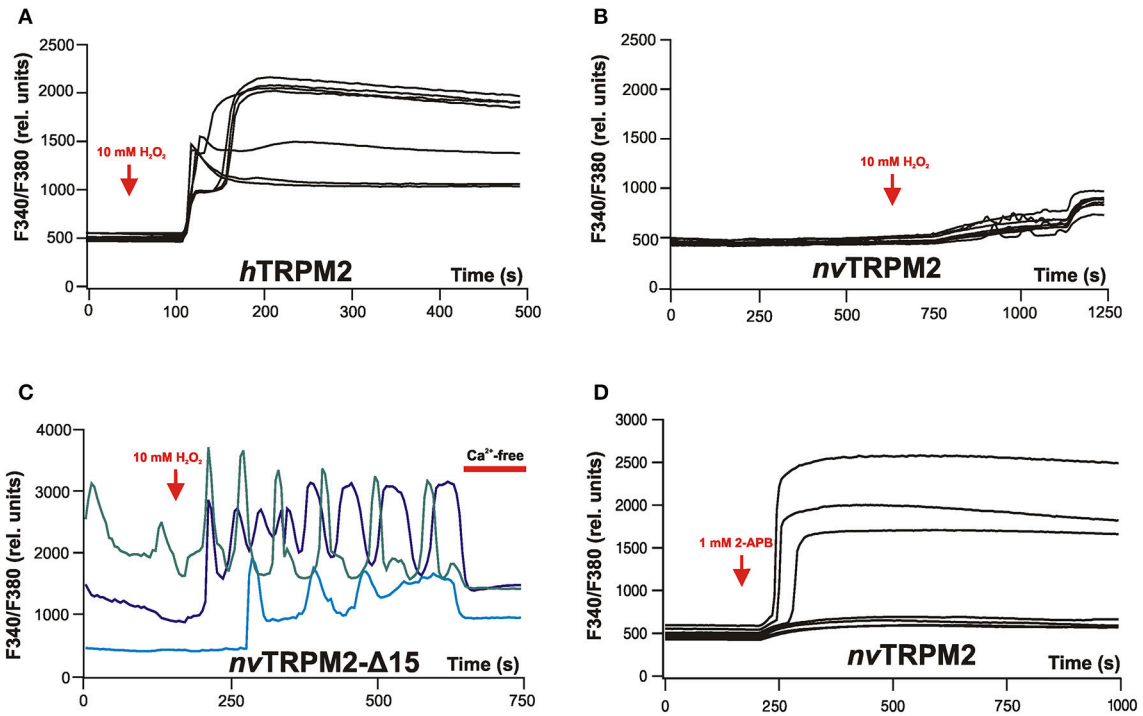

FIGURE 4 | Calcium imaging reveals typical effects on intracellular Ca^{2+} concentrations evoked by stimulation of either *h*TRPM2 or of *nv*TRPM2 channels in HEK-293 cells. **(A)** Characteristic changes of the intracellular Ca^{2+} concentration of cells transfected with human TRPM2 after extracellular stimulation of the cells with 10 mM H_2O_2. Note the plateau-like increases in $[Ca^{2+}]_i$. **(B)** Cells transfected with *nv*TRPM2 do not respond to oxidative stress. **(C)** Cells are transfected with a channel variant of *nv*TRPM2 where the ADPRase activity of the NUDT9H domain has been disrupted by a deletion. H_2O_2 induces characteristic oscillations of $[Ca^{2+}]_i$. For better distinction, the individual curves are highlighted in different colors. After replacing the standard bath solution (containing 1.2 mM Ca^{2+}) with a divalent-free bath solution (containing 10 mM EGTA) the oscillations stop. **(D)** Stimulation of *nv*TRPM2 by extracellular application of 2-APB (1 mM). A plateau-like increase in $[Ca^{2+}]_i$ results because 2-APB is an activator and prevents channel inactivation at the same time. Responses of non-transfected cells are shown as negative control. Figures are slightly modified from Kühn et al. (2015, 2017).

However, patch-clamp experiments have so far not revealed how inactivation can be temporarily reversed, which seems to be a prerequisite for oscillations. Moreover, this must happen in an extremely homogenous and synchronized manner within the total channel population of a cell. Cyclic regulation of the ADPRase activity of the NUDT9H domain cannot be an explanation because oscillations were observed exclusively in mutants where this region is dysfunctional. Ca^{2+}-dependent regulation of other ADPR-degrading enzymes is a theoretical possibility without experimental evidence.

So there are ample research opportunities for scientists fascinated by the biology of the sea anemone and by the signaling that *nv*TRPM2 may participate in. Our own interests are focused more on what is outlined in the following chapter.

LESSONS TO BE LEARNED FROM *nv*TRPM2 FOR THE GATING MECHANISM OF *H*TRPM2

When we started the studies on *nv*TRPM2, our long-lasting and general aim was gain of insight on the relation between structure and function of the human TRPM2 channel, with emphasis on gating as consequence of ADPR binding. We thought we were on a promising path when we achieved the functional expression of *nv*TRPM2 as ADPR-activated channel. Then we discovered that the NUDT9 homology region fulfills opposite functions in sea anemone and man, dampening the hope for learning with this approach how the C-terminus contributes to gating.

Obviously, evolution had used strongly divergent paths to create an ADPR-gated channel in cnidarians and mammals which could not be anticipated. In spite of the unexpectedly large functional inter-species discrepancies, our research on *nv*TRPM2 directed us toward experiments on the human C-terminus that reveal valuable information on the function of this domain so different from *Nematostella*.

Although many structural requirements for ADPR binding have been defined in *h*TRPM2 as well as in the NUDT9 enzyme, the most urgent question remains how ADPR binding to the channel creates the structural re-arrangement decisive for gating and which parts of the protein participate.

In a recent study, a detailed structural model for the binding of ADPR to the NUDT9H domain of the *h*TRPM2 channel was proposed (Yu et al., 2017). This model is basically guided by the crystal structure of the human NUDT9 enzyme (Shen et al., 2003). However, as promising as this approach may be,

a structural view on the isolated NUDT9H domain entails the risk of misinterpreting the situation in the full-length channel. This reservation does not concern a potential design of drugs that might modify the function of the NUDT9H domain. But this approach does not necessarily elucidate the structural basis of the interaction between the NUDT9H domain and the other parts of the channel. Hints that may shed light on this interaction derive from our experimental findings that certain C-terminal modifications of *h*TRPM2 interfere with channel function (Kühn et al., 2015, 2016). These findings demonstrate that not only subtle alterations within the NUDT9H region have a strong impact on the human channel but also manipulations of the C-terminus outside of this region. Importantly, these modifications appear to compromise the NUDT9H domain independently of the function that the region fulfills in each species. In *h*TRPM2, gating is prevented, whereas ADPR degradation is abolished in *nv*TRPM2.

In future, it will be a major challenge to integrate all the experimentally gained information on particular mutated single amino acids in *h*TRPM2 and *nv*TRPM2 into structural models that may help to explain the interaction of various parts of the whole protein on a mechanistic level.

With respect to the yet unknown interaction mode between NUDT9H and channel core in *h*TRPM2, the disturbance of the NUDT9H domain by modifications outside of it raises further questions. It should be studied in detail whether binding of ADPR is impeded or whether a subsequent step within the gating process is affected. Iordanov et al. (2016) have already presented evidence that within the NUDT9H region, about 20% of C-terminal sequence might represent an interface for the transduction of ligand binding to pore-opening. It is imagined that larger parts of the protein participate geometrically to orchestrate a fully functional interaction.

In general, the comparison of *nv*TRPM2 and *h*TRPM2 remains an attractive approach to delineate the structural basis for particular functional details, but these studies are only at their beginning.

CONCLUDING REMARKS

In the sea anemone *Nematostella vectensis* as well as in mammals, TRPM2 represents a cation channel activated by ADPR. This mode of channel activation is unique and not found for any other known channels. However, the mechanisms how ADPR achieves gating are remarkably distinct in the orthologs, and opposite tasks have been assigned to the NUDT9H domain. Hence, TRPM2 is a fascinating example how one gene in distantly related species has evolved in a strikingly divergent manner and still has gained analogous functional properties. At the same time, evolution has created critical but diametrically different roles for homologous parts of the protein. In the NUDT9 domain of *nv*TPM2, as opposed to the situation in *h*TRPM2, catalytic function is conserved and bears functional importance for channel function. Thus, *nv*TRPM2 can be considered a true and unquestionable chanzyme.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Neuropathic Pain: Delving into the Oxidative Origin and the Possible Implication of Transient Receptor Potential Channels

Cristina Carrasco[1], Mustafa Naziroğlu[2], Ana B. Rodríguez[1] and José A. Pariente[1]*

[1] Department of Physiology, Faculty of Sciences, University of Extremadura, Badajoz, Spain, [2] Neuroscience Research Center, Suleyman Demirel University, Isparta, Turkey

Currently, neuropathic pain is an underestimated socioeconomic health problem affecting millions of people worldwide, which incidence may increase in the next years due to chronification of several diseases, such as cancer and diabetes. Growing evidence links neuropathic pain present in several disorders [i.e., spinal cord injury (SCI), cancer, diabetes and alcoholism] to central sensitization, as a global result of mitochondrial dysfunction induced by oxidative and nitrosative stress. Additionally, inflammatory signals and the overload in intracellular calcium ion could be also implicated in this complex network that has not yet been elucidated. Recently, calcium channels namely transient receptor potential (TRP) superfamily, including members of the subfamilies A (TRAP1), M (TRPM2 and 7), and V (TRPV1 and 4), have demonstrated to play a role in the nociception mediated by sensory neurons. Therefore, as neuropathic pain could be a consequence of the imbalance between reactive oxygen species and endogen antioxidants, antioxidant supplementation may be a treatment option. This kind of therapy would exert its beneficial action through antioxidant and immunoregulatory functions, optimizing mitochondrial function and even increasing the biogenesis of this vital organelle; on balance, antioxidant supplementation would improve the patient's quality of life. This review seeks to deepen on current knowledge about neuropathic pain, summarizing clinical conditions and probable causes, the relationship existing between oxidative stress, mitochondrial dysfunction and TRP channels activation, and scientific evidence related to antioxidant supplementation.

Keywords: neuropathic pain, oxidative stress, inflammation, mitochondrial dysfunction, TRP channels, antioxidants

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****Correspondence:***
Cristina Carrasco
ccarom@unex.es

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INTRODUCTION

Currently, neuropathic pain (NP) is an underestimated socioeconomic health problem affecting millions of people worldwide. It has been recently redefined by the International Association for the Study of Pain as a "pain caused by lesion or disease of the somatosensory system" and it may appear in a wide range of conditions; it can be classified into peripheral or central NP, depending on anatomic location of the lesion or disease. Without a specific diagnostic tool, both clinicians and researchers might use a grading system with different levels of certainty about the presence

of NP ("possible," "probable," and "definite") in a patient; however, it should be mentioned that coexisting NP and other types of pains (such as nociceptive pain) can make difficult a reliable distinction (Treede et al., 2008; Jensen et al., 2011). The common symptoms of different types of NP are mechanical allodynia and hyperalgesia. Unlike nociceptive pain, commonly prescribed analgesics often fail in alleviating NP. Thus, it can become a chronic and hardly bearable condition, recently called refractory NP. In some cases, this most severe NP may lead to increased episodes of depression and suicide (Torrance et al., 2013; Hassler et al., 2014; Kawaguchi et al., 2014).

Further research is needed to understand underlying mechanisms of NP that allow to design individual and rational treatment strategies. Growing evidence points that mitochondrial dysfunction induced by oxidative and nitrosative stress, along with inflammation, constitute the physiopathological basis for the development of several diseases (Carrasco et al., 2015). Regarding to NP, this adverse context leads to peripheral and central sensitization. It must be considered that mammalian nerves are especially susceptible to free radicals, including oxygen (ROS) and nitrogen reactive species (RNS), due to their high content in phospholipids and axonal mitochondrion; in addition, neuronal antioxidant defenses are weak (Areti et al., 2014). Studies about antioxidant supplementation in animal models of NP point that hydroxyl ($^{\bullet}OH$) and superoxide ($O_2^{\bullet-}$) radicals and nitric oxide ($^{\bullet}NO$) might be involved in the physiopathology of this kind of pain (Kawaguchi et al., 2014). However, the way these messenger molecules regulate pain signaling is still poorly understood. In addition, overload intracellular calcium ion (Ca^{2+}) has also an important role in the etiology of NP. Ca^{2+} enters cells in different ways including cation channels. Voltage gated calcium channels and chemical channels (i.e., glutamate) are well known calcium channels (Kumar et al., 2014). Moreover, new calcium channels namely transient receptor potential (TRP) superfamily were discovered in eye cells of Drosophila flyers (Hardie, 2011; Naziroğlu, 2011). In different species, TRP superfamily is divided into 30 channels within seven subfamilies such as TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin), TRPV (vanilloid), and TPRN (NomPC), nevertheless there are 28 subfamilies within 6 subgroups in mammalian (Hardie, 2011; Naziroğlu, 2011; Uchida et al., 2017). At least, nine members of TRP superfamily are activated by oxidative stress including TRPM2, TRPM7, TRPA1, TRPC3, TRPC5, TRPC6, TRPV1, TRPV3, and TRPV4 (Ogawa et al., 2016). Dorsal root ganglion (DRG) neurons play an important role in the painful NP. There is no barrier between the DRG and blood and compounds with high molecular weight can easily diffuse into the DRG (Abram et al., 2006). Expression levels of TRPA1, TRPM2, TRPV1, and TRPV4 channels are high in the DRG and trigeminal ganglia neurons (Kobayashi et al., 2005; Obata et al., 2005; Fonfria et al., 2006; Naziroğlu, 2011). Hence, the TRPA1, TRPM2, TRPV1, and TRPV4 play an important role in the nociception mediated by sensory neurons, including the DRG (Materazzi et al., 2012; Özdemir et al., 2016; Kahya et al., 2017). Therefore, increased ROS/RNS levels induced by several clinical conditions may play a deleterious effect on different biomolecules (e.g., lipids, proteins and nucleic acids), organelles and antioxidant defenses, leading to exacerbate nitro-oxidative stress, mitochondrial dysfunction, glial activation and inflammatory response. Recent evidence also points out the possible implication of TRP channels in NP. Altogether, this adverse context is ultimately responsible of the typical painful symptoms of NP (**Figure 1**).

This review summarizes current knowledge about NP, focusing on clinical conditions and probable causes, the relationship existing among oxidative stress, mitochondrial dysfunction and TRP channels activation and scientific evidence related to antioxidant supplementation.

Spinal Cord Injury

Following spinal cord injury (SCI), individuals suffer not only motor dysfunction but also the development of chronic NP. Up to 80% of patients experience this condition within months after injury, which dramatically impairs their quality of life; thus, taking in account that this kind of NP is refractory to clinical treatments, depression and suicide are very frequently (Stillman et al., 2017). It is thought that after SCI, many neuroadaptation responses are implemented in dorsal horn, triggering central mechanisms which likely contribute to NP. Apart from dysfunction of neurons, other pathogenic events have been defined about post-SCI pain, including pro-inflammatory signaling, microglia activation and intracellular Ca^{2+} alteration; however, little is known about how oxidative stress may play a critical role in this condition (Hulsebosch et al., 2009; Due et al., 2014).

Evidence supports that after a central nervous system injury, increased extracellular glutamate levels activate several intracellular pathways including ROS formation; this change in redox status promotes a leukocyte mediated pro-inflammatory response that ultimately leads to the exacerbation of secondary damage (Hulsebosch et al., 2009). In this sense, some studies have implicated the lipid peroxidation byproduct (acrolein) in many neuropathological diseases and NP modalities (Shi et al., 2011). During inflammation and trauma acrolein is released, causing damage to biomolecules and altering several cellular processes in neurons, including mitochondria functionality. Consequently, this aldehyde is well known to be a potent oxidant, perpetuating a vicious cycle of oxidative stress which may partially explain its role as TRPA1 agonist. Due et al. (2014) have reported increased levels of acrolein within or near the injured site for at least 2 weeks following experimental SCI, a phenomenon which was correlated with the onset of sensory and behavioral hypersensitivity in rats. Moreover, exogenous administration of acrolein into rodent spinal cord also induced pain symptomatology, whereas the

Abbreviations: APN, Alcoholic peripheral neuropathy; CIPN, chemotherapy-induced peripheral neuropathy; DRG, dorsal root ganglion; ETC, mitochondrial electron transport chain; GSH, glutathione; NAC, N acetyl cysteine; NP, neuropathic pain; PARP-, poly (ADP-ribose) polymerase-1; PDN, peripheral diabetic neuropathy; PKC, protein kinase C; ROS, reactive oxygen species; RNS, reactive nitrogen species; SCI, spinal cord injury; TRP, transient receptor potential; TRPA1, transient receptor potential ankyrin 1; TRPM2, transient receptor potential melastatin 2; TRPV1, transient receptor potential vanilloid 1; TRPV4, transient receptor potential vanilloid 4.

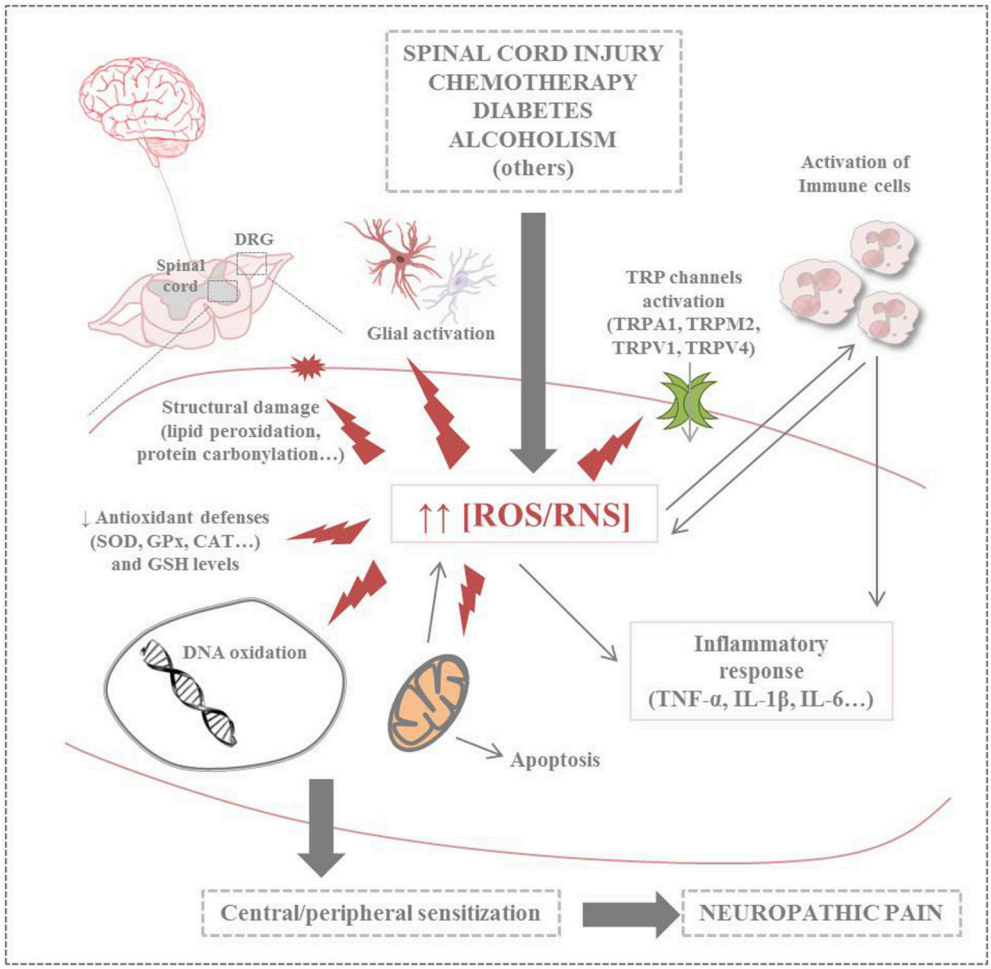

FIGURE 1 | Summary of harmful effects of nitro-oxidative stress on neuronal cells in neuropathic pain (CAT, catalase; GPx, glutathione peroxidase; GSH, glutathione; IL-1/6, interleukin 1/6; ROS/RNS, reactive oxygen species/reactive nitrogen species; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; TRP, transient receptor potential; TRPA1, transient receptor potential ankyrin 1; TRPM2, transient receptor potential melastatin 2; TRPV1/4, transient receptor potential vanilloid 1/4).

treatment with its scavenger hydralazine modestly diminished sensitivity to both tactile and thermal stimuli. Researchers also performed *in vitro* assays observing that acrolein increases neuronal excitation. Together, these findings strongly support the pro-nociceptive role of acrolein likely via TRPA1 activation, as deduced by increased levels of this receptor in sensory ganglia also observed in the referred study. But as authors mentioned, other TRP channels might also be sensitive to acrolein and contribute to SCI-induced NP. In accordance with these observations, Park et al. (2014) have also reported *in vivo* evidence about the crucial role of acrolein in the pathogenesis of spinal cord trauma and the potential use of hydralazine as analgesic. In this study, a significant reduction of acrolein levels, tissue damage, motor deficits and NP was observed in hydralazine-treated rats.

On the other hand, other mediators may also be part of the pathophysiological mechanisms triggered by oxidative stress in this kind of NP, such as aquaporin-1. Apart from mediating several physiological processes via water transport, aquaporin-1 must also play a not still well understood role in the etiology of different neuropathological conditions; it is supposed to contribute to some of the typical outcomes such as edema and cyst formation. In experimental models of SCI, this protein has been found to be significantly elevated for up to 11 months in sensory axons, neurons, astrocytes, and ependymal cells, despite consequent loss of nerve tissue at the site of injury. Experimental data point oxidative stress as one of the factors that contributes to aquaporin-1 up-regulation since administration of the antioxidant melatonin not only reduced protein levels but also mechanical allodynia and appearance of aquaporin-1-positive fibers below laminae I and II (Nesic et al., 2008).

More recently, *in vivo* proteomic approaches of SCI have demonstrated that peripheral nerve injury alters the expression and/or subcellular distribution of some specific dorsal horn proteins, for example those involved in nociceptive signaling, cellular metabolism, plasma membrane receptor trafficking, oxidative stress, apoptosis and degeneration (Lee et al., 2003; Kunz et al., 2005; Singh et al., 2009). In this sense, the TRP

superfamily's member TRPM4 should be mentioned. TRPM4 is a non-selective, Ca^{2+}-impermeable channel that exclusively transports monovalent cations. TRPM4 is activated by increased intracellular ATP concentration and oxidative stress, but it is inhibited by intracellular ATP depletion (Nilius et al., 2004; Simon et al., 2010). Involvement of the Cys1093 residue oxidation in TRPM4 channel activation has also a significant role in oxidative stress dependent (without ATP depletion) activation and desensitization of TRPM4. Involvement of hydrogen peroxide (H_2O_2) is well known for induction of necrosis; in endogenously expressed TRPM4 HeLa cells, H_2O_2 induction of both necrosis and apoptosis has been shown (Simon et al., 2010). TRPM4 has an important role for induction of neurological diseases. For example, involvement of TRPM4 in etiology of SCI was reported by result of recent papers (Gerzanich et al., 2009; Lee et al., 2014).

Chemotherapy-Induced Peripheral Neuropathy

Many first-line chemotherapy agents used in the current clinical practice, such as platinum-based anticancer drugs (i.e., cisplatin, oxaliplatin), proteasome/angiogenesis inhibitors (bortezomib/thalidomide), vinca alkaloids (i.e., vincristine, vinorelbine) and taxanes (i.e., paclitaxel, docetaxel) cause a dose-limiting side effect called chemotherapy-induced peripheral neuropathy (CIPN) (Han and Smith, 2013; Kerckhove et al., 2017). This kind of NP involves predominantly sensory nerves and occurs in a stocking-and-glove distribution. Depending on anticancer drugs, 38–100% of cancer patients are affected by CIPN and its symptoms (mainly allodynia and hypersensitivity) may persist from months to years following cessation of anticancer treatment, a phenomenon known as *coasting*. It seems that chemotherapy regimens (drug or combination of drugs administered and dosing), methods of pain assessment and the individual patient characteristics (presence of comorbidities associated with increased risk of neuropathy, such as diabetes, depression, insomnia or genetic particularities) are some of the several factors that may influence on the onset of CIPN and the severity of symptoms. In addition, most of the effective analgesics in NP failed to provide symptomatic relief of CIPN and often exhibit side effects (Han and Smith, 2013; Griffiths and Flatters, 2015). Thus, CIPN may limit the dosing, duration and effectiveness of the treatment, affecting survival and quality of life of the patient (Ji et al., 2013; Kerckhove et al., 2017).

In general, unlike other types of NP (including those induced by trauma and diabetes) axonal degeneration in peripheral nerves is not present in CIPN (Ji et al., 2013). For this reason, NP is suspected to be a complex phenomenon resulting from the interrelation of various mechanisms. It has been observed that anticancer drugs may cause neuronal damage in a variety of ways, such as nuclear and mitochondrial DNA damage, ion channel disturbances (i.e., calcium, sodium and potassium), impairment of axonal transport and inflammatory process (Han and Smith, 2013; Massicot et al., 2013; Kerckhove et al., 2017). More recently, experimental evidence points oxidative stress and mitochondrial dysfunction as one of the common pathophysiological mechanisms responsible of neurotoxicity in CIPN. Thus, overproduction of ROS and RNS may affect redox status toward oxidation, interfering with the antioxidant defense system and cell function. An increase in several markers of oxidative stress (lipid peroxidation, carbonylated proteins and DNA oxidation) in experimental models of CIPN has been observed; moreover, homozygous individuals for GSTP1 105Ile allele, that encode the oxidative stress regulatory enzyme glutathione S-transferase pi 1, tend to suffer this type of NP more frequently than other people (Han and Smith, 2013). At mitochondrial level, structural integrity and energy function of this organelle are affected, triggering the apoptosis cascade. Furthermore, inefficient autophagy/mitophagy of damaged biomolecules and organelles leads to a vicious cycle in the cells, which ultimately exacerbates the progression of the CIPN typical neurodegeneration. Consequently, inflammatory response is activated in both neurons and immune system cells (Massicot et al., 2013; Areti et al., 2014). Specific oxidative damage features in CIPN induced by most commonly used chemotherapeutic agents are detailed below.

Regarding to CIPN induced by the taxanes, Duggett et al. (2016) analyzed oxidative stress in DRG neurons of rats treated with paclitaxel at three time-points (prior to pain onset (day 7), during (peak pain) and at resolution of pain). Whereas researchers did not find any change in mitochondrial ROS or $O_2^{\bullet-}$ levels while studying entire neuronal populations, analysis of separately subpopulations of nociceptive neurons showed a statistically significant increase of ROS levels in isolectin B4-positive neurons from treated rats compared to control rats at the three time-points studied. For authors, these results indicate that neuronal antioxidant defenses seem to be initially overwhelmed, leading to ROS overproduction prior to pain onset; this phenomenon causes mitochondrial dysfunction with increased superoxide radical levels at peak pain, triggering signaling cascades that may underlie the coasting effect, such as apoptosis. It has been observed that the apoptotic process in paclitaxel induced-painful neuropathy is due to the permeability transition pore opening, with the consequent cytochrome c release and Ca^{2+} homeostasis dysregulation (Areti et al., 2014). In this sense, Griffiths et al. (Griffiths and Flatters, 2015) reported that the selective pharmacological modulation of the mitochondrial electron transport chain (ETC) (at level of complex I or III) *in vivo* could also reverse or attenuate the above-mentioned symptoms, but with deleterious effects on motor coordination in the case of complex I inhibition at 3 and 24 h after paclitaxel administration; however, only complex III inhibition before and during the chemotherapy exposure caused an effective relief of pain in prophylactic studies. According to authors, paclitaxel administration causes mitochondrial dysfunction as first oxidative event, resulting in excessive ROS levels which could not be counteracted by the weak antioxidant defenses of neurons; due to this fact, ROS-driven pain behaviors start. In addition, other studies have also demonstrated the involvement of TRP channels in paclitaxel-induced pain, which are well known to be predominantly expressed on this subpopulation of DRG neurons. In fact, a specific cellular signaling pathway has been described in mice, including the activation of protease-activated receptor 2

and downstream enzymes phospholipase C and protein kinases A and C by mast cell triptase, and resultant sensitization of TRPV1, TRPV4, and TRPA1 (Chen et al., 2011). Later, Materazzi et al. (2012) observed *in vitro* that paclitaxel administration induced oxidative stress byproducts which ultimately activate TRPA1 and TRPV4. Thus, pharmacological TRP channel targeting may offer a future possibility to attenuate paclitaxel-induced mechanical and thermal hypersensitivity in clinical practice. In summary, as Kerckhove et al. (2017) point the oxidative stress in taxanes induced-NP not only causes damage to neuronal and non-neuronal cells, but also macrophage activation, with the consequent overproduction of pro-inflammatory cytokines, such as TNF-α and IL-1β. In fact, Li et al. (2015) have recently reported that paclitaxel treatment may also trigger pro-inflammatory mechanisms in DRG neurons via toll-like receptor 4, which is also associated to the sensitization of TRPV1.

On the other hand, platinum compounds such as oxaliplatin have a strong neurotoxicity compared to other anticancer drugs; more than 90% of patients develop acute neuropathy which could become chronic in a 30–50% of the cases. However, the complex machinery underlying CIPN is poorly understood. To date, it has been observed that the treatment with this kind of antitumor agents causes ROS generation, mitochondrial dysfunction (including frataxin deficiency, mitochondrial DNA damage and defective components of ETC), loss in antioxidant enzymes, ion channels disturbances and nerve tissue damage (protein carbonylation and lipid peroxidation) (Areti et al., 2014; Kerckhove et al., 2017). It is thought that various receptors and molecular pathways must be involved in the sensitization of peripheral and central sensory nerves, and thus, in the chronicity of CIPN. Recently, Massicot et al. (2013) have described *in vitro* and *in vivo* biochemical effects after exposure to high concentrations of oxaliplatin, including neuronal activation of the purinoreceptor subtype 7, ROS and $^{\bullet}$NO production, lipid peroxidation, loss of mitochondrial transmembrane potential and further apoptosis via caspase 3 activation. Regarding to immune response, oxaliplatin-treated neurons release significant amounts of pro-inflammatory cytokines, mainly TNF-α and IL-6, whereas prostaglandin E2 levels were significantly higher in macrophagues exposed to the platinum compound than in control cells. These inflammatory mediators might stimulate nociceptors, leading to generation and further chronification of painful symptoms. Overall, authors suggest that oxidative stress along with purinoreceptor subtype 7/inflammasome pathway would play a persistent role in oxaliplatin-induced neurotoxicity and its transition from acute to chronic NP. In addition, purinoreceptor subtype 7 activation has been observed to cause caspase-1 activation, which is ultimately involved in the expression of cyclooxygenase 2 and prostaglandin E2 and IL-β production.

Recently, vinca alkaloids have demonstrated to exert their neurotoxicity via activation of spinal cord glia (i.e., astrocytes and microglia), offering an option for the treatment of CIPN through pharmacological antagonism of this phenomenon. For authors such as Ji et al. (2013) spinal astrocytic activation, but no microglial activation, seems to contribute to mechanical allodynia in vincristine-treated rats, leading to overexpression of IL-β. This pro-inflammatory mediator might bind to its endogenous receptor to induce N-methyl-D-aspartic acid receptor phosphorylation in spinal dorsal horn neurons; thus, neuronal activity and nociceptive signaling would be enhanced. On the contrary, other studies have reported both astrocytic and microglial activation (Sweitzer et al., 2006; Kiguchi et al., 2008) after vincristine exposure, as well as up-regulation of other inflammatory cytokines such as TNF-α (Kiguchi et al., 2008). As mentioned by Ji et al. (2013), methodological differences (i.e., animal model, dosage and route of administration, etc.) might be one of the reasons for this discrepancy. In any case, oxidative stress would trigger the glial activation, as well as other processes. A study performed *in vivo* has revealed that vincristine-induced ROS overproduction may also increase the activity of the enzyme dipeptidylpeptidase IV and decrease the levels of spinal endomorphin-2. Authors' hypothesis reveals that the loss of this kind of endogenous inhibitory signal might contribute to allodynia and central sensitization, with the subsequent development of chronic pain.

Finally, bortemozib and thalidomide are well known to act as proteasome/angiogenesis inhibitors. However, a proteasome-independent mechanism might also contribute to CIPN; thus, mitochondrial dysfunction may also be involved (Kerckhove et al., 2017). Recently, Zheng et al. (2012) have reported in rat sciatic nerve how bortemozib, as other antitumor drugs, causes significant deficits in complex I and II of ETC, as well as in ATP production, at two time points (pain onset -day 7- and peak pain -day 35-).

Therefore, scientific evidence highlights the importance of early oxidative stress in the CIPN onset. But the important question that remains unresolved is: what happens first after chemotherapy administration? Mitochondrial dysfunction or increased ROS levels? In any case, this context leads to a vicious cycle which compromises ATP production and neuronal viability (**Figure 2**). Experts agree that, experimentally, the period between the last injection of the antitumoral agent and the time of normal onset of pain is a crucial window for an effective ROS scavenging. Taking into account all the above-mentioned, monitoring oxidative stress related-parameters during the course of CIPN could be helpful in clinical practice (Areti et al., 2014). Moreover, some studies have demonstrated the effectiveness of antioxidant therapies in this kind of neuropathic pain. These promising results about antioxidant supplementation in CIPN will be discussed later.

Diabetic Neuropathy

Peripheral diabetic neuropathy (PDN) is the most common diabetic complication in patients with diabetes (both type 1 and type 2). It may appear as a painful and insensate neuropathy, compromising patient's functionality, mood and quality of life. Symptomatology (i.e., paresthesia, spontaneous pain, tactile allodynia and mechanical and thermal hypo/hyperalgesia) may improve and resolve spontaneously, or culminate in total loss of sensation and ultimately in foot ulceration and amputation. Current clinical strategies for the management of PDN include glycolic control and treatment with drugs such as tricyclic

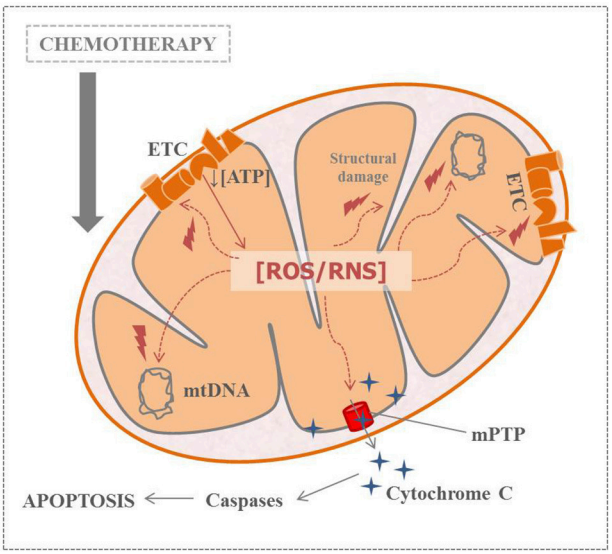

FIGURE 2 | Relation between nitro-oxidative stress, mitochondrial dysfunction and apoptosis in chemotherapy-induced peripheral neuropathy (ATP, adenosine triphosphate; ETC, electronic transport channel; mtDNA, mitochondrial DNA; mPTP, mitochondrial permeability transition pore; ROS/RNS, reactive oxygen species/reactive nitrogen species).

compounds, serotonin noradrenalin reuptake inhibitors, α-lipoic acid, anticonvulsants, opiates, membrane stabilizers and topical capsaicin; however, these therapeutic options are often inefficient, have significant side effects and its prescription depends on the presence of comorbidities (Obrosova et al., 2008; Pacher, 2008; Obrosova, 2009; Tesfaye, 2011; Ma et al., 2015).

Pathogenesis of PDN is poorly understood, but it is quite clear that hyperglycemia plays a vital role in the development of this diabetic complication. Experimental evidence points a multifactorial etiology, linking hyperglycemia with the activation of multiple cell phenomena such as polyol pathway, advanced glycation end-products formation, protein kinase C (PKC) and nuclear factor Kβ signaling, among many others (Pacher, 2008; Obrosova, 2009). It should be noted that, among other physiological effects, chronic hyperglycemia leads to an imbalance of the oxidative status, affecting central and peripheral nervous system; hence, nitro-oxidative stress is also thought to be one of the responsible factors of nervous degeneration that characterize PDN (Mirshekar et al., 2010; Tesfaye, 2011). In diabetic state, hyperglycemia leads to overproduction of free radicals (i.e., mitochondrial $O_2^{\bullet -}$, $^{\bullet}NO$ and peroxynitrite) mainly derived from glucose oxidation and lipid peroxidation, which cause oxidative damage to biomolecules. In particular, it is well known that oxidative DNA damage triggers the over-activation of the nuclear enzyme poly (ADP-ribose) polymerase-1 (PARP-1). An *in vivo* study performed by Obrosova (2009) demonstrated that administration of a PARP inhibitor counteract small sensory nerve fiber dysfunction and degeneration. Therefore, PARP-1 activation seems to play an important role in the pathogenesis of PDN in several ways, likely including the regulation of various important inflammatory pathways. In this sense, other studies have observed increased levels of TNF-α in diabetic animal tissues (Satoh et al., 2003; Skundric and Lisak, 2003). In addition, Ma et al. (2015) reported the role of mitochondrial bioenergetics deficits in PDN and its possible link with immune response. As authors highlighted, inflammatory signaling may lead to inhibition of ETC activity, through induction of changes in the phosphorylation state of proteins and the reduction of the mitochondrial membrane potential. Taking evidence in account, more studies are necessary to go deep into the participation of oxidative stress, mitochondrial dysfunction and inflammatory response in the development of PDN.

Alcoholic Peripheral Neuropathy

Long-term excessive consumption of alcohol may lead to a condition known as alcoholic peripheral neuropathy (APN). Like other types of NP, it is characterized by spontaneous pain, hyperalgesia and allodynia. Regarding to APN related-risk factors, duration and amount of total lifetime alcohol consumption have been demonstrated as the most determinants; interestingly, a higher prevalence has been found in women than men (Chopra and Tiwari, 2012). Although little is known about physiopathological mechanisms underlying APN, a combination of direct toxic effects of ethanol or its metabolites and nutritional deficiencies (mainly thiamine) may offer a plausible explanation to this complication.

To date, different molecular mechanisms (i.e., PKC and nuclear factor Kβ) (Dina et al., 2000), signaling pathways (i.e., MEK/ERK and apoptosis via caspase activation) (Jung et al., 2005; Dina et al., 2007), receptors (i.e., metabotropic glutamate and μ opioid receptors) (Miyoshi et al., 2007; Narita et al., 2007), nerve cells (i.e., astrocytes and microglia) (Narita et al., 2007) and neuroendocrine stress axis (i.e., sympatho-adrenal and hypothalamo-pituitary-adrenal axis) (Dina et al., 2008) have shown to be involved in the APN. Focusing oxidative stress, some studies have indicated that in heavy drinkers increased nitro-oxidative stress plays a pivotal role in the neuronal damage. This imbalance in redox status might be caused by acetaldehyde, a highly toxic and reactive metabolite derived from the biphasic catabolic conversion of ethanol to acetate, in particular, as a byproduct of the mitochondrial enzyme acetaldehyde dehydrogenase. In the liver, acetaldehyde is known to cause impairment of mitochondrial ETC and stimulation of inflammatory response, among other toxic effects (Chopra and Tiwari, 2012). Therefore, mitochondrial dysfunction may also lead to an inefficient detoxification and subsequent accumulation of acetaldehyde, worsening redox status and cytotoxic effects on biomolecules, including proteins, lipids and DNA. In this context, some *in vivo* studies have demonstrated that several oxidative markers are affected by following ethanol chronic administration. For example, glutathione (GSH) levels and GSH peroxidase activity have been observed to be diminished in the sciatic nerves of ethanol-fed rats compared to pair-fed rats; on the contrary, the amount of the lipid peroxidation product malondialdehyde increased in the same tissue (Bosch-Morell et al., 1998). More recently, Tiwari et al. (Tiwari et al., 2009, 2011) have confirmed significant increased levels of lipid peroxidation and marked decrease in GSH, superoxide dismutase

and catalase activities in the sciatic nerve of rats which were given alcohol for 10 weeks. Alcohol has been also found to enhanced production of hydrogen peroxide and $^{\bullet}OH$ like species (Dicker and Cederbaum, 1992). As mentioned above, ROS overproduction may lead to sensitization of dorsal horn cells, activation of spinal glial cells and inflammatory response, which ultimately activate PKC and nuclear factor Kβ translocation, MEK/ERK signaling and apoptosis.

Oxidative Stress Dependent TRP Channels and Pain

It is well known that ROS and RNS are produced in several physiological functions, such as mitochondrial and cytochromes P450 activities. These free radicals are scavenged by enzymatic and non-enzymatic antioxidants. At 2002, two different groups from Kyoto-Japan (Bautista et al., 2005) and Aachen-Germany (Kistner et al., 2016) reported activation of a TRP channel namely LTRPC2 (former name of TRPM2) by RNS and ROS. Today, 9 TRP channels (TRPA1, TRPC5, TRPM2, TRPM4, TRPM7, TRPV1, TRPV2, TRPV3, and TRPV4) are demonstrated to be activated by oxidative stress (Mori et al., 2016). Expression levels of these channels are very different in tissues and cells. For example, it has been observed that the expression levels of four TRP channels (TRPA1, TRPM2, TRPV1 and TRPV4) are high in neurons related to nociception. Hence, this section is focused on these four TRP channels.

TRPA1

Cysteine is a sulfur-containing amino acid in humans. Cysteine, as a source of thiol redox system, acts also as the main source of different antioxidants such as GSH, glutathione peroxidase, N acetyl cysteine (NAC) and α-lipoic acid. Hence, the cysteine groups are main target for ROS and RNS (Sen and Packer, 2000; Naziroğlu, 2007).

The TRPA1 channels are activated by different stimuli including chemicals (mustard oil and cinnamaldehyde) and cold body temperature (≤17°C). In addition, TRPA1 is also an oxidative stress-sensitive Ca^{2+}-permeable channel. Therefore, activation of TRPA1 in neurons by oxidative stress such as H_2O_2 was reported (Materazzi et al., 2012; Bai and Lipski, 2013; Toda et al., 2016). Furthermore, the TRPA1 channel is activated by depletion of intracellular GSH, although its activation in the DRG neurons was inhibited by antioxidants of thiol redox system, such as GSH and selenium (Materazzi et al., 2012; Özdemir et al., 2016; Kahya et al., 2017) (**Table 1**).

It is well known that increase of intracellular ROS, RNS and Ca^{2+} has main roles in etiology of pain processes (Kallenborn-Gerhardt et al., 2012; Ogawa et al., 2016). As it was mentioned above, the TRPA1 and TRPV4 channels are activated by different stimuli, including oxidative stress (Bai and Lipski, 2013). Involvement of TRPA1 channels in the etiology of pain processes has not been fully clarified yet, although there are some reports on TRPA1 activation-induced pain processes such as diabetic peripheral pain (Andersson et al., 2015; Jardín et al., 2017; Kahya et al., 2017) and SCI-induced pain (Park et al., 2015; Klafke et al., 2016) and chemotherapeutic agent-induced pain (Naziroglu and Braidy, 2017) through excessive ROS and RNS production in the rodents. In addition, it was reported in DRG neurons of wild type mice and TRPA1 knockout mice that activation of TRPV1 by chemotherapeutic agents induced excessive ROS production and mechanical allodynia. However, TRPA1 and TRPV4 antagonist treatments induced decrease on the allodynia and oxidative stress in the mice (Materazzi et al., 2012). In a previous study, the same group did not observe Ca^{2+} response effects induced by exposure to chemotherapeutic agents in cultured mouse DRG and Chinese hamster ovary (CHO) cell line (Nassini et al., 2011), although chemotherapeutic agent evoked an antioxidant GSH-sensitive Ca^{2+} response in the CHO cell line and DRG neuron. Results of a study indicated that chemotherapeutic agents-induced oxidative stress caused TRPA1 activation instead of direct channel targeting (Nassini et al., 2011). The report was confirmed by a recent study; thus, it was observed that chemotherapeutic agent-induced increase of TRPA1 expression, cell death and neuropathic pain in mice DRG was reduced by aluminum and GSH treatment (Lee et al., 2017). TRPA1 activator role of hydrogen sulfide through nitric oxide production was recently reported in DRG neuron too (Miyamoto et al., 2017), as shown in **Table 1**.

There is a synergic interaction between TRPA1 and TRPV1 on channel activation mechanisms in DRG, because TRPA1 is co-localized with 30–50% TRPV1 expressing neurons in rat and human DRG (Bautista et al., 2005). Therefore, the sensitization ratio of TRPA1 is affected by several factors, including oxidative stress and TRPV1 blocker (Kistner et al., 2016; Ogawa et al., 2016). On the subject, increased sensitization of human TRPA1 in DRG neuron was reported by inflammation and oxidative stress, although the increased sensitization in the neuron is decreased by antioxidant NAC and capsazepine (Kistner et al., 2016).

TRPM2

Another member of TRP superfamily is TRPM2. The enzyme (ADP ribose) pyrophosphatase in the C-terminal domain of TRPM2 contains is sensitive to ROS and RNS (Naziroğlu, 2007). TRPM2 channel in transfected cell lines is gated by extracellular and intracellular ROS, possibly by interacting with the ADP ribose pyrophosphatase enzyme in the tail of the protein C domain (Perraud et al., 2001; Hara et al., 2002; Wehage et al., 2002). Later, TRPM2 activator role of oxidative stress from ADPR was reported in transfected cells by single channel patch-clamp experiments (Naziroglu and Lückhoff, 2008). Presence of TRPM2 function in DRG neuron was firstly reported in 2011 (Naziroğlu et al., 2011a). It was highlighted that the excessive ROS production through activation of NADPH oxidase contributes to sensitization in DRG neuron for persistent pain induction (Kallenborn-Gerhardt et al., 2012). Result of a more recent study indicated involvement of NADPH oxidase on TRPM2 channel activation in DRG neuron (Naziroğlu, 2017) (**Table 1**). In addition to the TRPA1 channel, involvement of cysteine groups on the activation of TRPM2 channels in transfected human embryonic kidney (HEK-293) cells was reported (Mei et al., 2006). Then, protective roles of GSH and NAC as members of thiol redox system on TRPM2 channel and peripheral pain inhibition in DRG neuron were also reported (Naziroğlu et al., 2011b; Özgül and Naziroğlu, 2012; Sözbir and Naziroğlu, 2017) (**Table 1**). It seems that members of thiol redox system have

TABLE 1 | Role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) on the activation of transient receptor potential channels (TRPA1, TRPM2, TRPV1, and TRPV4) in the peripheral neuron.

Channel	ROS/RNS	Material	Value	Reference
TRPA1	H_2O_2,	Rat DRG	Protective role of GSH on peripheral pain through inhibition of cysteine oxidation.	Bosch-Morell et al., 1998; Materazzi et al., 2012
TRPA1	Nitric oxide	Rat DRG	Protective role of dithiothreitol, cysteine and GSH on peripheral pain through inhibition of nitric oxide production.	Naziroğlu, 2007; Tiwari et al., 2011
TRPM2	ADP-ribose and H_2O_2	Rat DRG	NADPH oxidase dependent activation of TRPM2	Naziroglu and Braidy, 2017
TRPM2	ADP-ribose and H_2O_2	Rat DRG	Protective role of GSH and NAC on peripheral pain and channel activation through inhibition of oxide stress.	Lee et al., 2017; Miyamoto et al., 2017; Naziroglu and Braidy, 2017
TRPV1	H_2O_2 and nerve growth factor	Rat DRG	Protective role of GSH, selenium and NAC on hyperalgesia and channel activation through inhibition of oxide stress.	Mei et al., 2006; Naziroğlu et al., 2011b; Kahya et al., 2017
TRPV1	H_2O_2	Mice DRG	Oxidative stress-induced inflammatory hyperalgesia	Sözbir and Naziroğlu, 2017
TRPV1	RNS and NADPH oxidase	Mice and Rat DRG	Oxidative stress-induced inflammatory hyperalgesia and channel activation	Caterina et al., 1997; Naziroglu and Braidy, 2017
TRPV4	H_2O_2 and ROS	Rat DRG	Protective role of GSH on peripheral pain through inhibition of cysteine oxidation	Dina et al., 2008; Materazzi et al., 2012
TRPV4	ROS	Rat DRG	Protective role of TRPV4 blockers on mechanical allodynia and oxidative stress	Materazzi et al., 2012

important roles on inhibition of oxidative stress-dependent TPM2 channel activation and peripheral pain in rodents.

TRPV1

A subfamily of TRP superfamily is vanilloid family. TRPV1 is a member of the vanilloid subfamily. The channel was firstly expressed in rats through activation of high temperature and pungent hot chili pepper component (capsaicin) in mice DRG neuron (Caterina et al., 1997). The channel can also be activated by different stimuli including low pH (<5.9), high temperature (>43°C) and oxidative stress leading to the perception of pain, and oxidative injury (Yoshida et al., 2006). As most of cation channel protein, TRPV1 channel protein contains six transmembrane domains. Similar to TRPA1 (Takahashi et al., 2011) and TRPM2 (Mei et al., 2006) membrane structure, oxidative alterations of multiple Cys residues in different cells are involved in this mode of TRPV1 activation by modifying (Yoshida et al., 2006; Chuang and Lin, 2009) and disulfide bond formation (Wang and Chuang, 2011). Therefore, the TRPV1 is activated in rat DRG neuron by depletion of intracellular GSH (Naziroglu et al., 2013), although hyperalgesia and the TRPV1 channel were inhibited in the DRG neurons of rats by treatment of thiol redox cycle members such as GSH, selenium and NAC (Khodorova et al., 2013; Naziroglu et al., 2013; Kahya et al., 2017) (**Table 1**).

Excessive ROS are produced in physiological functions such as mitochondrial function and phagocytic activity. During the killing bacteria and virus, ROS are used in the anti-inflammatory cells such as macrophages microphages and microglia. Therefore, there is a direct relationship between increased levels of ROS and inflammatory hyperalgesia (Oehler et al., 2017). Interactions between TRPV1 and long sustained thermal hypersensitivity in oxidative stress-induced inflammatory hyperalgesia of mouse hind paw were reported (Keeble et al., 2009). Therefore, there is a direct role of oxidative stress through activation of TRPV1 on hyperalgesia in DRG neuron of wild type and Nox1 deficient mice (Ibi et al., 2008) (**Table 1**). Niflumic acid is also a TRPV1 channel antagonist and it was reported that peripheral neuropathy by suppressing excessive ROS, RNS, inflammatory cytokine production and TRPV1 activation in neuropathic pain-induced rats were recovered by the niflumic acid treatment (Marwaha et al., 2016).

TRPV4

A member of TRP superfamily is TRPV4 and it was firstly described with mammalian osmo-transducer property (Liedtke et al., 2000). The channel is also activated by phorbol esters, low pH, citrate, arachidonic acid, exogenous chemicals (bisandrographolide A) and heat (24°C ≥) (Güler et al., 2002; Yoshida et al., 2006). In addition to the stimulators, activation of TRPV4 in neurons by oxidative stress such as H_2O_2 was reported (Materazzi et al., 2012; Bai and Lipski, 2013), although its activation in the DRG neurons was inhibited by GSH (Materazzi et al., 2012) (**Table 1**). It was also reported in DRG neurons of wild type and TRPV4 knockout mice that activation of TRPV4 by paclitaxel induced mechanical allodynia and excessive ROS production, although the allodynia and oxidative stress was partially decreased by the TRPV4 antagonist treatment (Materazzi et al., 2012) (**Table 1**).

Role of Other Factors on TRP Channels and Pain

In addition to the oxidative stress, several other factors play a role in the induction of CIPN with/without TRP channel activation, including glutamate receptors, neuropeptides, PKC and inflammation. Although their role in CIPN has been known for a long time, there are limited reports about the interaction between these factors and TRP channels in the literature. In this section, some brief information is given about other main factors and TRP channels activation related to pain induction in experimental animals.

1) Glutamate receptors and neuropeptides: Many types of ionotropic glutamate receptors were reported in literature. Presence of three types of ligand-gated glutamate ion channel receptors such as N-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, and kainite receptors has been known for a long time. Their involvement in CIPN was also investigated by numerous papers (Wu and Zhuo, 2009; Vécsei et al., 2015). On the other hand, substance P and calcitonin-gene-related-neuropeptide are well known neuropeptides in the induction of peripheral neuropathy and nociception. Their implication in CIPN through the overload of Ca^{2+} entry has been demonstrated (Hill and Oliver, 2007; Nassini et al., 2014). In addition, recent studies have pointed out the involvement of neuropeptides on TRP channel activation, such as TRPA1, TRPM8 and TRPV1 (Nassini et al., 2014; Chukyo et al., 2018). For example, it was observed that substance P was potentiated by the sensitization of TRPV1 (Marwaha et al., 2016).
2) PKC enzyme: Apart from the involvement of PKC on the induction of oxidative stress in neurons, both human and animal studies have linked this enzyme to several pain types through excessive Ca^{2+} entry and cation channel activation (Carozzi et al., 2015; Kumar et al., 2017). In this sense, the involvement of PKC in experimental models of CIPN was also evidenced by several papers, although mechanical hyperalgesia was decreased by the treatment with PKC inhibitors such as hypericin and calphostin C (Norcini et al., 2009). Sensitivities of nociceptive neurons to heat stimulation and TRPV1 activation were increased by the activation of PKC and inflammatory mediators (Khasar et al., 1999; Gao et al., 2016). There is also direct relationship between PKC and onset of mechanical hyperalgesia (Khasar et al., 1999; Hucho et al., 2006). It was reported that paclitaxel-induced mechanical hypersensitivity were increased in the DRG of mice by up-regulation of PKC (Dutra et al., 2015). Moreover, antioxidants such as quercetin were able to inhibit TRPV1 and PKC activations in a study of paclitaxel-induced peripheral neuropathy (Gao et al., 2016).
3) Inflammation: Brain and spinal glia neurons have a main role on homeostasis in central nervous system and peripheral nervous system. The glial cells have been shown to contribute to the development of chronic pain as results of surgery, inflammation, and SCI. Therefore, reduction of NP was observed by treatment of the glial activity (Carozzi et al., 2015). Involvement of oxidative stress-induced inflammation was reflected in other parts of this review (see "Chemotherapy-induced peripheral neuropathy" and "Spinal cord injury" sections). The increased glial neuron-induced inflammation induces pain through TRP channel activation in DRG and spinal cord neurons. For example, TRPV1 and TRPA1 are activated by several stimuli related with traumatic brain injury, including mechanical shear stress, leading to the release of substance P and inflammation (Corrigan et al., 2016). Enhanced expression and spinal inflammation-induced sensitization of TRPV1 and streptozotocin-induced thermal hyperalgesia and neuropathy were reported in rats (Bishnoi et al., 2011). Involvement of TRPV1 in the activation of spinal glia in mice with nociceptive, inflammatory and neuropathic pain was also reported (Chen et al., 2009).

Antioxidant Supplementation

In general, scientific evidence reinforces the future use of antioxidant supplementation in several pathological conditions. Taking in account previous works of our research group (Carrasco et al., 2013, 2014a,b) and other authors, both preventive and therapeutic uses of antioxidants have been reported to reduce not only oxidative stress related parameters but also inflammatory response and pain in several diseases. As mentioned earlier, it has been demonstrated that current drugs used in the management of different kinds of NP are ineffective and usually not safe for the patient. For this reason, antioxidant supplementation might be an alternative to take in account in clinical practice.

As an illustration, oral administration of molecular hydrogen may have therapeutic potential for the management of NP. Unlike other antioxidants, hydrogen reaches target organs easily, where selectively neutralizes $^{\bullet}OH$, and does not accumulate in living cells nor produce noxious metabolites (Kawaguchi et al., 2014). Naringenin, an abundant flavanone in citrus fruits (Kaulaskar et al., 2012) and genistein, a natural phytoestrogen from soybean (Valsecchi et al., 2008), have been exhibited analgesic, antioxidant and immunoregulatory properties in sciatic nerve injury models. α-lipoic acid treatment (600 mg/day) has also demonstrated to improve neuropathic symptoms (pain, burning, paresthesia, and numbness) and deficits in patients with NP (Tesfaye, 2011).

Concerning antioxidant supplementation in CINP, natural antioxidants such as curcumin (Al Moundhri et al., 2013), silibinin, α-tocopherol (Kerckhove et al., 2017), rutin and quercetin (Azevedo et al., 2013) have exhibited antinociceptive effects in oxaliplatin induced-CINP. For example, administration of the flavonoids rutin and quercetin has shown to diminish oxidative phenomena including lipid peroxidation, nitrosylation and iNOS expression, as well as pain symptomatology (thermal and mechanical allodynia) in treated mice compared to non-treated ones (Azevedo et al., 2013). Likewise, the thiol compound NAC has demonstrated to exert a beneficial effect in the treatment of oxaliplatin induced-CINP, significantly reducing inflammatory response (TNF-α, IL-1β and IL-6) in the neuroblastoma cell line SH-SY5Y; additionally, researchers also observed that NAC prevented apoptosis by inhibition of P2X7 receptor activation by blocking ROS production and caspase-3 activation. Interestingly,

in this study NAC exhibited the highest preventive effect compared to other drugs also tested such as ibuprofen and acetaminophen (Massicot et al., 2013). On the other hand, prophylactic treatment with acetyl-L-carnitine has demonstrated to prevent paclitaxel-, oxaliplatin- and bortezomib-induced mitochondrial dysfunction and pain (Zheng et al., 2012). In spite of the promising results, most clinical studies related to the use of antioxidants as chemotherapy adjuncts did not report on their impact on anticancer efficacy; hence, as Han et al. (Han and Smith, 2013) highlighted this is an important question which should be examined in greater depth. In this sense, previous *in vitro* studies performed by our research group have shown that co-administration of conventional chemotherapeutic agents with antioxidants such as melatonin enhances chemotherapeutic-induced cytotoxicity and apoptosis in different cancer cell lines (Uguz et al., 2012; Pariente et al., 2016, 2017a,b). In addition, since some studies have also recorded failure in antioxidant supplementation efficacy, optimal design of clinical trials in terms of targeted delivery of antioxidants, clinical pathology and concentration dependent dosage schedule is needed to go ahead in the knowledge and future application of this kind of treatment in CIPN (Kamat et al., 2008). Finally, as Areti et al. (2014) point out monitoring oxidative stress related-parameters (i.e., levels of malondialdehyde, GSH, superoxide dismutase and activities of mitochondrial enzymes such as citrate synthase and ATP synthase) during the course of CIPN could be helpful in clinical practice.

Future Consideration

Taking in account the scientific evidence summarized in this review, NP is a complex network of several molecular processes, including nitro-oxidative stress, immune response, and TRP channels activation, among others. Noteworthy, NP seems to be not only promoted by direct injury to neurons but also by TRP channels mediating damage in the surrounding tissue. However, the way how these actors and other factors (e.g., sodium channels, acid-sensing ion channels and synaptic receptors) are interconnected leading to noxious symptomatology remains to be unresolved.

Since 1990s, with the discovery of TRP channels, our understanding about nociception has changed. Most nociceptive TRP channels are predominantly expressed in peripheral sensory neurons, but there is also a significant expression in the central nervous system and other tissue and cell types (i.e., keratinocytes, vascular endothelial cells, bladder epithelial cells, fibroblasts and human dental pulp) (Mickle et al., 2016). Thus, we are still far from a complete understanding of the biology of nociception and its applicability in clinical practice. Current evidence points out the possibility that multiple nociceptive TRP channels are activated during pathological conditions, including the nine oxidative sensitive-TRP channels known until today. Although there are several reports on four oxidative sensitive-TRP channels reviewed in this paper, there is no report linking the remaining oxidative sensitive-TRP channels (TRPM4, TRPM7, and TRPC5) and pain in the peripheral neurons. In this sense, contradictory results have been obtained about the expression of certain TRP channels in DRG neurons, such as TRPV4; in addition, TRPV2-6 and TRPM3 expression at this level is unknown. Furthermore, information about some important aspects related to TRP channels, including their location, trafficking, functionality and overlapping in neurons and other kind of cells, both in physiological and pathophysiological states, is lacking. Besides their neuronal/plasma membrane location, it has been observed that a significant fraction of TRP channels is also present in organelles membranes which may be translocated as required by cells exposed to injury/inflammation (Mickle et al., 2016). Finally, it should be noted that some populations consume large amounts of capsaicin, which is well known to activate TRPV1 channels and to induce overload Ca^{2+} entry in hippocampal and DRG neurons (Kahya et al., 2017). In recent papers, we have reported the involvement of TRPV1 channels in the induction of epilepsy (Naziroğlu, 2015; Naziroglu and Övey, 2015). In this sense, some Turkish populations have been traditionally consuming high amounts of hot chili pepper (capsaicin) in food and it has been observed a high incidence of epilepsy in these areas (unpublished data). Thus, similar possible relationship between high amount hot chili pepper consumption and several peripheral pain inductions should be investigated by future studies. Furthermore, the relevance of TRPs in NP will remain elusive until experimental studies (including knockdown or knockout animal models) demonstrate that an increase in TRP activity by exogenous TRP activators produces NP.

In any case, TRP channels are now presented as attractive targets for the development of new-generation analgesics. Until today, there are many small molecule blockers of TRPV1 (AZD1386) (Clinical Trials, 2012), TRPA1 (GRC-17536) (Clinical Trials, 2014) and TRPV3 (SAR292833) (Clinical Trials, 2016), apart from topical TRPV1 agonists, such as zucapsaicin (Clinical Trials, 2011) and NGX 4010 (Mou et al., 2014), that have been tested in clinical trials of several NP conditions; to the best of our knowledge, NGX 4010 is the only compound that has been launched for clinical use in human post-herpetic neuralgia-NP conditions. Concerning to cancer pain, a phase I clinical trial is being carried out to determine the efficacy of periganglionic/intrathecal administration of the potent TRPV1 agonist resiniferatoxin in advanced cancer patients with bone pain (Clinical Trials, 2017). However, it is suspected that other nociceptive TRP channels may be involved in cancer pain. Therefore, this and other questions, such as efficacy and site of action of drugs targeting nociceptive TRP channels, will need to be answered in the next years.

CONCLUSIONS

NP is an underestimated socioeconomic health problem affecting millions of people worldwide, which incidence may increase in the next years due to chronification of several diseases such as cancer and diabetes. Nitro-oxidative stress and inflammatory response, with the consequent activation of TRP channels, seem to play a major role in the beginning and development of NP. Hence, it is now urgent to discover new, effective and safe strategies to prevent and/or treat this hardly bearable condition. Recent discoveries in different biomedical fields

point out the need to change paradigms about pharmacological management of diseases. From our point of view, therapeutic options must not only be directed to reach a molecular target, which ultimately would represent a fixed picture of the disease, but also to restore physiological global context in terms of nitro-oxidative stress and inflammatory response, just as the antioxidant treatment seems to act. Furthermore, increasing aging population and chronic diseases prevalence demand the development and implementation of antioxidant therapies in clinical practice. But it must not be forgotten that possible prevention of several diseases following a varied and balanced diet, as well as other healthy habits, is a reality nowadays. Health care institutions, clinicians and general population must be aware of the importance of nutrition, as source of natural antioxidants, in our physical and mental state, as much in health as in illness.

AUTHOR CONTRIBUTIONS

CC and MN wrote the review. AR and JP critically revised the work and approved its version to be submitted.

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REVIEW
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Thermo-Sensitive TRP Channels: Novel Targets for Treating Chemotherapy-Induced Peripheral Pain

Mustafa Nazıroğlu[1]* *and Nady Braidy*[2]

[1] Neuroscience Research Center, Suleyman Demirel University, Isparta, Turkey, [2] Centre for Healthy Brain Ageing, School of Psychiatry, University of New South Wales, Sydney, NSW, Australia

Abnormal Ca^{2+} channel physiology, expression levels, and hypersensitivity to heat have been implicated in several pain states following treatment with chemotherapeutic agents. As members of the Ca^{2+} permeable transient receptor potential (TRP), five of the channels (TRPV1-4 and TRPM2) are activated by different heat temperatures, and two of the channels (TRPA1 and TRPM8) are activated by cold temperature. Accumulating evidences indicates that antagonists of TRPA1 and TRPM8 may protect against cisplatin, oxaliplatin, and paclitaxel-induced mitochondrial oxidative stress, inflammation, cold allodynia, and hyperalgesia. TRPV1 was responsible from the cisplatin-induced heat hyperalgesia and mechanical allodynia in the sensory neurons. TRPA1, TRPM8, and TRPV2 protein expression levels were mostly increased in the dorsal root (DRG) and trigeminal ganglia by these treatments. There is a debate on direct or oxaliplatin-induced oxidative cold stress dependent TRPA1 and TRPV4 activation in the DRG. Involvement of molecular pathways such as cysteine groups, glutathione metabolism, anandamide, cAMP, lipopolysaccharide, proteinase-activated receptor 2, and mitogen-activated protein kinase were also indicated in the oxaliplatin and paclitaxel-induced cold allodynia. In this review, we summarized results of five temperature-regulated TRP channels (TRPA1, TRPM8, TRPV1, TRPV2, and TRPV4) as novel targets for treating chemotherapy-induced peripheral pain

Keywords: allodynia, chemotherapeutic agents, hyperalgesia, oxidative stress, thermo sensitive TRP channels

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***Correspondence:**
Mustafa Nazıroğlu
mustafanaziroglu@sdu.edu.tr

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INTRODUCTION

Chemotherapeutic agents such as taxanes (paclitaxel, docetaxel) and platinum analogs (cisplatin, carboplatin, oxaliplatin) are used in treatment of several cancer types. Taxanes inhibit progression of mitosis through stabilization of tubulin in the treatment of solid tumors (Sharma et al., 2007). However, platinum derived chemotherapeutic drugs inhibit DNA synthesis and repair through cross-linking of DNA strands and are used for the treatment of several cancer types such lung carcinoma, testicular cancer, ovarian cancer, etc. (Kelland, 2007). However, severe painful neuropathy is a main complication of these cancer agents. Several peripheral neuropathies such as numbness, tingling, and chronic pain distributed in a distal stocking-and-glove pattern have been reported in patients treated with a variety of chemotherapeutic agents. The etiology of painful neuropathy remains unclear. Current analgesic drugs cannot completely alleviate the pain,

although they may provide partial analgesic effects in some patients. Severe painful neuropathy due to chemotherapeutic agents has pushed some patients to suicide (Lester and Yang, 1996; Bauduer et al., 2000). Therefore, discovery of novel therapeutic agents against chemotherapy-induced painful neuropathy is an urgent subject.

In the etiology of pain and neuropathy, calcium ion (Ca^{2+}) overload plays an important role. Ca^{2+} enters into cells by different ways including cation channels. Voltage gated calcium channels (VGCC) and chemical channels (i.e., glutamate) are well-known calcium channels (Kumar et al., 2014). However, new calcium channels namely the transient receptor potential (TRP) superfamily were discovered in eye cells of drosophila flys (Hardie, 2011; Nazıroğlu, 2011). Today, the TRP superfamily contains 28 channels with 7 different subgroups (Nazıroğlu, 2011; Uchida et al., 2017).

Dorsal root ganglion (DRG) neurons have important roles in the pathobiology of neuropathic pain. There is no barrier between the DRG and blood, and compounds with a high molecular weight can easily diffuse into the DRG (Abram et al., 2006). The TRPA1, TRPV1 and TRPV4 channels are mainly expressed in the DRG and trigeminal ganglia neurons (Kobayashi et al., 2005; Obata et al., 2005; Fonfria et al., 2006; Nativi et al., 2013; Yazğan and Nazıroğlu, 2017). Hence, the TRPA1, TRPV1 and TRPV4 have been associated with pain transmission of sensory neurons, including the DRG (Materazzi et al., 2012; Kahya et al., 2017).

Some peripheral primary afferent fibers are affected by low and high temperature changes and are called thermoreceptors. So far, 11 TRP channels in mammalian cells have been identified as thermosensitive TRP (thermo-TRP) channels (Uchida et al., 2017). Two TRP channels (TRPV1 and TRPV2) are activated by high temperatures (43°C≥ and 55°C≥, respectively). Five TRP channels (TRPV1-4 and TRPM2) are activated by different heat temperatures, although two of TRP channels (TRPA1 and TRPM8) are activated by cold (≤17°C) and (≤25°C) cool temperatures, respectively (Caterina et al., 1999; Xu et al., 2002; Story et al., 2003; Bandell et al., 2004; Nazıroğlu and Ozgül, 2012). In addition, the remaining **two** channels, TRPM3 and TRPC5 are noxious heat and cold sensors, respectively (Vriens et al., 2011; Zimmermann et al., 2011). In addition, TRPV1, TRPA1, and TRPV4 are also oxidative stress-sensitive Ca^{2+}-permeable channels. Therefore, activation of TRPA1 and TRPV4 in neurons by oxidative stress such as H_2O_2 has been previously reported (Bai and Lipski, 2010; Materazzi et al., 2012; Toda et al., 2016). However, activation of TRPA1 and TRPV4 in the DRG neurons was inhibited by members of the cysteine antioxidant redox cycle such as glutathione (GSH) and selenium (Materazzi et al., 2012; Kahya et al., 2017).

Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium ion; CAPS, capsaicin; CHO, Chinese hamster ovary; CPZ, capsazepine; DRG, dorsal root ganglion; fMLP, N-formylmethionine peptides such as formylmethionyl- leucyl phenylalanine; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; PAR2, proteinase-activated receptor 2; PARP-1, Poly-ADPR polymerase 1; ROS, reactive oxygen species; TRP, transient receptor potential; TRPA1, transient receptor potential ankyrin 1; TRPM8, transient receptor potential melastatin 8; TRPV1, transient receptor potential vanilloid 1; TRPV4, transient receptor potential vanilloid 4.

Owing to the high expression levels of TRPA1, TRPM8, TRPV1, TRPV2, and TRPV4 in the DRG (Bridges et al., 2003; Obata et al., 2005; Fonfria et al., 2006; Nativi et al., 2013), these channels represent novel targets for the management of chemotherapy-induced neuropathic pain. Present information on chemotherapy-induced neuropathic pain in human and experimental animals suggests the involvement of at least five thermo-TRP channels (TRPA1, TRPM8, TRPV1, TRPV2, and TRPV4; **Table 1**). In this review, we summarized the potential role of the five thermo TRP channels as novel targets for treating chemotherapy-induced neuropathic pain.

CHEMOTHERAPEUTIC AGENTS

There is limited information on chemotherapeutic agents and thermo-TRP channels in pain induction, although the mode of action of chemotherapeutic agents has been well-established (**Figure 1**).

Cisplatin

Cisplatin is an effective anti-tumor drug that is used in the treatment of several cancers such as ovarian, bladder, and testicular cancers (Kelland, 2007). It acts by crosslinking DNA it inhibits DNA replication and repair mechanisms through formation of DNA-platinum products (Dzagnidze et al., 2007). However, it also induces several adverse effects such as mechanical allodynia, hyperalgesia, and toxicity in neurons including DRG. Cisplatin produces a cumulative toxic effect on peripheral nerves, and 30–40% of cancer patients receiving this agent experience neuropathic pain (Khasabova et al., 2012). Apoptosis, oxidative stress and necrosis pathways in the cancer cells through TRP channel stimulation are also activated by cisplatin treatment (Sakalli Çetin et al., 2017). Cisplatin-induced neuropathy and apoptosis of sensory neurons were attributed to uptake of the drug into the DRG affecting large myelinated sensory nerve fibers (Ta et al., 2006). The damage and injury to DRG neurons could be partially decreased by different adjuvant therapies such as anandamide (as an endogenous cannabinoid), which attenuates hyperalgesia in neuropathic pain (Khasabova et al., 2012). Therefore, the prevention or reduction in the neurotoxic effects of cisplatin remains of major clinical significance in cancer patients.

Oxaliplatin

Oxaliplatin is a platinum complex containing agent and is the most commonly using anti-tumor agent for the treatment of several cancer types such as colorectal cancer (Kelland, 2007). Oxaliplatin has less complications than cisplatin, including a lower incidence of hematotoxicity and manageable gastrointestinal toxicity compared to other platinum-based chemotherapeutics. However, neurotoxicity remains a very common complication in patients treated with oxaliplatin, because it has a long terminal half-life (Descoeur et al., 2011; Zhao et al., 2012). Acute and chronic pain has been reported in patients treated with oxaliplatin. Acute neuropathies are characterized as accrual numbness, paresthesia, dysesthesia, and peripheral pain following 1–12 h of treatment. One

TABLE 1 | Roles of TRPA1, TRPM8, TRPV1, TRPV2, and TRPV4 channels on chemotherapeuty-induced peripheral pain in experimental animals.

Channel	Agent	Material	Value/Effect	References
TRPA1	Oxaliplatin	Rat DRG	Increased cAMP level and channel sensitization	Anand et al., 2010
TRPA1	Oxaliplatin	Mice DRG- trigeminal ganglion	Increased channel expression level	Ta et al., 2010
TRPA1	Oxaliplatin	Mice DRG and CHO	Oxidative and cold allodynia	Nassini et al., 2011
TRPA1	Oxaliplatin	Mice DRG	Increased channel sensitization but no effect on cold hyperalgesia	Chen et al., 2011
TRPA1	Oxaliplatin	Mice DRG	Increase of channel protein expression level but no change of cold hypersensitivity	Descoeur et al., 2011
TRPA1	Oxaliplatin	Mice DRG and CHO	Oxidative and cold allodynia	Materazzi et al., 2012
TRPA1	Oxaliplatin	Mice DRG	No thermal hyperalgesia but cold allodynia	Park et al., 2015
TRPA1	Oxaliplatin	Rat DRG	increase of PAR2 and channel activation	Tian et al., 2015
TRPA1	Oxaliplatin	Rat DRG	Increase of nocifensive behaviors and channel mRNA expression levels	Mizuno et al., 2014
TRPA1	Oxaliplatin	Mice DRG	Increase of channel protein expression and cold hypersensitivity	Yamamoto et al., 2016
TRPA1	Oxaliplatin	Mice DRG	Decrease of oxaliplatin-induced TRPA1 expression, cell death and neuropathic pain by GSH treatment	Lee et al., 2017
TRPA1	Cisplatin	Mice DRG- trigeminal ganglion	Increased channel expression level	Ta et al., 2010
TRPA1	Paclitaxel	Rat DRG	TRPA1-stimulated transmitter release was increased or decreased as concentration and exposure time dependent	Pittman et al., 2014
TRPM8	Cisplatin	Mice DRG- trigeminal ganglion	Increased channel expression level	Ta et al., 2010
TRPM8	Oxaliplatin	Mice DRG	Increase of channel expression and cold allodynia	Gauchan et al., 2009
TRPM8	Oxaliplatin	Mice DRG- trigeminal ganglion	No change channel expression level	Ta et al., 2010
TRPM8	Oxaliplatin	Mice DRG	No change in the TRPM8 protein expression but increase of cold hypersensitivity	Descoeur et al. (2011)
TRPM8	Oxaliplatin and oxalate	Rat DRG	Increase of TRPM8 mRNA and protein expression levels	Kawashiri et al., 2012
TRPM8	Oxaliplatin	Rat DRG	Increase of nocifensive behaviors and channel mRNA expression levels	Mizuno et al., 2014
TRPV1	Cisplatin	Mice	Mechanical hyperalgesia but no pronociceptive role of TRPV1 in toxic neuropathy	Bölcskei et al., 2005
TRPV1	Cisplatin	Mice DRG- trigeminal ganglion	Increased TRPV1 protein expression level	Ta et al., 2010
TRPV1	Cisplatin	Rat DRG	No changes in TRPV1 protein expression	Hori et al., 2010
TRPV1	Cisplatin	Mice DRG	Increase of TRPV1 protein expression	Khasabova et al., 2012
TRPV1	Oxaliplatin	Mice DRG- trigeminal ganglion	No change channel expression level but thermal hyperalgesia and mechanical allodynia	Ta et al., 2010
TRPV1	Oxaliplatin	Mice DRG	Increase of channel sensitization but no effect on cold hyperalgesia	Chen et al., 2011
TRPV1	Oxaliplatin	Mice DRG	Increased channel sensitization	Wainger et al., 2015
TRPV1	Paclitaxel and vinorelbine	Rat DRG	No effect on paclitaxel and vinorelbine-induced substance P production	Miyano et al., 2009
TRPV1	Paclitaxel	Mice DRG	Increase of channel expression and thermal hyperalgesia.	Hara et al., 2013
TRPV1	Paclitaxel	Rat DRG	TRPV1-stimulated transmitter release was increased or decreased as concentration and exposure time dependent	Pittman et al., 2014
TRPV1	Paclitaxel	Human and rat DRG	TRPV1 stimulation via Toll-like receptor 4 signaling	Li et al., 2015
TRPV1	5-FU	Rat	Channel activation and pain induction by TRPV1 but not TRPA1	Chen et al., 2013
TRPV2	Cisplatin	Rat DRG	Increased TRPV2 protein expression in the small DRG but not in DRG innervating gastrocnemius muscle	Hori et al., 2010
TRPV4	Paclitaxel	Rat DRG	Hyperalgesia though activation of $\alpha 2\beta 1$ integrin and Src tyrosine kinase pathways	Alessandri-Haber et al., 2008
TRPV4	Paclitaxel	Rat DRG	No effect	Alessandri-Haber et al., 2004
TRPV4	Oxaliplatin	Mice DRG	Increase of channel sensitization but no effect on cold hyperalgesia	Chen et al., 2011
TRPV4	Oxaliplatin	Mice DRG	Oxidative but not cold allodynia	Materazzi et al., 2012

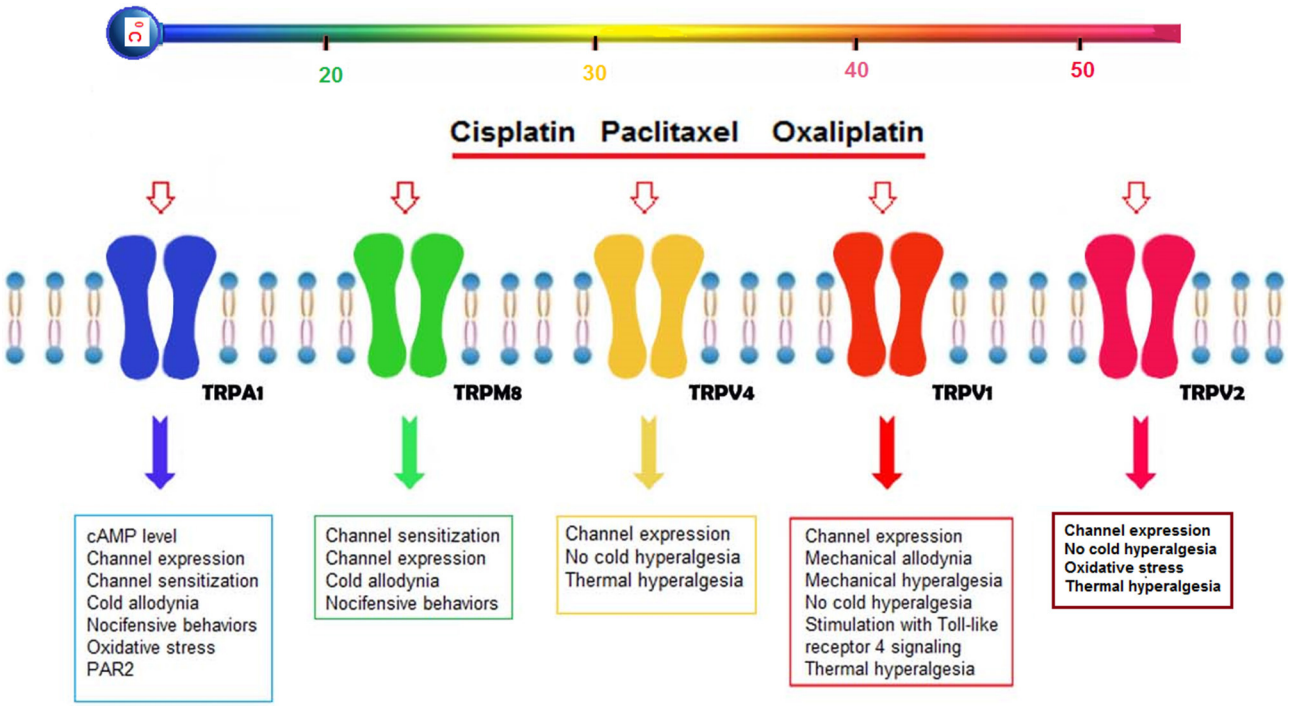

FIGURE 1 | Possible effects of cisplatin, oxaliplatin, and paclitaxel on thermo-TRP channels (TRPA1, TRPM8, TRPV1, TRPV2, and TRPV4) in the DRG neurons. Three chemotherapeutic agents (cisplatin, oxaliplatin, and paclitaxel) induce severe peripheral pain adverse effect in treatment of cancer patients. Reports on chemotherapy-induced pain in peripheral nerves were focused on five thermo-TRP channels (TRPA1, TRPM8, TRPV1, TRPV2, and TRPV4), because their expression levels are mostly high in the peripheral neurons. Activation of the five thermo-TRP channels by the cisplatin, oxaliplatin and paclitaxel lead to changes on levels of channel expression, channel sensitization, nociceptive behaviors, oxidative stress, mechanical, heat and cold hypersensitivity (Anand et al., 2010; Ta et al., 2010; Hara et al., 2013). In addition, the levels are induced by activation of some secondary molecular mechanisms such as glutathione (GSH) (Lee et al., 2017), proteinase-activated receptor 2 (PAR2) (Tian et al., 2015), cAMP (Anand et al., 2010), and Toll-like receptor 4 (TLR4) signaling (Meseguer et al., 2014).

of main complication in the treatment of oxaliplatin in the cancer patients is increased cold sensitivity (Kelland, 2007). Oxaliplatin is metabolized to oxalate, and dichloro (1,2-diaminocyclohexane)platinum are produced during the metabolism of oxaliplatin (Nakagawa and Kaneko, 2017). Cold hyperalgesia and mechanical allodynia of oxaliplatin is attributed to oxalate and dichloro (1,2-diaminocyclohexane)platinum (Sakurai et al., 2009; Nakagawa and Kaneko, 2017). Additionally, these metabolites are also responsible for oxaliplatin-induced cold oxidative stress (Nakagawa and Kaneko, 2017).

Paclitaxel

One of the most common chemotherapeutic agents is paclitaxel which was originally isolated from Pacific Yew tree *Taxus brevifolia* Nutt (Wani et al., 1971). Paclitaxel has been mostly used in treatment of lung, ovarian, head, neck and breast cancer (Chen et al., 2011). In paclitaxel treatment, the division of cancer cells is inhibited through dynamic assembly or disassembly of the mitotic spindle (Marupudi et al., 2007). Hypersensitive reactions such as bronchospasm, pulmonary edema and neuropathy occur during treatment with paclitaxel (Shepherd, 2003; Sisignano et al., 2016). Recent studies have suggested the involvement of mitochondrial oxidative stress and overload Ca^{2+} entry through VGCC and TRP channels (Materazzi et al., 2012; Duggett et al., 2016; Sekiguchi et al., 2016), although the exact mechanism of neuropathic pain induced by paclitaxel remains to be elucidated.

CHEMOTHERAPEUTIC AGENTS AND THERMO-TRP CHANNELS

As already mentioned, chemotherapeutic agent can cause painful neuropathy that is usually resistant to analgesic drugs (Hara et al., 2013; Oehler et al., 2017). In addition to chronic neuropathy, paclitaxel is also associated with an acute pain syndrome (Chen et al., 2011), although its exact mechanism remains unclear. Accumulating evidence on chemotherapy-induced pain and hypersensitivity through activation of cation channels such as TRPA1, TRPM8, TRPV1, and TRPV4 focused on two main subjects, oxidative stress, and Ca^{2+} overload (**Figure 2**).

TRPA1

TRPA1 is a member of ankyrin subfamily in the TRP superfamily. There are 6 domains and 4 pores in the structure of the TRPV1 channel. TRPV1 is activated by different stimuli such as oxidative stress, chemicals such as mustard oil and cinnamaldehyde, and cold body temperature ($\leq$17°C).

Excessive reactive oxygen species (ROS) and low levels of antioxidants play a pivotal role in the pathobiology of cancers

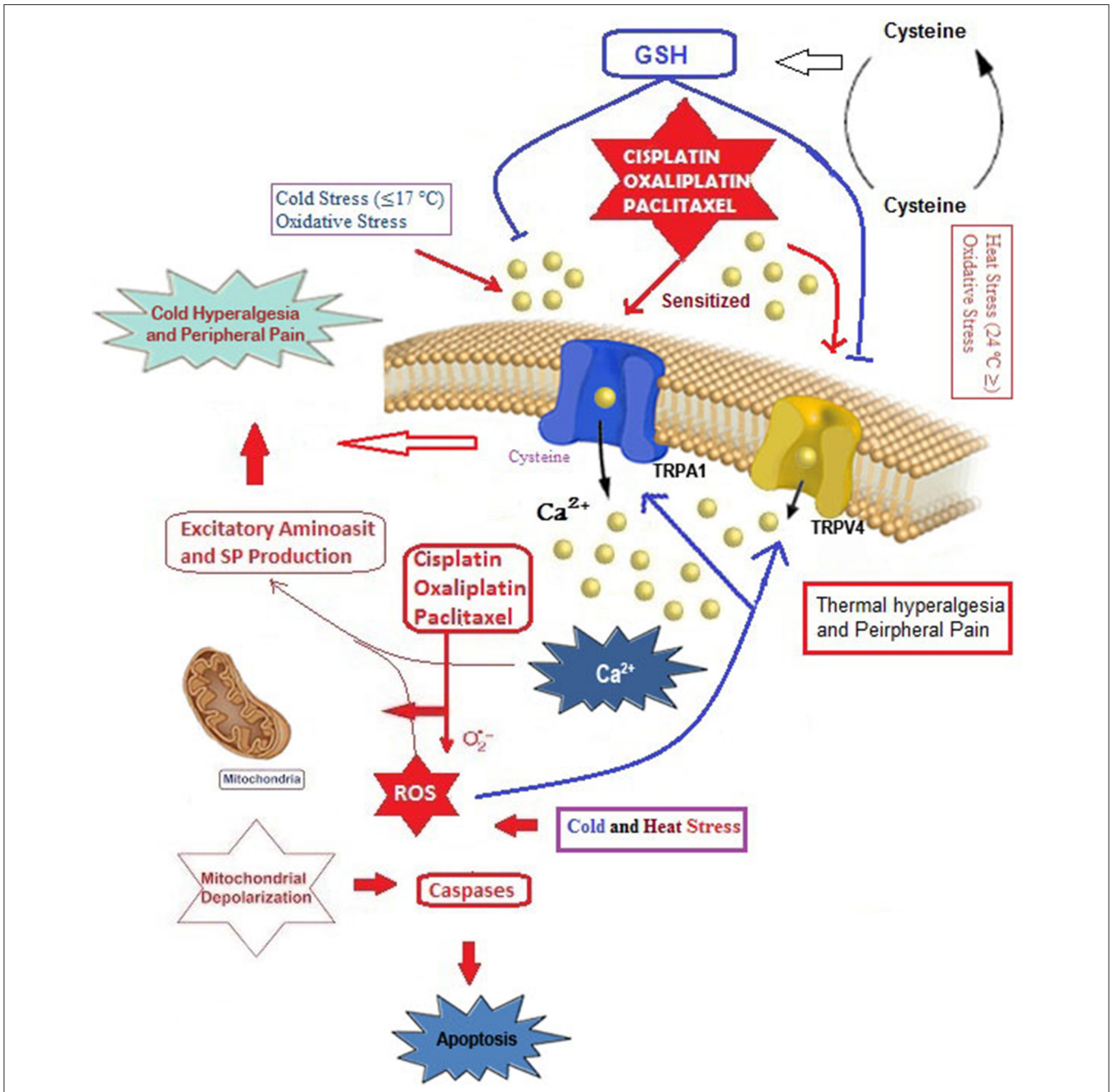

FIGURE 2 | Possible molecular pathways of cisplatin, oxaliplatin and paclitaxel on oxidative stress-dependent TRPA1 and TRPV4 activation in the DRG neurons. Cysteine groups are main target of oxidative stress in cellular membranes and membrane of TRPA1 has rich content of cysteine groups (Takahashi et al., 2011). TRPA1 and TRPV4 are oxidative stress-sensitive Ca^{2+}-permeable channels. The cisplatin, oxaliplatin, and paclitaxel can results in augmented TRPA1 and TRPV4, leading to Ca^{2+} influx through direct channel activation or excessive production of oxidative stress and induction of apoptosis through depolarization of mitochondrial membranes. Overload Ca^{2+} influxes induce pain through substance P (SP) and excitatory amino acid production. Glutathione (GSH) is synthetized from cysteine redox cycle. Protective role of GSH on TRPA1 and TRPV4 through oxaliplatin and paclitaxel-induced oxidative stress in DRG neuron was reported (Materazzi et al., 2012). The molecular pathway may be a cause of chemotherapy-induced peripheral pain and this subject warrants further investigation.

(Koçer and Nazıroğlu, 2013; Koçer et al., 2014). As already mentioned, the TRPA1 and TRPV4 channels are activated by different stimuli, including oxidative stress (Bai and Lipski, 2010). Involvement of cysteine residues and the antioxidant, dithiothreitol in the N domain of TRPA1, were indicated by a mass spectrometry study (Macpherson et al., 2007). Activation of TRPA1 though reversible covalent or oxidative modifications of the cysteine residues in DRG of wild and TRPA1 knockout

mice were reported (Andersson et al., 2008; Salazar et al., 2008). Activations of TRPA1 and TRPV4 were reported in the DRG of wild type and TRPA1 knockout mice by cold exposure and paclitaxel-induced excessive reactive oxygen species (ROS) production and mechanical allodynia, although the allodynia and oxidative stress was partially decreased by the TRPA1 (HC-030031) and TRPV4 (HC-067047) antagonist treatments (Materazzi et al., 2012). However, another study did not observe significant differences in calcium response as an effect of oxaliplatin or cisplatin exposure in cultured mouse DRG and naïve Chinese hamster ovary (CHO) cell line (Nassini et al., 2011), although cisplatin and oxaliplatin evoked an antioxidant GSH-sensitive calcium response in the CHO cell line and DRG neuron. Result of another study indicated that a single dose of oxaliplatin produced mechanical and cold hyperalgesia in wild type rats. In addition, mechanical and cold allodynia were induced in wild type mice but not in TRPA1 knockout mice (Nassini et al., 2011). The study also reported that cisplatin and oxaliplatin caused TRPA1 activation via excessive ROS production instead of direct channel targeting (Nassini et al., 2011). In addition, it was reported that cisplatin and oxaliplatin-induced neuropathy was treated through inhibition of platinum accumulation in the DRG of rats (Holmes et al., 1998) and patients with cancer, using GSH (Cascinu et al., 2002). Result of a recent study indicated that oxaliplatin-induced increase in TRPA1 expression, cell death and neuropathic pain in the DRG of mice were decreased by treatment with aluminum and GSH (Lee et al., 2017).

In addition to cysteine oxidation in the N domain of TRPA1, inhibition of prolyl hydroxylases pathway through decreased oxygen levels on the activation of TRPA1 in the vagal and sensory neurons of mice was also reported (Takahashi et al., 2011). The inhibition of prolyl hydroxylase (PHD) induced hydroxylation of a proline residue in the N-terminal ankyrin repeat domain induces activation of TRPA1 through induction of hypoxia (Nakagawa and Kaneko, 2017). TRPA1 activation through PHD inhibition on oxaliplatin-induced cold hypersensitivity has been previously investigated. The study showed oxaliplatin, and dimethyl oxalate as a membrane-permeable oxalate analog induced TRPA1 sensitization to ROS by inhibiting PHD -mediated hydroxylation of the Pro394 residue on human TRPA1 (Miyake et al., 2016).

During inflammation, p38 mitogen-activated protein kinase (MAPK) has a significant role in the development and maintenance of neuropathic pain. The involvement of TRPA1 through activation of p38 MAPK in oxaliplatin-induced acute cold hypersensitivity in mice DRG neuron was recently reported (Yamamoto et al., 2016). The involvement of N-formylmethionine peptides such as formylmethionyl-leucyl phenylalanine [fMLP] in the induction of acute pain and mechanical allodynia through activations of TRPA1 and TRPV1 in mice were indicated by fMLP treatment (Chiu et al., 2013). Lipopolysaccharide (LPS) is a toxic by-product of bacterial lysis and mechanical allodynia is induced through activation of TRPA1 and through activation of the Toll-like receptor 4 (TLR4) signaling pathways in mice exposed to LPS treatment (Meseguer et al., 2014).

Hypersensitivity to mechanical stimuli is called "mechanical allodynia," while a thermal stimulus is called "thermal hyperalgesia." Chemotherapy-induced peripheral neuropathy has been widely investigated in experimental animals as mechanical allodynia and thermal hyperalgesia. Results of TRPA1, TRPM8, and TRPV1 on mechanical allodynia and thermal hyperalgesia are conflicting. For example, induction of a cold hypersensitivity through activation of TRPM8 but not TRPA1 in mice DRG neurons was reported following acute oxaliplatin treatment (Descoeur et al., 2011). However, on the contrary, the involvement of TRPA1 but not TRPM8 and TRPV1 was reported by Zhao et al. (2012) in oxaliplatin induced acute neuropathy in DRG neurons. No significant difference was also reported between oxaliplatin and vehicle groups for thermal hyperalgesia at 42, 47, and 52°C, although the presence of cold allodynia through TRPA1 activation was reported in oxaliplatin-treated mice (Park et al., 2015).

Proteinase-activated receptor 2 (PAR2) is a member of PAR subfamily of G protein-coupled receptors and activation of these receptors regulates several pathophysiological processes including inflammation and pain (Wu et al., 2017). The role of PAR2 on oxaliplatin-induced TRPA1 activation and peripheral pain induction was recently investigated in rat DRGs by Tian et al. (2015). The induction of mechanical hyperalgesia and cold hypersensitivity through increased PAR2 activation was also reported in the same study (Tian et al., 2015). Similarly, it has been demonstrated that inhibition of PAR2 increased oxaliplatin-induced cold sensitivity, and blockade of the TRPV1 channel induced little effects on oxaliplatin-induced cold hypersensitive in superficial dorsal horn of the rat spinal cord (Chen et al., 2015). The results of both studies suggest the involvement of PAR2 in TRPA1 activation induced cold allodynia, but not TRPV1-induced cold hypersensitive in oxaliplatin-treated rats. In addition, increase of channel sensitizations in TRPA1, TRPV1, and TRPV4 was reported in DRG of mice by paclitaxel treatment, although paclitaxel-induced cold hyperalgesia was not decreased by treatment with TRPA1, TRPV1, and TRPV4 channel antagonists (Chen et al., 2011).

The involvement of increased intracellular cAMP levels on TRP sensitization mediated neuronal damage was reported (Anand et al., 2010). Results of several studies indicated that cancer patients are very sensitive to cold after oxaliplatin treatment. In addition to chemicals and oxidative stress, cold body temperature ($\leq$17°C) activates TRPA1. Therefore, the TRPA1 acts as a "cold sensor" which is increased by pain induction (Yamamoto et al., 2016), although there is inconsistent evidence for its role in cold detection (Bandell et al., 2004; Bautista et al., 2005; Anand et al., 2010). In addition, there is synergic interaction between TRPA1 and TRPV1 on channel activation mechanisms in DRG, because TRPA1 is colocalized with 30–50% TRPV1 expressing neurons in rat and human DRG (Bautista et al., 2005). The sensitization ratio of TRPA1 and TRPV1 are affected by several factors, including chemotherapeutic agents. Increased TRPA1 and TRPV1 channel

sensitization was reported in peripheral neurons of oxaliplatin-treated mice (Anand et al., 2010; Wainger et al., 2015). Increased protein expression levels of TRPA1 (Ta et al., 2010; Descoeur et al., 2011; Nassini et al., 2011; Zhao et al., 2012; Yamamoto et al., 2016), TRPV1 (Descoeur et al., 2011; Nassini et al., 2011), and TRPM8 protein (Gauchan et al., 2009; Ta et al., 2010; Descoeur et al., 2011) were reported in DRG and trigeminal ganglion by acute oxaliplatin and cisplatin treatments, but conflicting reports are also present on the expression levels of TRPA1, TRPM8 (Zhao et al., 2012), and TRPV1 (Ta et al., 2010; Zhao et al., 2012) in the DRG neurons.

Goshajinkigan is a traditional Japanese medicine and it has been previously used for the treatment of numbness of the extremities, low back pain, and diabetic neuropathy. The effect of Goshajinkigan on neuropathy through inhibition or stimulation of TRPA1, TRPM8, and TRPV1 channels in oxaliplatin-induced neuropathy rat model was recently investigated (Mizuno et al., 2014). Enhanced nociceptive behaviors and DRG mRNA expression levels of TRPA1 and TRPM8 but not TRPV1 mRNA in the oxaliplatin-treated rats were also decreased following treatment with goshajinkigan.

There are some notable differences on cold dependent-activation of TRPA1 channel between humans and rodents. For example, cold dependent activation of TRPA1 was reported in rat and mouse but not in humans or rhesus monkeys (Chen et al., 2013). Co-expression and synergic interactions between TRPV1 and TRPA1 were also observed in nociceptive neuronal fibers in rats with oral ulcerative mucositis–induced spontaneous pain following treatment with 5-fluorouracil (5-FU), and the TRPV1 but not TRPA1 was activated by 5-FU treatment (Chen et al., 2013).

TRPM8

TRPM8 is expressed in a distinct subset of nociceptors, including DRG neurons and the channel is activated by cool temperature (<25°C), menthol, icilin (Nazıroğlu and Ozgül, 2012; Okazawa et al., 2014). As already mentioned, hypersensitivity of cold stimuli in patients can occur after infusion of oxaliplatin into cancer patients. Oxaliplatin induced-cold allodynia and increases in TRPM8 mRNA levels in the DRG of rats were also reported (Gauchan et al., 2009; Ta et al., 2010). However, oxaliplatin-induced cold hypersensitivity in neuropathic pain models were decreased by deletion of the TRPM8 gene and treatments of TRPM8 and TRPV1 antagonists, but not by a TRPV1 antagonist (5′-iodoresiniferatoxin) treatment (Gauchan et al., 2009; Ta et al., 2010). Consistent with these reports, one study reported oxaliplatin-induced induction of cold hyperalgesia and increased TRPM8 mRNA levels (3, 5, and 8 days of oxaliplatin treatment) in the DRG of rats (Kawashiri et al., 2012). Furthermore, they observed oxalate-induced increase of TRPM8 protein in the DRG (Kawashiri et al., 2012).

Voltage gated calcium channels (VGCC) are very selective to Ca^{2+} and they are activated by increases in voltage but they are inhibited by a decrease of intracellular and cell membrane voltage changes. Based on their threshold of voltage-dependent activation, they were divided into two subgroups as high-voltage activated channels (HVA) and low-voltage-activated (LVA) channels (Kumar et al., 2014). HVA channels can be further subdivided into 5 types (L-, P/Q-, N-, and R-type) according to their biophysical, pharmacological, and molecular features. Interactions between TRPM8 and molecular pathways or other calcium channels were also investigated in DRG neurons of oxaliplatin-treated experimental animals. Oxaliplatin-induced cold hyperalgesia was increased by stimulation of the L-type channel, nuclear factor of activated T-cell and TRPM8, although the cold hyperalgesia was decreased by VGCC blocker treatments. In addition, TRPM8 mRNA and protein expression levels in the L4-6 DRG of oxaliplatin treated rats were increased following oxaliplatin treatment (Kawashiri et al., 2012).

TRPV1

TRPV1 is a member of vanilloid subfamily of the TRP superfamily. The channel was firstly expressed in rats through activation of high temperature and pungent hot chili pepper component (capsaicin) in mice DRG (Caterina et al., 1997). In addition to capsaicin and high temperature (>43°C), the channel can be activated by different stimuli including low pH (<5.9), oxidative stress leading to the perception of pain, and oxidative injury (Tominaga et al., 1998; Yoshida et al., 2006; Nazıroğlu, 2015). Apart from mice, the channel was also expressed in DRG of different mammalian animals and human (Hayes et al., 2000), and also has six transmembrane domains. Cysteine groups as a source of thiol redox system act as the main source of different antioxidants such as GSH, glutathione peroxidase and alpha lipoic acid (Sen and Packer, 2000). Hence, the cysteine groups represent the main target of ROS and reactive nitrogen species (RNS) (Nazıroğlu, 2007). In addition to TRPA1 (Takahashi et al., 2011), it was reported that oxidative alterations of multiple Cys residues in different cells are involved in this mode of TRPV1 activation through modifying the extracellular (Yoshida et al., 2006) or intracellular Cys residues (Chuang and Lin, 2009) and disulfide bond formation (Wang and Chuang, 2011). In addition, results of a recent study indicated heterogeneous subunit composition of TRPV1 through heterogeneous modification of Cys-258 residues in the human TRPV1 tetrameric complex in disulfide bond of the channels (Ogawa et al., 2016). Therefore, the TRPV1 is activated in DRG (Nazıroğlu et al., 2013), hippocampus (Övey and Nazıroğlu, 2015) of rats by depletion of intracellular GSH, although the channel was inhibited in cells following treatment with thiol redox cycle members such as GSH, selenium and N acetyl cysteine (Nazıroğlu et al., 2013, 2014; Kahya et al., 2017).

ROS are produced in physiological levels as part of normal mitochondrial function and phagocytic activity. During the removal of bacteria and viruses, ROS are produced by anti-inflammatory cells such as macrophages microphages and microglia. Therefore, there is direct relationship between increased levels of ROS and inflammatory hyperalgesia (Oehler et al., 2017). Interaction between TRPV1 and long sustained thermal hypersensitivity (but not mechanical hypersensitivity) in oxidative stress-induced inflammatory hyperalgesia of the mouse hind paw has been previously reported (Keeble et al., 2009). Therefore, there is a direct role of ROS through activation of TRPV1 on hyperalgesia in the DRG neuron (Ibi et al., 2008).

Cisplatin-induced TRPV1 channel expressions were investigated in DRG neuron by Hori et al. (2010) and they observed no change on the frequency of TRPV1-positive cells in DRG neurons with different diameter by cisplatin treatment. The roles of mechanical hyperalgesia in TRPV1 knockout mouse and pronociceptive role of TRPV1 in mild burn (51°C for 15 s) injury was also reported in another study (Bölcskei et al., 2005), but they observed no pronociceptive role of TRPV1 in cisplatin-induced toxic neuropathy.

Reports on cisplatin-induced thermal sensitivity in rodents are conflicting. For example, cutaneous mechanical allodynia and hyperalgesia but not noxious thermal sensitivity was reported by cisplatin treatment (Hori et al., 2010). Some studies reported induction of mechanical and cold stimuli hyperalgesia and allodynia associated with minor motor disorders (Authier et al., 2003), whereas other studies (De Koning et al., 1987; Tredici et al., 1999) reported no effects in the responses to thermal stimulation after cisplatin treatment. In a recent study (Zhao et al., 2012), TRPV1-mediated nociceptive behaviors are not affected by cisplatin, paclitaxel and oxaliplatin. In addition, the numbers of capsaicin-sensitive DRG neurons were not changed by oxaliplatin treatment and the authors concluded that there is no role of TRPV1 on oxaliplatin-induced acute peripheral neuropathy in the DRG neurons. Consistent with the report, induction of thermal hyperalgesia through increased TRPV1 expression in the DRG after paclitaxel treatment was observed, although the hyperalgesia was decreased by TRPV1 treatment (Hara et al., 2013). In addition, the TRPV1 activator role of paclitaxel via stimulation of TLR4 signaling was reported in DRG neurons of human and paclitaxel-treated rats (Li et al., 2015).

In a study, diameters of TRPV1 remained unchanged in mice DRG neurons following cisplatin treatment, although the occurrence of TRPV1 in the neurons was increased by cisplatin treatment (Khasabova et al., 2012). In contrary to the report, no protective effect of TRPV1 (AMG9810) or TRPA1 (HC030031) antagonists on cisplatin-evoked mechanical and cold allodynia in rats was reported in another study (Guindon et al., 2013). Induction of mechanical hyperalgesia, and cold allodynia (via 10°C water) in rat models of cisplatin-induced peripheral neuropathy were reported (Authier et al., 2003; Nassini et al., 2011). Similar result was observed by Ta et al. (2009) and increased thermal hyperalgesia to cold was reported in cisplatin-treated mice. However, some authors attributed the direct effect of cisplatin to TRPA1 instead of TRPV1 in the neuron, because TRPA1 receptors are required for the development of cisplatin-evoked mechanical allodynia in mice (Nassini et al., 2011; Khasabova et al., 2012).

Increased intracellular Ca^{2+} concentrations induced release of excessive substance P from the central and peripheral nerve terminals of DRG neurons in response to noxious stimuli (Sacerdote and Levrini, 2012). The role of VGCC blockers and TRPV1 channel was also investigated on paclitaxel- and vinorelbine (a chemotherapeutic drug)-induced substance P release in DRG neuron of rats and no role of TRPV1 on the substance P release was observed in the DRG (Miyano et al., 2009).

The involvement of oxaliplatin on the release of calcitonin gene-related peptide from rat sensory neurons in culture was recently reported (Pittman et al., 2014). In addition, they reported that TRPA1 and TRPV1 channel activation-induced transmitter release were increased or decreased according to the concentration and exposure time of the drug and in peptidergic DRG neurons with small diameter by paclitaxel treatment.

TRPV2

Another member of TRP superfamily is the TRPV2 and the channel is also a member of thermosensitive TRP channels and it is activated by a very high-threshold heat temperature (>52°C; Ahluwalia et al., 2002). There are limited data and reports on the physiological role of the TRPV2 channel in the literature. Cisplatin-induced TRPV2 channel expressions were investigated in DRG neuron (Hori et al., 2010) and increased of TRPV2 protein expression in the small-cell of L5 positive DRG neurons but not in L5 DRG cells innervating gastrocnemius muscle was reported following cisplatin administration (Hori et al., 2010). Increase of highly noxious temperatures (>56°C)-induced TRPV2 protein expression levels in peripheral thermal of neuron via the transduction of pain hypersensitivity (Shimosato et al., 2005). Because selective TRPV2 antagonists are not commercially available, further mechanistic studies including TRPV2 knockout mouse might be needed to determine the exact involvement of TRPV2 in cisplatin-induced neuropathy.

TRPV4

As a member of TRP superfamily, TRPV4 was firstly described with mammalian osmotransducer property (Liedtke et al., 2000). Several activators of TRPV4 such as low pH, citrate, phorbol esters, arachidonic acid, oxidative stress, and exogenous chemicals (bisandrographolide A) have been described (Güler et al., 2002; Alessandri-Haber et al., 2004; Materazzi et al., 2012). Additionally, TRPV4 is activated by heat (24°C≥) (Güler et al., 2002) and the channel is also a member of thermo-TRP group. Enhanced nociception in neuropathic pain was reported by heat activation of TRPV4. Therefore, TRPV4 is essential for inflammatory thermal hyperalgesia (Davis et al., 2000), but not for normal heat sensation (Caterina et al., 2000).

Induction of hyperalgesia through activation of α2β1 integrin and Src tyrosine kinase pathways in rat DRG neuron was reported in the TRPV4 knockout mice by paclitaxel treatment (Alessandri-Haber et al., 2008). However, similar results were not shown in hind paw and DRG of rats by the same study, and TRPV4 did not act an essential role in paclitaxel-induced nociceptive behavioral responses to mechanical and hypotonic stimulation in the hind paw (Alessandri-Haber et al., 2004). It was reported in DRG neurons of wild type and TRPV4 knockout mice that TRPA1 and TRPV4 are activated by paclitaxel-induced mechanical allodynia and excessive ROS production but not cold exposure, although the allodynia and oxidative stress was partially decreased by treatment with a TRPV4 (HC-067047) antagonist (Materazzi et al., 2012). RN1734 is also a TRPV4 antagonist (Vincent et al., 2009) and inhibition of TRPV4 did

not alter nociceptive baseline in control mice, and mechanical allodynia and heat are partially reserved by RN1734.

CONCLUSION AND FUTURE SUBJECTS

Accumulating evidence suggests that neuropathic pain and painful neurotoxicity in the rodents are increased by selected chemotherapeutic agent through increased sensitization of TRPA1, TRPM8, and TRPV1. In addition, antagonists of TRPA1 and TRPM8 were able to attenuate cisplatin, oxaliplatin, and paclitaxel-induced mitochondrial oxidative stress, inflammation, cold allodynia, and hyperalgesia, although TRPV1 was responsible for cisplatin-induced heat hyperalgesia and mechanical allodynia in sensory neurons. TRPA1, TRPM8, and TRPV2 protein expression levels were mostly increased in the DRG and trigeminal ganglia neurons by chemotherapeutic agents. There is a debate on direct or oxaliplatin-induced oxidative cold stress dependent TRPA1 and TRPV4 activation in the DRG. Involvement of molecular pathways such as cysteine group, GSH, anandamide, cAMP, lipopolysaccharide, proteinase-activated receptor 2, and mitogen-activated protein kinase were also indicated in oxaliplatin and paclitaxel-induced cold allodynia. Therefore, there is growing evidence for the potential role of TRP channel inhibitors as modulators of chemotherapy-induced neuropathic pain in the clinic.

A new member of the TRP superfamily is TRPM2. The enzyme ADPR pyrophosphatase in the C-terminal domain of TRPM2 is sensitive to ROS and RNS (Wehage et al., 2002; Nazıroğlu, 2007; Nazıroğlu and Lückhoff, 2008). It is well-known that excessive ROS production contributes to sensitization in persistent pain of DRG neuron (Kallenborn-Gerhardt et al., 2012). In addition, results of recent studies have suggested the involvement of warm temperature on the activation of TRPM2 channels in the rat DRG neurons (Tan and McNaughton, 2016). To our knowledge, there is no study of the interaction between TRPM2 channel and chemotherapeutic agents in DRG neurons. Future studies should investigate the interactions between TRPM2 and other oxidative stress-dependent TRP channels such as TRPM7 and TRPC5 in the DRG neuron following exposure to chemotherapeutic agents. There is no report on interactions between remaining thermo-TRP channels such as TRPV3 and TRPM3 and chemotherapeutic agents in the peripheral neurons. The interaction should be also clarified in primary neurons.

AUTHOR CONTRIBUTIONS

MN formulated the present hypothesis and he was responsible for writing the report. NB made critical revision for the manuscript. The original figures were produced by MN.

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Distinct Mechanism of Cysteine Oxidation-Dependent Activation and Cold Sensitization of Human Transient Receptor Potential Ankyrin 1 Channel by High and Low Oxaliplatin

Takahito Miyake[1], Saki Nakamura[1], Zhao Meng[1], Satoshi Hamano[2], Keisuke Inoue[2], Tomohiro Numata[3], Nobuaki Takahashi[2], Kazuki Nagayasu[1], Hisashi Shirakawa[1], Yasuo Mori[2], Takayuki Nakagawa[4] and Shuji Kaneko[1]*

[1] Department of Molecular Pharmacology, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan, [2] Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto, Japan, [3] Department of Physiology, Graduate School of Medical Sciences, Fukuoka University, Fukuoka, Japan, [4] Department of Clinical Pharmacology and Therapeutics, Kyoto University Hospital, Kyoto, Japan

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***Correspondence:**
Takayuki Nakagawa
tknakaga@kuhp.kyoto-u.ac.jp

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Oxaliplatin, a third-generation platinum-based chemotherapeutic agent, displays unique acute peripheral neuropathy triggered or enhanced by cold, and accumulating evidence suggests that transient receptor potential ankyrin 1 (TRPA1) is responsible. TRPA1 is activated by oxaliplatin via a glutathione-sensitive mechanism. However, oxaliplatin interrupts hydroxylation of a proline residue located in the N-terminal region of TRPA1 via inhibition of prolyl hydroxylase (PHD), which causes sensitization of TRPA1 to reactive oxygen species (ROS). Furthermore, PHD inhibition endows cold-insensitive human TRPA1 (hTRPA1) with ROS-dependent cold sensitivity. Since cysteine oxidation and proline hydroxylation regulate its activity, their association with oxaliplatin-induced TRPA1 activation and acquirement of cold sensitivity were investigated in the present study. A high concentration of oxaliplatin (1 mM) induced outward-rectifier whole-cell currents and increased the intracellular Ca^{2+} concentration in hTRPA1-expressing HEK293 cells, but did not increase the probability of hTRPA1 channel opening in the inside-out configuration. Oxaliplatin also induced the rapid generation of hydrogen peroxide, and the resultant Ca^{2+} influx was prevented in the presence of glutathione and in cysteine-mutated hTRPA1 (Cys641Ser)-expressing cells, whereas proline-mutated hTRPA1 (Pro394Ala)-expressing cells showed similar whole-cell currents and Ca^{2+} influx. By contrast, a lower concentration of oxaliplatin (100 μM) did not increase the intracellular Ca^{2+} concentration but did confer cold sensitivity on hTRPA1-expressing cells, and this was inhibited by PHD2 co-overexpression. Cold sensitivity was abolished by the mitochondria-targeting ROS scavenger mitoTEMPO and

was minimal in cysteine-mutated hTRPA1 (Cys641Ser or Cys665Ser)-expressing cells. Thus, high oxaliplatin evokes ROS-mediated cysteine oxidation-dependent hTRPA1 activation independent of PHD activity, while a lower concentration induces cold-induced cysteine oxidation-dependent opening of hTRPA1 via PHD inhibition.

Keywords: TRPA1, oxaliplatin, reactive oxygen species, cold hypersensitivity, prolyl hydroxylase, cysteine oxidation, peripheral neuropathy

INTRODUCTION

Oxaliplatin (L-OHP), a third-generation platinum-based agent, is frequently used to treat locally advanced and metastatic cancers of the colon or rectum. However, it increases the incidence of chemotherapy-induced peripheral neuropathy (CIPN), often resulting in chemotherapeutic dose delay or treatment discontinuation (Falcone et al., 2007; Miltenburg and Boogerd, 2014). In addition to cumulative and chronic CIPN after multiple chemotherapy cycles, oxaliplatin induces a peculiar acute CIPN, characterized by paresthesia, dysesthesia, or acral numbness, in ∼90% of patients during or within hours of infusion. Acute CIPN is specific to oxaliplatin and often triggered or exacerbated by cold exposure (Wilson et al., 2002; Miltenburg and Boogerd, 2014; Cavaletti and Marmiroli, 2015).

The mechanisms underlying L-OHP-induced chronic CIPN can be explained, at least in part, by neurotoxicity in peripheral sensory neurons due to mitochondrial dysfunction and generation of reactive oxygen species (ROS) (Joseph and Levine, 2009; Di Cesare Mannelli et al., 2012; Azevedo et al., 2013) following accumulation of platinum in the dorsal root ganglia (DRG) (Screnci et al., 2000; Cavaletti et al., 2001). By contrast, L-OHP-induced acute CIPN is recognized as a channelopathy. A body of evidence suggests that it is caused by alteration of the kinetics of the axonal voltage-gated Na^+ channel (Sittl et al., 2012; Deuis et al., 2013) and/or activation of transient receptor potential ankyrin 1 (TRPA1) (Nassini et al., 2011; Zhao et al., 2012).

TRPA1 is a polymodal cation channel that plays a pivotal role as a nociceptor (Wu et al., 2010; Viana, 2016). This channel is opened by a large number of irritant chemicals (Bandell et al., 2004; Jordt et al., 2004). TRPA1 is also activated by oxidative stimuli such as, ROS and hyperoxia (Takahashi et al., 2008, 2011). TRPA1 activation evoked by most irritant chemicals and oxidative stimuli is caused by covalent or oxidative modification of cysteine residues in the N-terminal region (Hinman et al., 2006; Macpherson et al., 2007). On the other hand, we previously identified another mechanism for TRPA1 activation; a decrease in oxygen concentration diminishes the activity of prolyl hydroxylases (PHDs) and relieves TRPA1 from the PHD-dependent hydroxylation of a proline residue (Pro^{394}) located within the N-terminal ankyrin repeat domain, leading to hypoxia-induced activation (Takahashi et al., 2011; So et al., 2016).

It is reported that both L-OHP and cisplatin activate TRPA1 via glutathione-sensitive mechanisms (Nassini et al., 2011). However, we previously demonstrated that L-OHP and its characteristic metabolite oxalate enhance the responsiveness of TRPA1, which may contribute to the cold hypersensitivity induced by L-OHP in mice, but this is not the case for cisplatin and paclitaxel (Zhao et al., 2012). We also demonstrated the molecular mechanism: L-OHP and oxalate inhibit PHD activity, which augments the sensitivity of human TRPA1 (hTRPA1) to ROS by inhibiting hydroxylation of Pro^{394}. Furthermore, we found that use of a PHD inhibitor or a hTRPA1 mutant lacking the hydroxylation-susceptible Pro^{394} residue induces hTRPA1 sensitization to ROS, which enables cold-insensitive hTRPA1 to sense cold by detecting cold-evoked ROS production (Miyake et al., 2016). Therefore, the cold-induced indirect activation of hTRPA1 that is sensitized by PHD inhibition may be responsible for L-OHP-induced acute CIPN triggered by cold, although whether L-OHP actually endows cold sensitivity to hTRPA1-expressing cells has not been clarified. Thus, L-OHP is likely to activate and sensitize TRPA1, but whether the L-OHP-induced TRPA1 activation and sensitization is due to the oxidation of cysteine residues and/or inhibition of proline hydroxylation remains unknown.

In this study, we investigated whether and how cysteine oxidation and/or inhibition of proline hydroxylation contribute to the L-OHP-induced hTRPA1 activation and sensitization *in vitro*. A high concentration of L-OHP evoked cysteine oxidation-dependent hTRPA1 activation, independent of hydroxylation of the PHD-targeted proline residue, while a subthreshold concentration of L-OHP endowed hTRPA1 with cysteine oxidation-dependent cold sensitivity through PHD inhibition.

MATERIALS AND METHODS

Reagents

L-OHP and allyl isothiocyanate (AITC) were purchased from Wako Pure Chemical Industries (Osaka, Japan). N-tert-butyl-α-phenylnitrone (PBN), cremophore EL, 2-aminoethoxydiphenyl borate (2-APB), poly-L-lysine, and D-mannitol were purchased from Sigma-Aldrich (St. Louis, MO). The 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) was acquired from Dojindo Laboratories (Kumamoto, Japan). The mitoTEMPO was obtained from Santa Cruz (Dallas, TX). Peroxy Green 1 (PG-1) was synthesized previously (Miyake et al., 2016) according to the literature (Miller et al., 2007). Other drugs and chemicals were obtained from Nacalai Tesuque (Kyoto, Japan).

Plasmids

Constructs consisting of recombinant hTRPA1, its cysteine mutants (C633S, C641S, C665S), its proline mutant (P394A), or the human PHD2 cDNA in the pCIneo expression vector were

prepared previously (Takahashi et al., 2011). The pEGFP-C3 was purchased from Clontech Laboratories (Madison, WI).

Cell Cultures and Transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX I (10566-016, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Sigma) and maintained at 37°C in a humidified incubator set at 5% CO_2. HEK293 cells were co-transfected with recombinant plasmids and pEGFP-C3 using SuperFect Transfection Reagent (Qiagen, Hilden, Germany) or Lipofectamine 2000 (Life Technologies). Two days after transfection, cells were placed onto coverslips coated with poly-L-lysine and used in electrophysiological recording or fluorometric imaging.

Electrophysiology

Electrophysiological recordings were performed with a pipette made from a glass capillary (outer diameter, 1.5 mm) with an internal filament (Narishige, Tokyo, Japan) pulled using a P-87 micropipette puller (Sutter, Novato, CA). Access resistance ranged from 2 to 5 MΩ when the pipette was filled with pipette solution described below. For whole-cell patch-clamp recordings, the bath solution contained 100 mM NaCl, 2 mM $CaCl_2$, and 10 mM HEPES (adjusted to pH 7.4 with NaOH and 300 mOsm with D-mannitol), and the pipette solution contained 100 mM Cs-aspartate, 5 mM BAPTA, 1.4 mM Ca-gluconate (30 nM free Ca^{2+}), 2 mM $MgSO_4$, 2 mM $MgCl_2$, 4 mM Na_2-ATP, 10 mM $Na_5P_3O_{10}$, and 10 mM HEPES (adjusted to pH 7.4 with CsOH and 300 mOsm with D-mannitol). Current-voltage relationships were measured using voltage ramps (−100 to +100 mV over 100 ms) applied every 10 s. The membrane potential was set at 0 mV. Access resistance values were compensated by 70%. For inside-out patch-clamp recordings, the bath solution contained 50 mM Cs-aspartate, 50 mM CsCl, 10 mM EGTA, 1 mM $CaCl_2$ (10 nM free Ca^{2+}), 1 mM $MgCl_2$, 10 mM $Na_5P_3O_{10}$, and 10 mM HEPES (adjusted to pH 7.4 with CsOH and 300 mOsm with D-mannitol), and the pipette solution contained 100 mM CsCl, 1 mM $MgCl_2$, 1 mM EGTA, and 10 mM HEPES (adjusted to pH 7.4 with CsOH and 300 mOsm with D-mannitol). The membrane potential was set at +80 mV. Data were filtered at 2.9 kHz. Experiments were conducted at room temperature. Patch-clamp recordings were performed using an EPC-10 patch-clamp amplifier (HEKA Instruments, Lambrecht, Germany) and PATCHMASTER software (HEKA). AITC (100 μM) was used to validate the expression of hTRPA1. The representative trace was obtained at least three independent experiments.

Measurement of Intracellular Ca^{2+} Concentration ($[Ca^{2+}]_i$)

Cells on coverslips were loaded for 30–40 min with 5 μM Fura-2 acetoxymethyl ester (Fura-2 AM; Dojindo Laboratories) in Krebs-Ringer solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES (pH adjusted to 7.4 with NaOH) containing 0.005% cremophore EL. Fluorescence images were captured every 5 s using alternating excitation at 340 and 380 nm and emission at 510 nm with an AQUACOSMOS/ORCA-AG imaging system (Hamamatsu Photonics, Shizuoka, Japan). For the pretreatment, L-OHP (100 μM) was added to the culture medium 2 h before loading. MitoTEMPO (10 μM, 10 mM stock solution in DMSO was diluted with the Fura-2 contained Krebs-Ringer solution) was preloaded with Fura-2 loading. Note that all drugs used for pretreatments were removed by washing before Ca^{2+} imaging experiments. Experiments were conducted at room temperature unless otherwise stated. Cold stimulation was performed with an SC-20 dual in-line solution heater/cooler and a CL-100 temperature controller (Warner Instruments, Hamden, CT). The velocity of the cooling ramp is about 3.75°C/min. The ratio of the fluorescence intensity obtained by excitation/emission at 340 nm/510 nm (F_{340}) to the fluorescence intensity obtained by excitation/emission at 380 nm/510 nm (F_{380}), namely, F_{340}/F_{380}, was calculated to quantify the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Cells with an F_{340}/F_{380} ratio >1.5 at baseline were excluded. Statistical analysis of the change in the ratio, ΔRatio (F_{340}/F_{380}), was performed as follows; in **Figure 3**, the average ΔRatio (F_{340}/F_{380}) during 0–2 min after 2-APB-application was used; in **Figure 5**, the ΔRatio (F_{340}/F_{380}) at 2 min after cold stimulation was used. AITC (100 μM) or 2-APB (100 μM) was used to validate the expression of hTRPA1.

Measurement of Intracellular H_2O_2 Level

Intracellular H_2O_2 level was measured using PG-1, a fluorescent probe with high selectivity for H_2O_2 (Miller et al., 2007). Cells on coverslips were loaded for 30–40 min with 5 μM PG-1 in HEPES-buffered saline containing 107 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 11.5 mM D-glucose, and 20 mM HEPES (pH adjusted to 7.4 with NaOH). Fluorescence images were captured every 20 s using alternating excitation at 488 nm and emission at 510 nm with the AQUACOSMOS/ORCA-AG imaging system (Hamamatsu Photonics). Experiments were conducted at room temperature. The fluorescence intensity obtained with excitation/emission of 488 nm/510 nm relative to the values obtained at 0 min (F/F_0) was calculated to validate the intracellular H_2O_2 concentration. The $\Delta F/F_0$ obtained from each cells at 15 min was used for statistical analysis.

Statistical Analysis

The data are presented as means ± S.E.M. from *n* independent experiments or cells. Statistical significances were calculated using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). The data in **Figures 2B,C**, **3C,D**, **5B,C** were compared using unpaired Student's *t*-tests (vs. Ctrl in **Figure 2C**, vs. WT in **Figures 3C,D**, **5C**). The data in **Figures 1D**, **5A** were compared using one-way analyses of variance (ANOVA), followed by Tukey's multiple comparisons test. In all cases, $P < 0.05$ were considered statistically significant.

RESULTS

A High Concentration of L-OHP Induces hTRPA1 Activation

To investigate whether L-OHP activates hTRPA1, we performed whole-cell patch-clamp recording and fura-2-based intracellular

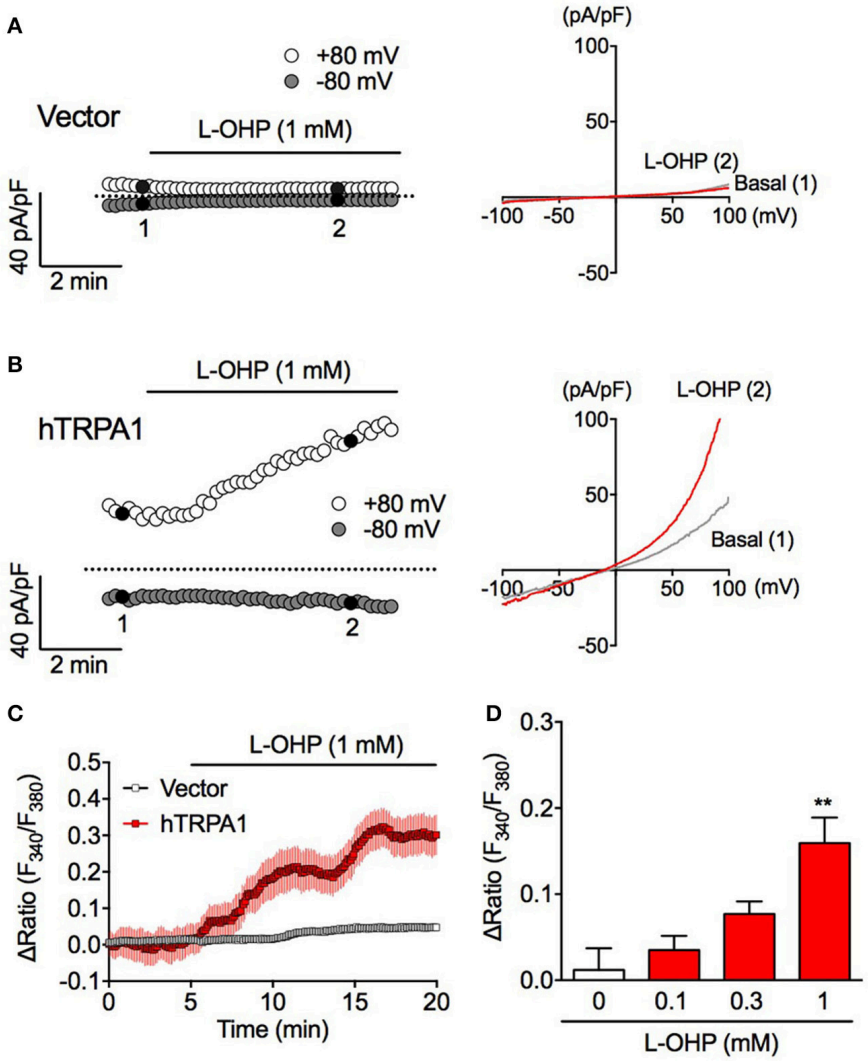

FIGURE 1 | High concentration of L-OHP induces hTRPA1 activation. **(A,B)** Effect of L-OHP (1 mM) on the whole-cell currents in HEK293 cells transfected with control **(A)** or hTRPA1 **(B)** vector. Left traces are representative whole-cell recordings. The current-voltage relationships were acquired at the time indicated by black-filled circle in **(A,B)**, respectively. Membrane potential was set at 0 mV. **(C)** Effect of L-OHP on the intracellular Ca^{2+} concentration in HEK293 cells transfected with control or hTRPA1 vector (n = 26–53 cells). Left panel shows representative traces of intracellular Ca^{2+} imaging. Panel **(D)** shows the statistical analysis for concentration-dependent effect of L-OHP (0.1, 0.3, and 1 mM). n = 4–8 independent experiments. $^{**}P < 0.01$ vs. the vehicle-treated hTRPA1-expressing cells (Veh). Panels **(C,D)** are expressed as mean ± S.E.M. All of the experiments were performed at room temperature.

Ca^{2+} imaging experiments. In vector-transfected HEK293 cells, L-OHP (1 mM) did not induce a membrane current (**Figure 1A**). Meanwhile, in hTRPA1-expressing cells, L-OHP (1 mM) induced gradually increasing TRPA1-like outward-rectifier currents (**Figure 1B**). Intracellular Ca^{2+} imaging revealed that L-OHP (1 mM) also significantly increased $[Ca^{2+}]_i$ in hTRPA1-expressing cells in a concentration-dependent manner (**Figure 1C**). However, no statistically significant increase was observed at lower concentrations of L-OHP (0.1 or 0.3 mM). These results indicate that L-OHP activates hTRPA1 when present at a concentration of at least 1 mM.

High L-OHP-Induced ROS Generation Is required for hTRPA1 Activation

To clarify whether L-OHP activates hTRPA1 directly, we performed inside-out patch-clamp recording experiments for removing intracellular components. We held the membrane potential at +80 mV, at which point weak voltage-gated hTRPA1 is easy to open. Although AITC (100 μM) increased the probability of hTRPA1 opening, a high concentration of L-OHP (1 mM) had no effect (**Figure 2A**), suggesting that high L-OHP activates hTRPA1 indirectly. Since L-OHP reportedly induces mitochondrial dysfunction and triggers ROS generation (Zheng et al., 2011), we examined the effect of high L-OHP (1 mM) on the

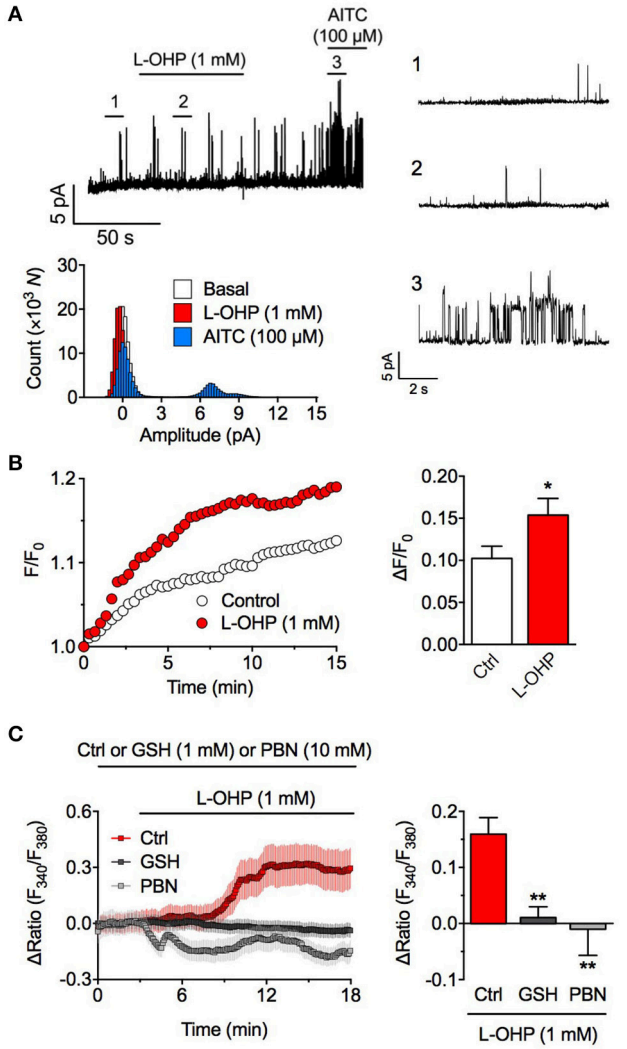

FIGURE 2 | High concentration of L-OHP activates hTRPA1 via ROS generation. **(A)** Effect of L-OHP (1 mM) on the open probability of hTRPA1 in inside-out recordings from hTRPA1-expressing HEK293 cells. Left upper panel shows a representative trace, right panels show the magnified traces at the time point as indicated in the left trace, and left lower panel shows their histogram. Membrane potential was set at +80 mV. Note that even at positive voltage, weak voltage-dependent TRPA1 was not activated by L-OHP. **(B)** Effect of L-OHP (1 mM) on the intracellular H_2O_2 concentration in HEK293 cells. Left panel shows representative traces of intracellular H_2O_2 imaging using PG-1, and right panel shows its statistical analysis. $n = 28$–32 cells from two independent experiments. $^*P < 0.05$ vs. vehicle-treated hTRPA1-expressing cells (Ctrl). **(C)** Effect of pretreatment with glutathione (1 mM) or PBN (10 mM) on the L-OHP-evoked $[Ca^{2+}]_i$ increase in hTRPA1-expressing HEK293 cells. Left panel shows representative traces of intracellular Ca^{2+} imaging ($n = 22$–34 cells), and right panel shows its statistical analysis ($n = 5$–8 independent experiments). $^{**}P < 0.01$ vs. non-treated hTRPA1-expressing cells (Ctrl). All of the experiments were performed at room temperature. All data except for **(A)** are expressed as mean ± S.E.M. GSH; glutathione.

intracellular ROS level using the H_2O_2-specific indicator PG-1 (Miller et al., 2007). In HEK293 cells, high L-OHP induced H_2O_2 generation within 15 min (**Figure 2B**). Furthermore, the high L-OHP-evoked $[Ca^{2+}]_i$ increase was significantly suppressed in the presence of the antioxidants glutathione (1 mM) or PBN (10 mM) (**Figure 2C**). These results suggest that high L-OHP does not directly activate hTRPA1 but rather triggers ROS generation, which causes glutathione-sensitive hTRPA1 activation.

High L-OHP-Evoked hTRPA1 Activation Is Regulated by Cysteine Oxidation and Is Independent of PHDs

Since ROS-induced TRPA1 activation is caused by oxidative modulation of the cysteine residues in the N-terminal region of TRPA1 (Takahashi et al., 2008; **Figure 3A**), we compared the high L-OHP-evoked $[Ca^{2+}]_i$ increase in HEK293 cells expressing wild-type hTRPA1 (hTRPA1-WT) or hTRPA1 cysteine mutants (hTRPA1-C633S, C641S, C665S) in which each ROS or oxygen-sensitive cysteine residue was replaced with serine, which is a well-characterized strategy to investigate the redox sensitivity of cysteine residues in TRPA1 (Macpherson et al., 2007; Takahashi et al., 2008, 2011). Among the hTRPA1 cysteine mutants, hTRPA1-C641S showed a significantly weaker response to high L-OHP (**Figures 3B,C**), while the responses of the other mutants (C633S and C665S) were comparable with hTRPA1-WT. By contrast, the response to 2-APB (100 μM), a cysteine oxidation-independent TRPA1 agonist (Hinman et al., 2006; Hu et al., 2009b), did not differ between hTRPA1-WT and its mutants (**Figures 3B,D**).

To investigate whether PHD inhibition is involved in high L-OHP-evoked hTRPA1 activation, we examined whether high L-OHP activates a PHD inhibition-insensitive hTRPA1 mutant hTRPA1-P394A. In whole-cell patch-clamp recordings, L-OHP (1 mM) successfully induced TRPA1-like outward-rectifier currents in hTRPA1-P394A expressing cells (**Figure 4A**). In Ca^{2+} imaging experiments, both hTRPA1-WT and hTRPA1-P394A showed a $[Ca^{2+}]_i$ increase induced by high L-OHP, and we did not observed any difference between them [**Figure 4B**, $n = 6$ independent experiments, $P = 0.233$ vs. hTRPA1-WT (**Figure 1D**, 1 mM L-OHP treated group, $n = 8$ independent experiments)]. These results suggest that high L-OHP activates hTRPA1 in a cysteine oxidation-dependent manner, while L-OHP-induced PHD inhibition is not involved in this phenomenon.

A Low Concentration of L-OHP Endows Cold Sensitivity of hTRPA1 via Both PHD Inhibition and Cysteine Oxidation

We recently reported that PHD inhibition causes hTRPA1 sensitization to ROS, which allows hTRPA1 to sense cold indirectly via cold-induced ROS generation (Miyake et al., 2016). Consistently, cold stimulation had minimal effect on $[Ca^{2+}]_i$ in non-treated hTRPA1-expressing cells in the present study, while pretreatment with a relatively low concentration of L-OHP (100 μM) for 2 h significantly increased $[Ca^{2+}]_i$ compared with that in non-treated hTRPA1-expressing cells. The cold-evoked $[Ca^{2+}]_i$ increase following low L-OHP pretreatment

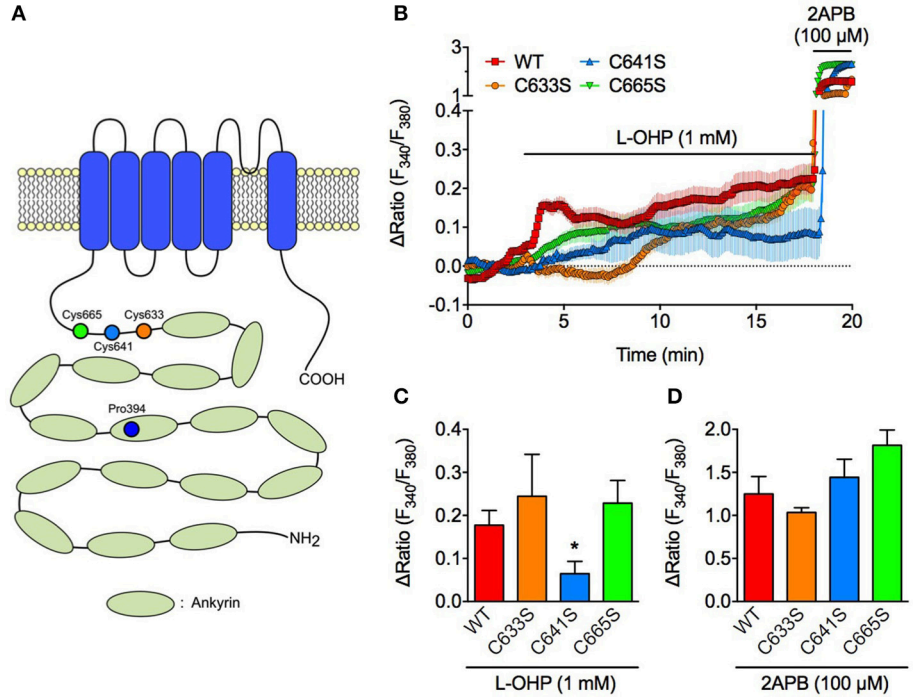

FIGURE 3 | High concentration of L-OHP activates hTRPA1 via Cys641 modification. Effect of L-OHP (1 mM) on the intracellular Ca^{2+} concentration in HEK293 cells expressing hTRPA1 cysteine mutants (hTRPA1-C633S, C641S, and C665S). 2-APB (100 μM) was used to validate the expression of hTRPA1 cysteine mutants. **(A)** Schematic diagram illustrates the location of cysteine and proline residues of hTRPA1 that we focused in this study. **(B–D)** Representative traces of intracellular Ca^{2+} imaging (n = 42–92 cells) **(B)** and its statistical analysis for L-OHP- and 2-APB-evoked $[Ca^{2+}]_i$ increase **(C,D)** (n = 5–10 independent experiments). Note that we used 2-APB instead of AITC, since AITC activates TRPA1 via Cys oxidation and not suitable for examining the full activation of TRPA1 in **(D)**. *$P < 0.05$ vs. wildtype hTRPA1-expressing cells (WT). All of the experiments were performed at room temperature. All data are expressed as mean ± S.E.M.

was partially but significantly inhibited abolished in cells co-expressing hTRPA1 and PHD2 (**Figure 5A**) and in hTRPA1-expressing cells pretreated with the mitochondria-targeting ROS scavenger mitoTEMPO (10 μM; **Figure 5B**). To investigate whether hTRPA1 cysteine residues are involved in the low L-OHP-endowed cold-evoked $[Ca^{2+}]_i$ increase, we performed the same experiments using hTRPA1 cysteine mutants. The cold-evoked $[Ca^{2+}]_i$ increase following low L-OHP pretreatment was significantly smaller in hTRPA1-C641S and hTRPA1-C665S mutants than that in hTRPA1-WT, while there was no difference between hTRPA1-WT and the hTRPA1-C633S mutant (**Figure 5C**). These results suggest that the low L-OHP-endowed cold sensitivity of hTRPA1 is dependent on both PHD inhibition and cysteine oxidation, and cold-induced mitochondrial ROS generation is important for the cold-evoked activation of hTRPA1 sensitized by low L-OHP.

DISCUSSION

Among others, TRPA1 is activated through dual mechanisms: covalent or oxidative modification of cysteine residues, and inhibition of hydroxylation of a proline residue in the N-terminal region (Takahashi et al., 2011). The results of the present study showed that a high concentration of L-OHP (≥1 mM) evoked hTRPA1 activation via ROS-mediated cysteine oxidation, independently of PHD inhibition, while both mechanisms are responsible for the cold-induced activation of hTRPA1 sensitized by the low concentration of L-OHP. The high concentration of L-OHP activated hTRPA1 in whole-cell patch clamp recordings and intracellular Ca^{2+} imaging experiments, but not membrane-excised inside-out patch clamp recordings, indicating that the high concentration of L-OHP affects cellular components other than hTRPA1 itself, and indirectly activates hTRPA1. Furthermore, glutathione and PBN suppressed the L-OHP (1 mM)-induced activation of hTRPA1, suggesting that the high concentration of L-OHP induces ROS production that is followed by the activation of hTRPA1 (**Figure 6**). We previously reported that the low concentration of L-OHP increases the sensitivity of hTRPA1 to ROS via PHD inhibition (Miyake et al., 2016). In this study, we further found that the L-OHP (100 μM)-pretreated hTRPA1-expressing cells showed larger response to cold compared with the control hTRPA1-expressing cells, which was not observed when we used PHD2-overexpressing cells, and was partially inhibited by the pretreatment with mitoTEMPO. The L-OHP dependent cold response was suppressed in ROS-sensitive cysteine-mutated hTRPA1-expressing cells, indicating that the pretreatment of L-OHP (100 μM) allows hTRPA1 to sense cold in the same mechanisms we revealed before (Miyake et al., 2016) via modification to cysteine residues (**Figure 6**).

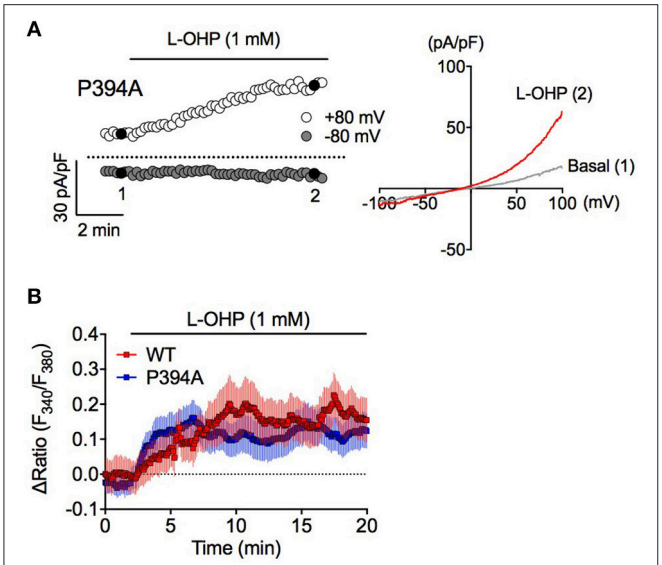

FIGURE 4 | High L-OHP evoked hTRPA1 activation is independent of PHD modification. **(A)** Effect of L-OHP (1 mM) on the whole-cell currents in HEK293 cells transfected with hTRPA1-P394A mutant vector. Left traces are representative whole-cell recordings. The current-voltage relationships were acquired at the time indicated by black-filled circle, respectively. Membrane potential was set at 0 mV. **(B)** Representative traces of intracellular Ca^{2+} imaging of HEK293 cells transfected with hTRPA1-WT or hTRPA1-P394A mutant vector (n = 26–50 cells). Panel **(B)** is expressed as mean ± S.E.M. All of the experiments were performed at room temperature.

TRPA1 can be activated by heavy metals such as, gold, zinc, and cadmium via cysteine modification (Hu et al., 2009a; Gu and Lin, 2010; Hatano et al., 2013; Miura et al., 2013). However, the concentration of the platinum compound L-OHP required for hTRPA1 activation (1 mM) is much higher than that reported previously for other heavy metals (μM) (Hu et al., 2009a; Hatano et al., 2013; Miura et al., 2013). The possibility that L-OHP directly activates hTRPA1 was ruled out since high L-OHP was unable to evoke TRPA1 activation in inside-out patch-clamp recording experiments. Rather, the results indicated indirect activation of hTRPA1 through L-OHP-induced ROS generation since (1) we detected an increase in intracellular H_2O_2 production following high L-OHP treatment, and (2) high L-OHP-evoked hTRPA1 activation was blocked by the membrane-impermeable antioxidant glutathione, consistent with a previous report (Nassini et al., 2011). It is reported that L-OHP induces apoptosis via mitochondrial damage (Gourdier et al., 2004), which triggers ROS generation (Bishop et al., 2010). Taken together, our findings suggest that high L-OHP triggers ROS generation, presumably by mitochondria, resulting in activation of TRPA1.

Cysteine oxidation is one of the most common mechanisms for TRPA1 activation by various agonists, and Cys633, Cys641, and Cys665 are crucial for activation by electrophiles (Hinman et al., 2006; Ibarra and Blair, 2013). Among these, Cys641 and Cys665 are important for activation by low concentrations of H_2O_2 (Takahashi et al., 2008) and nitric oxide (Kozai et al., 2014). Cys641 is also important for activation by zinc (Hu et al.,

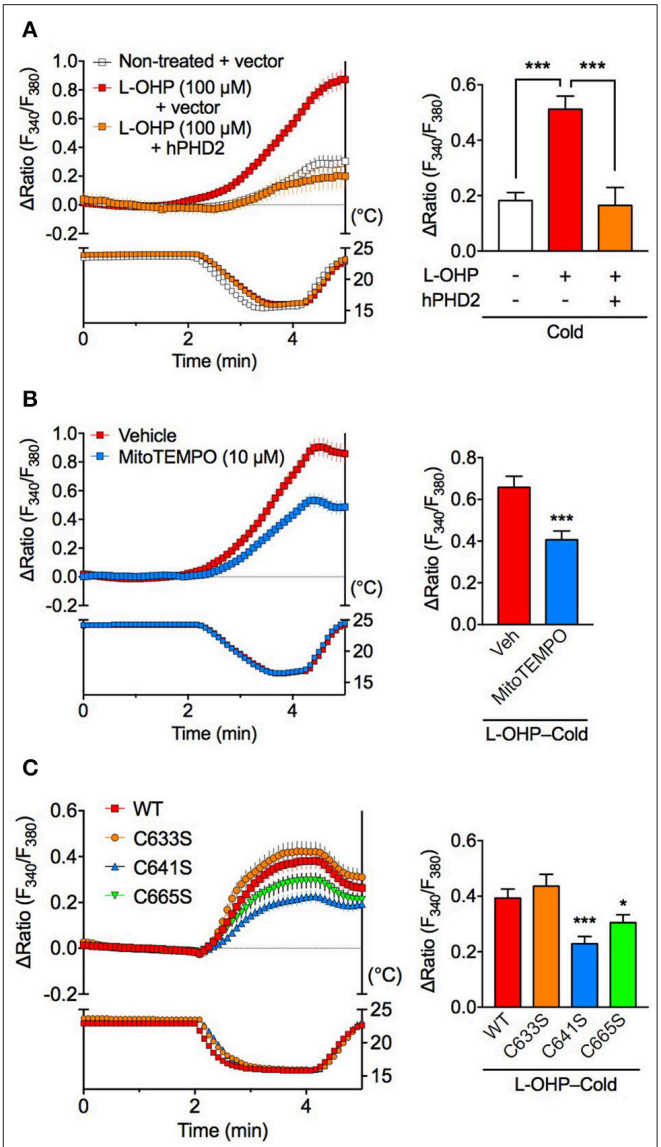

FIGURE 5 | Cold-induced activation of hTRPA1 sensitized by low L-OHP is dependent on PHD inhibition and cysteine oxidation. Effects of PHD2 overexpression **(A)**, a mitochondria-targeted ROS scavenger **(B)** or hTRPA1 cysteine mutants **(C)** on the cold-evoked $[Ca^{2+}]_i$ increase following pretreatment with L-OHP (100 μM) for 2 h in hTRPA1-expressing HEK293 cells were investigated. **(A)** Left panel shows representative traces of intracellular Ca^{2+} imaging from hTRPA1-expressing HEK293 cells co-transfected with or without human PHD2 (top) and the temperature of the recording solution (bottom). Right panel shows its statistical analysis (n = 47–83 cells from two independent experiments). ***P < 0.001. **(B)** Left panel shows representative traces of intracellular Ca^{2+} imaging from L-OHP-treated hTRPA1-expressing HEK293 cells pretreated with vehicle or mitoTEMPO (10 μM, loading with Fura-2 Ca^{2+} indicator; top) and the temperature of the recording solution (bottom). Right panel shows its statistical analysis (n = 94–95 cells from two independent experiments). ***P < 0.001 vs. vehicle (0.1% DMSO)-treated cells (Veh). **(C)** Left panel shows representative traces of intracellular Ca^{2+} imaging from L-OHP-treated HEK293 cells expressing wildtype hTRPA1 (WT) or hTRPA1 cysteine mutants (hTRPA1-C633S, C641S, and C665S; top) and the temperature of the recording solution (bottom). Right panel shows its statistical analysis (n = 202–266 cells from three independent experiments). *P < 0.05, ***P < 0.001 vs. WT. All data are expressed as mean ± S.E.M.

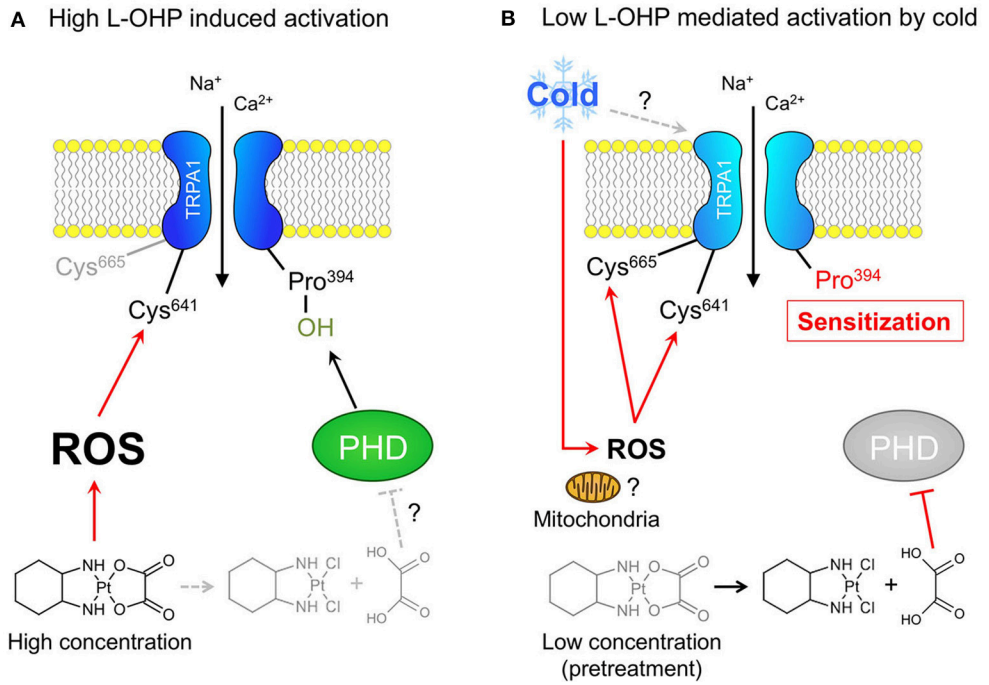

FIGURE 6 | Different concentration of L-OHP elicits cysteine oxidation-dependent activation or cold sensitization of hTRPA1. **(A)** The mechanism underlying the high L-OHP-induced hTRPA1 activation. L-OHP induces ROS generation (presumably via mitochondria), and the resultant ROS activate hTRPA1 via oxidation of Cys641. **(B)** The mechanism underlying the low L-OHP-mediated hTRPA1 activation by cold. The long exposure of L-OHP (more than 1 h) causes the inhibition of PHDs by oxalate, a metabolite of L-OHP, which results in the sensitization of hTRPA1. The sensitized hTRPA1 is activated by ROS produced by mitochondria following cold exposure via oxidation of Cys 641 and Cys665.

2009a) and cadmium (Miura et al., 2013), whereas Cys633 is involved in activation by HNO (Eberhardt et al., 2014) and the gold compound auranofin (Hatano et al., 2013). In the present study, we showed that mutation of Cys641 inhibited high L-OHP-evoked hTRPA1 activation. This result further supports indirect hTRPA1 activation by high L-OHP through ROS generation, although mutating Cys665 had no effect, which may partially contradict this finding. This paradox may indicate some additional roles of L-OHP in the high L-OHP-induced hTRPA1 activation, but further investigations are required. The fact that mutation of Cys633 did not affect high L-OHP-evoked hTRPA1 activation may suggest that platinum does not directly activate hTRPA1 in a cysteine-dependent manner, like gold.

Hydroxylation of the proline residue in the N-terminal region of TRPA1 by PHDs is critical for regulating TRPA1 activity (Miyake et al., 2016). Although L-OHP and its metabolite oxalate can inhibit PHDs (Miyake et al., 2016), the present results suggest that PHDs are not involved in the high L-OHP-evoked hTRPA1 activation. This apparent discrepancy may be explained by the previous observation that induction of TRPA1 sensitization in mouse DRG neurons by low L-OHP pretreatment requires more than 1 h (Zhao et al., 2012). Thus, it is likely that inhibition of PHDs and induction of TRPA1 sensitization by L-OHP and/or oxalate may be slower than the high L-OHP-induced rapid ROS generation that activates TRPA1. It is probable that cysteine oxidation by ROS, rather than inhibition of proline hydroxylation by L-OHP and/or oxalate and subsequent delayed sensitization of hTRPA1, contributes to hTRPA1 activation evoked by high L-OHP.

We previously reported that PHD inhibition by a PHD inhibitor dimethyloxalylglycine (DMOG) sensitizes hTRPA1 to ROS and induces channel opening at cold temperatures. Furthermore, similar cold hypersensitivity is also observed in the mice treated with DMOG, which is also inhibited by a TRPA1 antagonist HC030031 (Miyake et al., 2016). Consistently, in the present study, pretreatment with a relatively low concentration of L-OHP potentiated the cold sensitivity of hTRPA1. Furthermore, this L-OHP-induced cold sensitivity was significantly reduced when treated with a mitochondria-targeting ROS scavenger, suggesting that ROS generated from mitochondria during cold exposure contributes to the L-OHP-induced cold sensitivity, similar to DMOG. Consistent with our results, previous *in vivo* experiments showed that L-OHP-induced cold hypersensitivity was attenuated by a single acute administration of a ROS scavenger (Miyake et al., 2016) or a mitochondria-targeting ROS scavenger (Toyama et al., 2014). Furthermore, the results obtained from Cys641 and Cys665 hTRPA1 mutants confirmed that these residues are responsible for activation by H_2O_2 (Takahashi et al., 2008) and contribute to the indirect cold sensitivity of hTRPA1 induced by low L-OHP. These results suggest that ROS presumably generated from mitochondria during cold exposure oxidize cysteine residues in

the N-terminal region of hTRPA1, thereby activating hTRPA1 following exposure to low L-OHP pretreatment. However, in this study, mitoTEMPO did not completely inhibit the cold-induced increase of $[Ca^{2+}]_i$ in the low L-OHP-treated hTRPA1 expressing cells. This mitoTEMPO-insensitive component may be ROS-independent but still PHD-dependent, since the over-expression of PHD2 completely inhibited the cold-induced hTRPA1 activation and hTRPA1-P394A mutant (that mimics a constitutively PHD-inhibited condition) shows a weak ROS-independent cold sensitivity (Miyake et al., 2016). By contrast, the mutation demonstrated that Cys633 had no effect on the indirect cold sensitivity of hTRPA1, which may indicate that platinum itself is not involved in this phenomenon. This hypothesis is consistent with our previous findings that other platinum-based chemotherapeutic agents such as, cisplatin and carboplatin do not induce acute cold hypersensitivity (Zhao et al., 2012).

The concentration of L-OHP in commercial infusions is about 1.25 mM, while the calculated blood concentration in patients is $<100\,\mu M$ (Chalret du Rieu et al., 2014). Although L-OHP accumulates in the DRG and peripheral nerves (Screnci et al., 2000; Cavaletti et al., 2001), the concentration of L-OHP required to evoke TRPA1 activation (1 mM) appears to be too high to explain L-OHP-induced acute CIPN. In addition, a high concentration of cisplatin, which does not induce acute CIPN, also activates TRPA1 via ROS generation (Nassini et al., 2011). Thus, high L-OHP-evoked TRPA1 activation via ROS generation is unlikely to be responsible for acute CIPN following L-OHP treatment. Interestingly, delayed mechanical, thermal, and cold hypersensitivity following repeated administration of L-OHP in rodents is prevented by some antioxidants (Joseph and Levine, 2009; Di Cesare Mannelli et al., 2012; Azevedo et al., 2013) and a TRPA1 blocker (Nassini et al., 2011). Furthermore, TRPA1 activation via ROS is associated with mechanical hypersensitivity induced by cisplatin (Nassini et al., 2011) and some other classes of chemotherapeutic agents such as, paclitacel (Materazzi et al., 2012), bortezomib (Trevisan et al., 2013), and vincristine (Old et al., 2014). Thus, ROS-mediated TRPA1 activation may be a common mechanism for cumulative and chronic CIPN.

In conclusion, we further clarified the molecular details of how L-OHP activates or sensitizes hTRPA1. L-OHP exhibited complex concentration-dependent effects on hTRPA1; high L-OHP evoked hTRPA1 activation in a proline-independent manner, while low L-OHP sensitized hTRPA1 in a proline-dependent manner. This finding implies that the same chemical agent can function via different molecular mechanisms to regulate target proteins in a concentration-dependent manner. Nevertheless, the present results provide experimental evidence that TRPA1 blockage may be of clinical benefit for CIPN patients treated with L-OHP.

AUTHOR CONTRIBUTIONS

TM, TNa, and SK designed the project. TM, SN, ZM, SH, and KI performed the experiments. TM, SN, ZM, SH, HS, KN, and TNa analyzed the data; TNu, NT, and YM provided materials and technical advices. TM, TNa, and SK wrote the manuscript. SK supervised the experiments and finalized the manuscript.

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Inhibition of the TRPM2 and TRPV1 Channels through *Hypericum perforatum* in Sciatic Nerve Injury-induced Rats Demonstrates their Key Role in Apoptosis and Mitochondrial Oxidative Stress of Sciatic Nerve and Dorsal Root Ganglion

Fuat Uslusoy[1], *Mustafa Nazıroğlu*[2,3,4]* *and Bilal Çiğ*[3,4]

[1] Department of Plastic Reconstructive and Aesthetic Surgery, Faculty of Medicine, Suleyman Demirel University, Isparta, Turkey, [2] Neuroscience Research Center, Suleyman Demirel University, Isparta, Turkey, [3] Department of Biophysics, Faculty of Medicine, Suleyman Demirel University, Isparta, Turkey, [4] Department of Neuroscience, Institute of Health Sciences, Suleyman Demirel University, Isparta, Turkey

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***Correspondence:**
Mustafa Nazıroğlu
mustafanaziroglu@sdu.edu.tr

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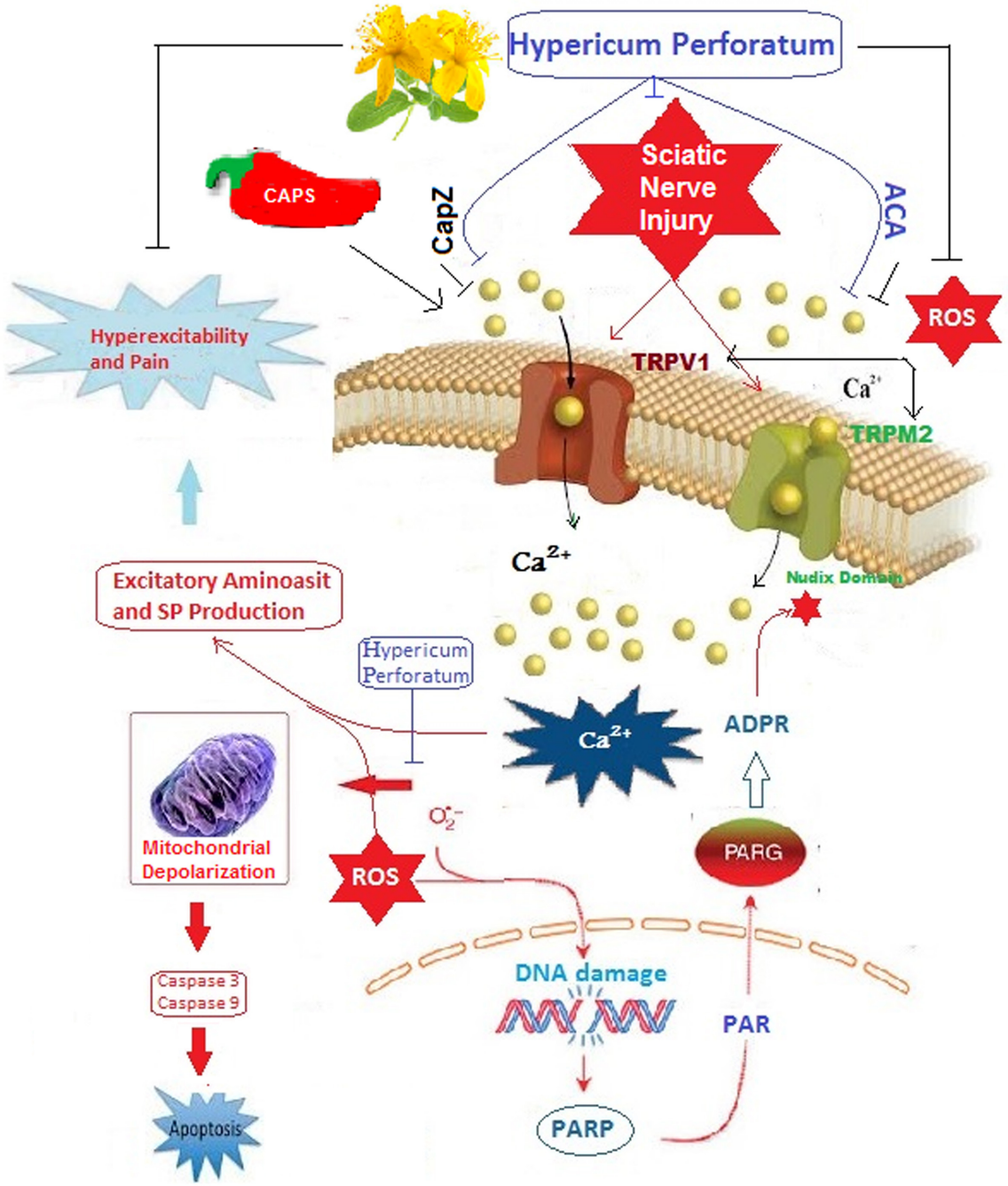
Sciatic nerve injury (SNI) results in neuropathic pain, which is characterized by the excessive Ca^{2+} entry, reactive oxygen species (ROS) and apoptosis processes although involvement of antioxidant *Hypericum perforatum* (HP) through TRPM2 and TRPV1 activation has not been clarified on the processes in SNI-induced rat, yet. We investigated the protective property of HP on the processes in the sciatic nerve and dorsal root ganglion neuron (DRGN) of SNI-induced rats. The rats were divided into five groups as control, sham, sham+HP, SNI, and SNI+HP. The HP groups received 30 mg/kg HP for 4 weeks after SNI induction. TRPM2 and TRPV1 channels were activated in the neurons by ADP-ribose or cumene peroxide and capsaicin, respectively. The SNI-induced TRPM2 and TRPV1 currents and intracellular free Ca^{2+} and ROS concentrations were reduced by HP, N-(p-amylcinnamoyl) anthranilic acid (ACA), and capsazepine (CapZ). SNI-induced increase in apoptosis and mitochondrial depolarization in sciatic nerve and DRGN of SNI group were decreased by HP, ACA, and CapZ treatments. PARP-1, caspase 3 and 9 expressions in the sciatic nerve, DRGN, skin, and musculus piriformis of SNI group were also attenuated by HP treatment. In conclusion, increase of mitochondrial ROS, apoptosis, and Ca^{2+} entry through inhibition of TRPM2 and TRPV1 in the sciatic nerve and DRGN neurons were decreased by HP treatment. The results may be relevant to the etiology and treatment of SNI by HP.

Keywords: apoptosis, *Hypericum perforatum*, sciatic nerve injury, mitochondrial oxidative stress, TRPM2, TRPV1



GRAPHICAL ABSTRACT | Poly (ADP-ribose) polymerase (PARP) catalyzes the transfer of ADP-ribose (ADPR) in nucleus during the DNA repair processes. TRPM2 channel is gated by ADPR and reactive oxygen species (ROS) through activation of ADP-ribose (ADPR) pyrophosphate enzyme in its nudix domain motif although it was inhibited by N-(p-amylcinnamoyl)anthranilic acid (ACA). TRPV1 channel is also activated by ROS and capsaicin (CAPS) but it is inhibited by capsazepine (CapZ).

INTRODUCTION

Calcium ion (Ca^{2+}) is an important messenger in neurons of the body (Kumar et al., 2014). Many physiological functions such as muscle metabolism, neuronal recovery, mitochondrial-reactive oxygen species (ROS) production, and apoptosis were regulated by the intracellular free Ca^{2+} ($[Ca^{2+}]_i$) concentration (Nazıroğlu, 2007; Kumar et al., 2014). The Ca^{2+} passes the cell membrane with several channels such as chemical gated and voltage gated calcium channel (VGCC). Apart from the well-known channels, transient receptor potential (TRP) cation channel family was recently discovered in different cells. Some subfamilies of the TRP family, such as TRP melastatin 2 (TRPM2) and TRP vanilloid 1 (TRPV1) are activated by oxidative stress (Tominaga and Tominaga, 2005; Nazıroğlu, 2011, 2015). The activator of TRPM2 channel was firstly discovered through activation of adenosine diphosphate ribose (ADPR) pyrophosphatase enzyme of C-terminal tail in the Nudix box motif of the channel by intracellular ADPR (Perraud et al., 2001) and extracellular H_2O_2 (Hara et al., 2002). Then, ADPR-independent activation mechanism of TRPM2 channel was indicated in a cell line by a single channel patch-clamp study (Nazıroğlu and Lückhoff, 2008). Activation of TRPV1 channel is firstly indicated in dorsal root ganglion neuron (DRGN) by capsaicin (Caterina et al., 2000). Then, involvement of oxidative stress on activation of the TRPV1 channel through activation of NADPH oxidase pathway was indicated by a cell line study (Susankova et al., 2006) and DRGN (Ding et al., 2016) studies. TRPM2 and TRPV1 expression levels are high in the DRGNs and overload Ca^{2+} entry through the channels involved in neuropathic pain (Isami et al., 2013; Pecze et al., 2013; Akpınar et al., 2016) and apoptotic (Hara et al., 2002) processes. The association between overload Ca^{2+} entry through TRPM2 and TRPV1 and peripheral pain intensity has been reported in sciatic nerve injury (SNI)-induced rats (Dolu et al., 2016). Accumulating evidence indicated that importance of oxidative stress, the TRPM2 and TRPV1 channels in DRGN and sciatic nerve injuries has been increasing in experimental animal and human (Facer et al., 2007; Frederick et al., 2007; Haraguchi et al., 2012). On the subject, it was reported that expression levels of TRPM2 and TRPV1 are increased in sciatic nerve and DRGN by spinal cord injury (SCI) and SNI (Frederick et al., 2007; Szigeti et al., 2012; Matsumoto et al., 2016).

It was demonstrated that inflammatory, mechanical injury, and ischemia induces excessive production of ROS, Ca^{2+} entry, and apoptosis through VGCC in neurodegenerative diseases such as SCI and SNI (Fisunov et al., 2000). Association between overload Ca^{2+} entry and excessive production of ROS has also been well-known in neurodegenerative disease (**Graphical abstract**). Involvement of excessive ROS production and overload Ca^{2+} entry has been emphasized in the generation of neuropathic pain after traumatic injuries (Genovese et al., 2006; Özdemir et al., 2016). Based on these findings, some dietary antioxidants have been tested for their clinical efficacy in treating oxidative stress, apoptosis, and Ca^{2+} entry because they acted to be safe and well-tolerated (Genovese et al., 2006; Alipour et al., 2014; Özdemir et al., 2016). *Hypericum perforatum* (HP) is also known as St John's worth which has been used as a popular plant medicine for treatment of several diseases such as skin wounds, burns, and mental depression (Stojanović et al., 2013). Antioxidant and ROS scavenger effects of flavonoids are well-known for a long time and the main component of HP is flavonoids such as hyperforin, pseudohyperforin, rutin, quercetin, and quercitrin (Kusari et al., 2009; Stojanović et al., 2013). A protective effect of HP on sciatic nerve in rats was recently reported (Mohammadi et al., 2012). Antioxidant and ROS scavenger effects of HP both in DRGN of SCI-induced rats (Uchida et al., 2008; Nazıroğlu et al., 2014a) and neutrophil of patients with inflammatory diseases (Nazıroğlu et al., 2014b,c) were reported. Recently, we observed modulator role of HP on apoptotic, inflammatory and oxidative stress values in muscle, blood and brain of SNI-induced rats (Uslusoy et al., 2017). Therefore, HP may attenuate oxidative stress, apoptosis and Ca^{2+} entry through modulation of TRPM2 and TRPV1 in DRGN and sciatic neurons of SNI-induced rats.

SNI-induced apoptosis and oxidative stress may be reduced by $[Ca^{2+}]_i$ concentration through modulation of TRPM2 and TRPV1 channels by HP. To our knowledge, there is no report of HP on apoptosis, oxidative stress and Ca^{2+} entry in SNI-induced rats. The aim of the current study is to determine the molecular mechanism of HP on apoptosis, oxidative stress and Ca^{2+} entry through TRPV1 and TRPM2 regulation in the sciatic nerve and DRGN after SNI.

MATERIALS AND METHODS

Animal

We used 40 female Wistar rats (aged between 3–4 months old) in the current study. The animals were housed two per cage, under controlled conditions of room temperature (22°C) and humidity (65–70%), on a 12 h light-dark cycle and allowed free access to commercial feed and tap water. Accessing the feed of the operated animals was facilitated to the rats though using specific cage apparatus in sham and SNI-induced groups for recovery days (3 days) of the operations.

Hypericum perforatum (HP) Extract

The HP extract was purchased from Indena (Indena Industria Derivati Naturali) S.p.A. Viale Ortles, Milan, Italy. The extract was mainly containing 0.10–0.30% total hypericin, 6.0% flavonoids, and 6.0% hyperforin (Özdemir et al., 2016; Uslusoy et al., 2017).

Study Groups

The rats were equally divided into five groups ($n = 8$) as follows: The control group had no SNI and treatment. They received one ml of 0.9% w/v saline solution via gastric gavage for 4 weeks.

Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium ion; ACA, N-(p-amylcinnamoyl) anthranilic acid; CAPS, capsaicin; CapZ, capsazepine; CPx, cumene hyroperoxide; DHR, dihydrorhodamine; DRGN, dorsal root ganglion neuron; HP, *Hypericum Perforatum*; PARP-1, Poly-ADPR polymerase 1; ROS, reactive oxygen species; SCI, spinal cord injury; SNI, sciatic nerve injury; TRP, transient receptor potential; TRPC6, transient receptor potential canonical 6; TRPM2, transient receptor potential melastatin 2; TRPV1, transient receptor potential vanilloid 1; WC, whole cell.

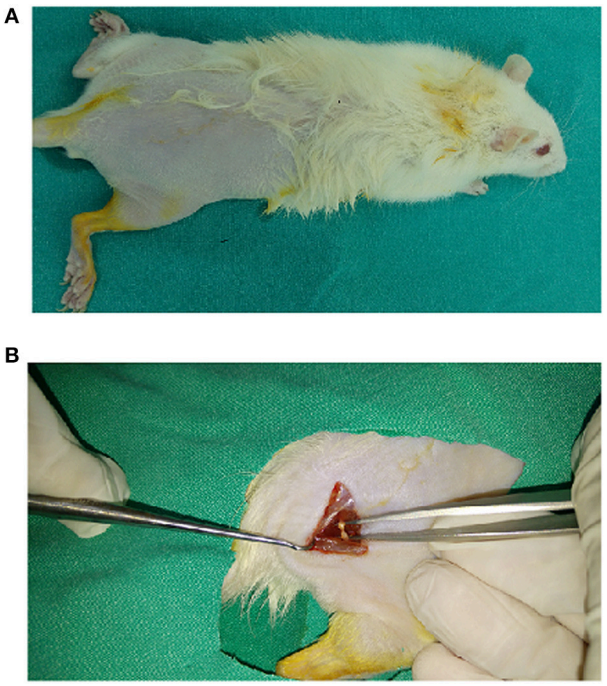


FIGURE 1 | Induction of sciatic nerve injury in right leg of the rats. After anesthesia **(A)**, the common sciatic nerve of the right hind paw was exposed the middle of thigh by blunt dissection through the biceps femoris **(B)**. For sciatic nerve crush, a hemostatic sterile clamp was used. The sciatic nerve was crushed for a total of 30 s. Then, the wound was closed with a 2.0 suture and rats were allowed to recover in the postoperative room. In sham-operated rats, the same surgical procedure was followed, the connective tissue was freed, and no ligatures were applied.

In the sham group, they exposed the same surgical procedure of SNI group, but no ligatures were applied to right leg (**Figure 1**). In sham+HP group, exposed same procedure of sham group, but the rats were supplemented HP. In the SNI group, they exposed the same surgical procedure of SNI group and ligatures were also applied to right leg. In SNI+HP group, exposed same procedure of SNI group, but the rats were supplemented HP. The HP (30 mg/kg/day) was dissolved in ml of 0.9% w/v saline and it was administrated to the rats via gastric gavage for 4 weeks (Özdemir et al., 2016; Uslusoy et al., 2017). SNI in the SNI group was induced in the rats according to method of Bennett and Xie (1988). In the SNI+HP group, the rats received oral HP (30 mg/kg/day). In the SNI+HP, the rats received HP (same as the sham+HP group) after SNI induction (same as the SNI group).

Twelve hours after the last HP dose administration, all rats were decapitated in accordance with Suleyman Demirel University (SDU) experimental animal legislation. The skin, muscle (Musculus piriformis), sciatic nerve, and DRGN samples were isolated as described in a previous study (Özdemir et al., 2016). In patch-clamp experiment and $[Ca^{2+}]_i$ concentration assays, the DRGNs were further treated with cumene hyroperoxide (CPx) (0.1 mM) or ADPR (1 mM) and capsaicin (0.01 mM) for activation of TRPM2 and TRPV1 channels, respectively and they were also inhibited the TRPM2 channels blockers, N-(p-amylcinnamoyl) anthranilic acid (ACA and 0.025 mM) and TRPV1 blocker, capsazepine (CapZ and 0.1 mM).

Induction of SNI and Preparation of Sciatic Nerve and DRGNs

Briefly, the rats were anesthetized by cocktail of xylazine (12.5 mg/kg) and ketamine (100 mg/kg) via intraperitoneally and the common sciatic nerve of the right hind paw was exposed the middle of thigh by blunt dissection through the biceps femoris. For sciatic nerve crush, a hemostatic sterile clamp was used. The sciatic nerve was crushed for a total of 30 s. Then, the wound was closed with a 2.0 suture and rats were exposed to recover in the postoperative room. For excusing the effects of anesthetics and surgical operation on the investigated values, we induced sham group. In sham-operated rats, the same surgical procedure was followed, the connective tissue was freed, and no ligatures were applied. After 3 days of the surgical operation, all animals received gentamicin (5 mg/kg, i.p.) to prevent sepsis.

The DRGN (T13-L5) were carefully dissected from peripheral nerve roots (Nazıroğlu et al., 2014a). The neurons were incubated in DMEM with 1% penicillin-streptomycin in 500 ml of DMEM. The connective tissue was removed and ganglia were treated with collagenase IV (0.28 ml in DMEM), and tyripsin (25,000 units/ml in DMEM for 45 min at 37°C and in an atmosphere containing 95% air and 5% CO_2. After dissociation with a sterile syringe, the DRGN suspension of medium and high size was obtained by centrifuged at 1,500 g and the medium and high size neurons were removed for the analysis (Akpınar et al., 2016).

Measurement of $[Ca^{2+}]_i$ Concentration in Sciatic Nerve and DRGN

In $[Ca^{2+}]_i$ measurement, extracellular buffer was contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid (HEPES), and 5 mM glucose (pH 7.4). Lysis buffer (pH 7.5) contained 20 mM Tris X-100, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM EGTA, 0.1% Triton X-100, 2.5 mM sodium pyrophosphate.

The sciatic nerve and DRGNs (10^6/ml) were allowed to recover in RPMI-1640 medium for 1 h before being loaded with 2 mM fura-2-AM for 30 min in a water-jacketed cuvette (37°C) with continuous magnetic stirring (Espino et al., 2010). Fluorescence was monitored with a Carry Eclipsys (Inc, Sydney, Australia) spectrofluorometer immediately after 0.1 ml cell suspension was added to 0.9 ml Ca^{2+}-containing extracellular medium, by recording excitation signals at 340 and 380 nm and emission signal at 505 nm at 1 s intervals. For calibration of $[Ca^{2+}]_i$, maximum, and minimum fluorescence values were obtained by adding the detergent Triton X-100 (0.1%) and the Ca^{2+} chelator EGTA (10 mM) sequentially at the end of each experiment. Calculation of the $[Ca^{2+}]_i$ concentrations were described in previous studies (Espino et al., 2010; Akpınar et al., 2016), assuming a Kd of 155 nM. The $[Ca^{2+}]_i$ concentrations in TRPM2 and TRPV1 experiments were recorded by using

the integral of the rise in $[Ca^{2+}]_i$ for 125 s after addition of cumene hyroperoxide (CPx and 0.1 mM) or capsaicin (0.01 mM) (Akpınar et al., 2016; Demirdaş et al., 2017), respectively. The $[Ca^{2+}]_i$ concentration is expressed as nanomolar (nM) taking a sample every second as previously described (Espino et al., 2010).

Electrophysiology

We used whole-cell mode of patch-clamp techniques (EPC10 patch-clamp set, HEKA, Lamprecht, Germany) was used in the DRGN of current studies (Akpınar et al., 2016; Özdemir et al., 2016). Resistances of whole cell recording electrodes were adjusted to about 3–6 MΩ by a puller (PC-10 Narishige International Limited, London, UK). We used standard extracellular bath and pipette solutions as described in previous studies (Nazıroğlu and Lückhoff, 2008; Akpınar et al., 2016). The intracellular Ca^{2+} concentration was held as 1 μM instead of physiological concentration (0.1 μM) in TRPM2 experiments because the channels are activated by presence of high intracellular Ca^{2+} concentration (McHugh et al., 2003). Holding potential of the patch-clamp analyses in the DRGNs was −60 mV. Voltage clamp technique was used in the analyses and current-voltage (I-V) relationships were obtained from voltage ramps from −90 to +60 mV applied over 200 ms. All experiments were performed at room temperature (22 ± 1°C).

In the experiments, TRPM2 channels are gated by ADPR (1 mM in patch pipette) although they were inhibited by ACA (0.025 mM). In a recent study, we observed activation of TRPV1 channels by medium level (0.01 mM) CAPS instead of low (0.001 mM) CAPS (Nazıroğlu, 2017). Therefore, TRPV1 channels were activated by adding extracellular (in patch chamber) CAPS (0.010 mM), and the channels were inhibited by administration of capsazepine (CapZ and 0.1 mM) into patch chamber through extracellular buffer. For the analysis, the maximal current amplitudes (pA) in a DRGN were divided by the cell capacitance (pF), a measure of the cell surface. The results in the patch clamp experiments are the current density (pA/pF).

Intracellular ROS Production Measurement

Dihydrorhodamine (DHR) 123 is an uncharged and nonfluorescent intracellular ROS production indicator. It can easily pass across cell membranes where it is oxidized to cationic rhodamine 123 which localizes in the mitochondria and exhibits green fluorescence. The sciatic nerve and DRGNs were incubated with 20 μm DHR 123 at 37°C for 25 min (Bejarano et al., 2009). The fluorescence intensities of the rhodamine 123 were assayed (excitation; 488 nm and emission; 543 nm) by using an automatic microplate reader (Infinite pro200; Tecan Inc, Groedig, Austria). The results were expressed as fold-increase over the pretreatment level.

Mitochondrial Membrane Potential (JC-1) Analyses

The mitochondrial membrane potential (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide, JC-1) was determined by JC-1 dye as described in previous studies (Bejarano et al., 2009; Espino et al., 2010). The JC-1- loaded sciatic and DRGNs neurons at 37° C for 45 min were excited at 488 nm and emission was detected at 590 nm (JC-1 aggregates) and 525 nm (JC-1 monomers). Values were calculated from emission ratios (590/525) and they are presented as fold-increase.

Cell Viability Assay

To determine the cell viability after SNI induction and HP treatment, we used to cell viability analyses as 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in the neurons as described elsewhere (Demirdaş et al., 2017). After incubation for 60 min with medium containing MTT solution (5 mg/ml), removed the neurons and dissolved the resulting MTT formazan in DMSO. Absorbance values were recorded in a spectrophotometer at 490 nm (UV-1800, Shimadzu, Kyoto, Japan). The data are presented as the fold increase over the pretreatment level (experimental/control).

Assay for Apoptosis, Caspase 3, and 9 Activities

The apoptosis levels were determined by using the spectrophotometer and a commercial kit of Biocolor Ltd. (Northern Ireland) as described in a previous study (Demirdaş et al., 2017). The method is based on loss of asymmetry in membranes of apoptotic neurons.

The determinations of caspase 3 and caspase 9 activities in the sciatic nerve and DRGN neurons were performed in the microplate reader (Infinite pro200) by using caspase 3 (N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) and caspase 9 (His-Asp-7-amino-4-methylcoumarin) substrates. Details of the assays were indicated in recent studies (Akpınar et al., 2016; Özdemir et al., 2016). The substrate cleavage was measured at 360 nm (excitation) and 460 nm (emission). Values were calculated as fluorescence units/mg protein. The data are expressed as fold-increase.

Western Blot Analyses

Standard procedures are used in the Western Blot analyses of sciatic nerve, DRGN, muscle, and skin (Akpınar et al., 2016; Özdemir et al., 2016). In the analyses, caspase 9 (p35/p10 Polyclonal Antibody), caspase 3 (p17-specific Polyclonal Antibody), beta actin (polyclonal antibody) and Poly-ADPR polymerase 1 (PARP-1) (polyclonal antibody) were purchased from (Proteintech, USA) although secondary antibodies (Rabbit IgG, HRP-linked whole anti-Aβ, from donkey) were purchased from GE Healthcare (Amersham, UK). Relative levels of immunoreactivity in ECL Western HRP Substrate (Millipore Luminate Forte, USA) were quantified using Syngene G:Box Gel Imagination System (UK). Rabbit anti-β-actin (1:2000) was used as an internal control for the concentration of proteins loaded. The data are expressed as relative density over the control level.

Statistical Analyses

All data were represented as means ± standard deviation (SD). The data were analyzed by using 17.0 version of SPSS statistical program (Chicago, Illinois, USA). $P \leq 0.05$ was considered to indicate a statistically significant difference. Presence of significance in the five groups was once detected by LSD-test. Then *p*-value levels of significances in the data were analyzed by using Mann-Whitney *U*-test.

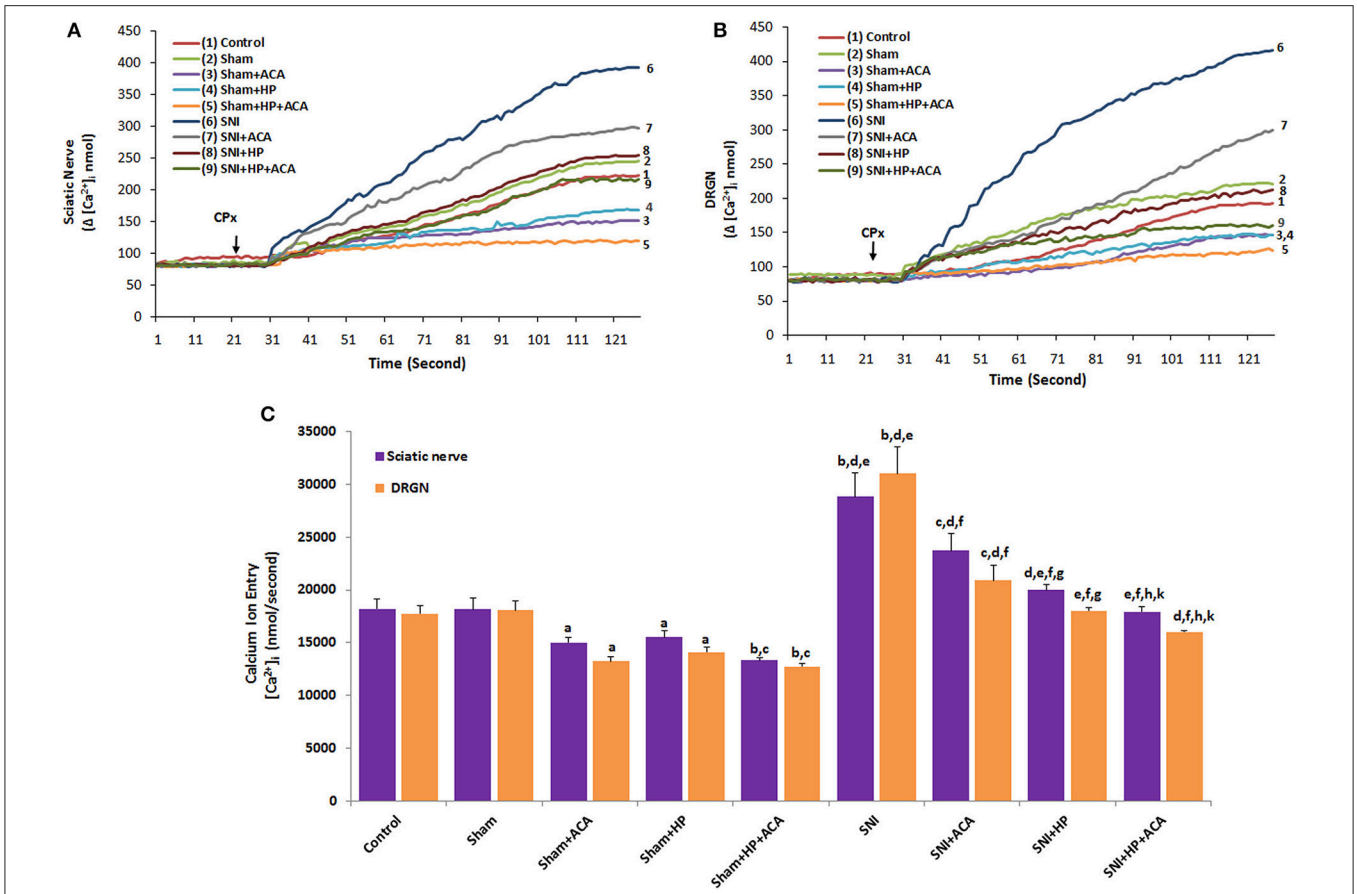


FIGURE 2 | Effect of *Hypericum perforatum* (HP) treatment on $[Ca^{2+}]_i$ concentration and TRPM2 in sciatic nerve (A) and dorsal root ganglion neurons (DRGNs) **(B)** of control, sham and SNI-induced rats. ($n = 8$ and mean ± SD). The animals received oral HP for 4 weeks. Then, these dissected neurons of control, sham and SNI were further *in vitro* treated with CPx (0.1 mM) and ACA (0.025 mM) before loading Fura-2 for 125 s. $^{a}p \leq 0.05$ and $^{b}p \leq 0.001$ vs. control and sham groups. $^{c}p \leq 0.05$ and $^{d}p \leq 0.001$ vs. sham+ACA and sham+HP groups. $^{e}p \leq 0.001$ vs. sham+HP+ACA group. $^{f}p \leq 0.001$ vs. SNI group. $^{g}p \leq 0.05$ and $^{h}p \leq 0.001$ vs. SNI+ACA group. $^{k}p \leq 0.05$ vs. SNI+HP group **(C)**.

RESULTS

Effects of HP on TRPM2 Channel Activation-Induced $[Ca^{2+}]_i$ Concentration in Sciatic Nerve and DRGNs of SNI-Induced Rats

TRPM2 channel was discovered as first candidate of oxidative stress dependent TRP channels because it has oxidative sensitive ADPR pyrophosphatase enzyme in C domain (Perraud et al., 2001). HP is containing antioxidant flavonoids in its content (Kusari et al., 2009; Stojanović et al., 2013). In etiology of SNI, oxidative stress has main role and HP may modulate the oxidative stress dependent-activated TRPM2 in sciatic nerve and DRGN of SNI-induced rats. For clarifying the modulator role of HP in the neurons, the neurons of HP supplemented rats were further *in vitro* stimulated by CPx (0.1 mM) (**Figures 2A,B**). Addition of CPx caused a significant rise in $[Ca^{2+}]_i$ concentration of sciatic nerve and DRGNs of SNI group which is attributed to activation of Ca^{2+}-permeable TRPM2 channels. This rise in intracellular $[Ca^{2+}]_i$ concentration was markedly ($p \leq 0.001$) higher in the SNI group than in the control and sham groups (**Figure 2C**). We observed low level of $[Ca^{2+}]_i$ concentration of the neurons in HP and ACA treated group. The $[Ca^{2+}]_i$ concentration in the neurons was significantly lower in the sham+HP ($p \leq 0.05$), sham+ACA ($p \leq 0.05$), and sham+HP+ACA ($p \leq 0.001$) groups than in control and sham groups. The $[Ca^{2+}]_i$ concentration in the neurons was low in SNI+HP and SNI+ACA and SNI+HP+ACA groups as compared to as compared to SNI only ($p \leq 0.001$). It seems that HP modulated the SNI-overload $[Ca^{2+}]_i$ concentration through regulation of TRPM2 in the neurons.

Effects of HP on TRPV1 Channel Activation-Induced $[Ca^{2+}]_i$ Concentration in Sciatic Nerve and DRGNs of SNI-Induced Rats

Stimulation of CAPS caused a significant rise in $[Ca^{2+}]_i$ concentration of sciatic nerve and DRGNs of SNI group which is attributed to activation of Ca^{2+}-permeable TRPV1 channels (**Figures 3A,B**). **Figure 3C** showed that, comparing with the control and sham groups, despite of the fact that

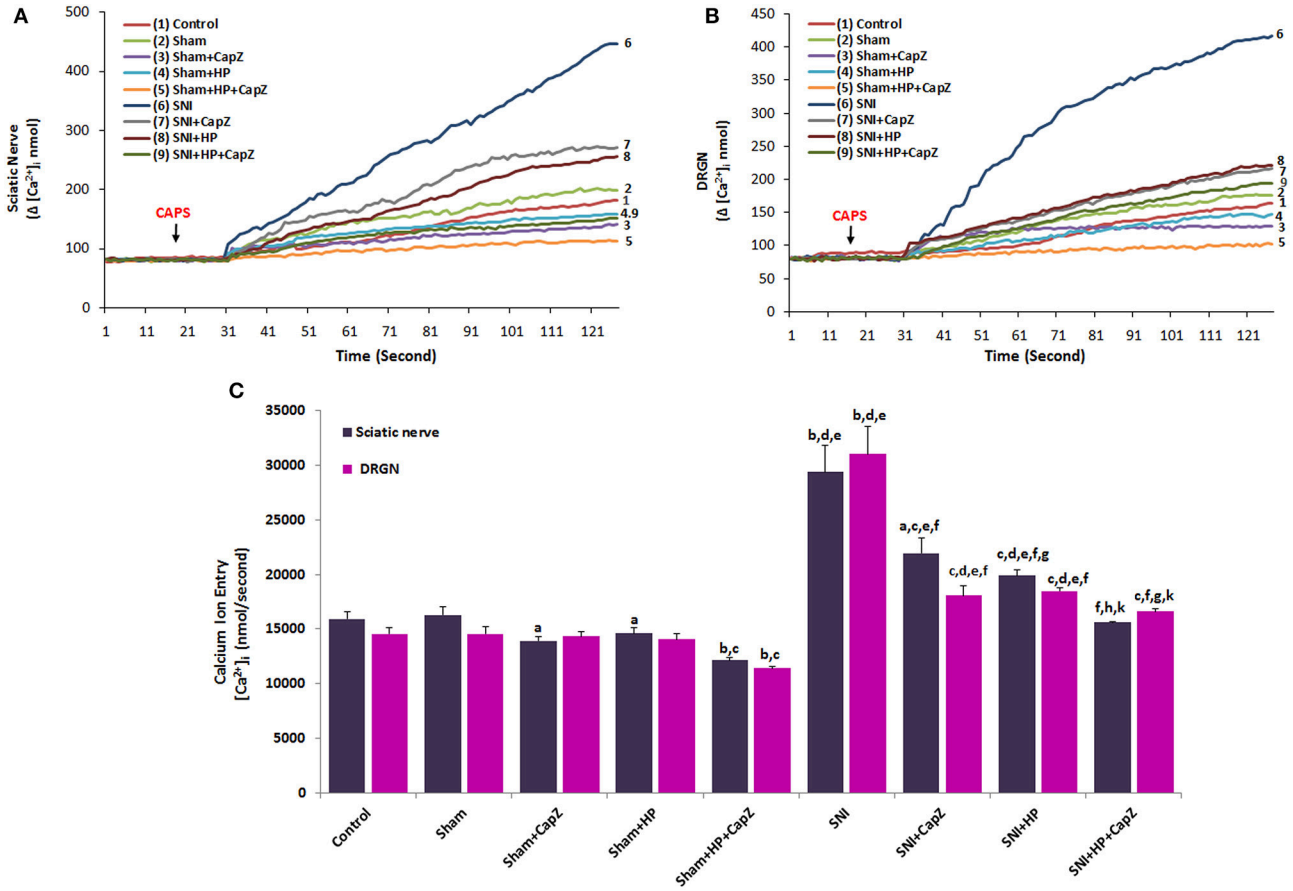


FIGURE 3 | Effect of *Hypericum perforatum* (HP) treatment on $[Ca^{2+}]_i$ concentration and TRPV1 in sciatic nerve (A) and dorsal root ganglion neuron (DRGN) **(B)** of control, sham and SNI-induced rats. ($n = 8$ and mean ± SD). The animals received oral HP for 4 weeks. Then, these dissected neurons of control, sham and SNI were further *in vitro* treated with CAPS (0.01 mM) and CapZ (0.1 mM) before loading Fura-2 for 125 s. $^{a}p \leq 0.05$ and $^{b}p \leq 0.001$ vs. control and sham groups. $^{c}p \leq 0.05$ and $^{d}p \leq 0.001$ vs. sham+CapZ and sham+HP groups. $^{e}p \leq 0.001$ vs. sham+HP+CapZ group. $^{f}p \leq 0.001$ vs. SNI group. $^{g}p \leq 0.05$ and $^{h}p \leq 0.001$ vs. SNI+CapZ group. $^{k}p \leq 0.05$ vs. SNI+HP group **(C)**.

the concentration of $[Ca^{2+}]_i$ was higher in SNI group, the TRPV1 inhibitors (HP and CapZ) could efficiently decrease the concentration of $[Ca^{2+}]_i$ which was induced by SNI induction ($p \leq 0.001$). We observed low level of $[Ca^{2+}]_i$ concentration of the neurons in HP and CapZ treated group. The $[Ca^{2+}]_i$ concentration in the neurons was significantly lower in the sham+HP ($p \leq 0.05$), sham+CapZ ($p \leq 0.05$) and sham+HP+CapZ ($p \leq 0.001$) groups than in control and sham groups. The $[Ca^{2+}]_i$ concentration in the neurons was low in SNI+HP and SNI+CapZ and SNI+HP+CapZ groups as compared to as compared to SNI only ($p \leq 0.001$). It seems that HP modulated the SNI-overload $[Ca^{2+}]_i$ concentration through regulation of TRPV1 in the neurons.

Effects of HP on ADPR-Induced TRPM2 Currents in DRGN of SCI-Induced Rats

The effects of antioxidant HP for TRPM2 channels activated by ADPR are indicated in **Figure 4**. ADPR (1 mM) induced a current in murine DRGNs (**Figures 4B,C**). There was no current in absence of ADPR (**Figure 4A**). Current densities of the DRGNs were markedly ($p \leq 0.001$) higher in the SNI+CAPS group (**Figure 4F**) than in the control ($p \leq 0.001$), control+CAPS ($p \leq 0.05$) and control+CAPS+CapZ ($p \leq 0.001$) groups. There was no activation of TRPM2 channel in HP (**Figure 4D**) and SNI+HP (**Figure 4E**) groups and the current densities in the DRGNs were significantly ($p \leq 0.001$) lower in HP and SCI+HP groups as compared to the SCI group (**Figure 3**). These results indicate that up-regulation of TRPM2 channel activity through HP treatment may be critical for SNI-mediated overload Ca^{2+} entry and intracellular ROS production in the DRGNs.

Effects of HP on CAPS-Induced TRPV1 Currents in DRGNs of Control and SNI-Induced Rats

The murine DRGNs were activated by capsaicin (**Figures 5B–D**). The CAPS-induced currents were reversibly and partially blocked by CapZ and $NMDG^{+}$ replacement instead of Na^{+}. There was

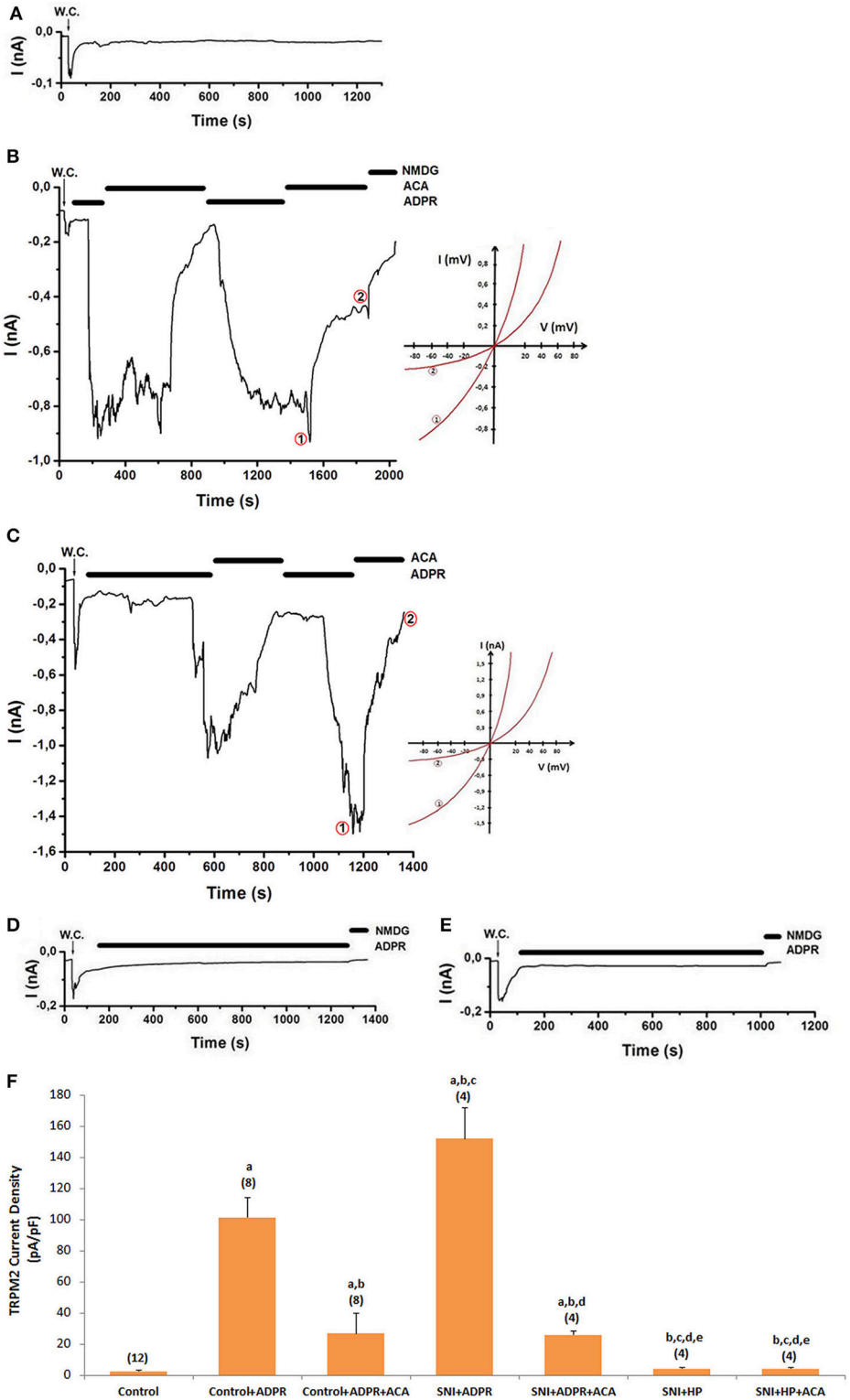


FIGURE 4 | Effects of HP on TRPM2 channel activation in dorsal root ganglion neuron (DRGN) of control and SNI-induced rat. The TRPM2 currents in DRGN were stimulated by intracellular ADPR (1 mM in patch pipette) but they were inhibited by extracellular ACA (0.025 mM) in the patch-chamber. W.C.: Whole cell. Control (without SCI induction and stimulation): Original recordings from control neuron **(A)**. **(B)**. Control+ADPR group (without SCI induction). **(C)**. SNI group (with SCI induction). **(D)**. SCI+HP group: The rats received HP after SCI induction. **(E)**. HP group: The rats received HP without SCI induction. **(F)**. TRPM2 channel current densities in the DRGN. The numbers in parentheses indicated n numbers of groups were indicated by numbers in parentheses. ($^{a}p \leq 0.001$ vs. control. $^{b}p \leq 0.001$ vs. control+ADPR group. $^{c}p \leq 0.001$ vs. control+ADPR+ACA group. $^{d}p \leq 0.001$ vs. SNI+ADPR group. $^{e}p \leq 0.001$ vs. SNI+ADPR+ACA group).

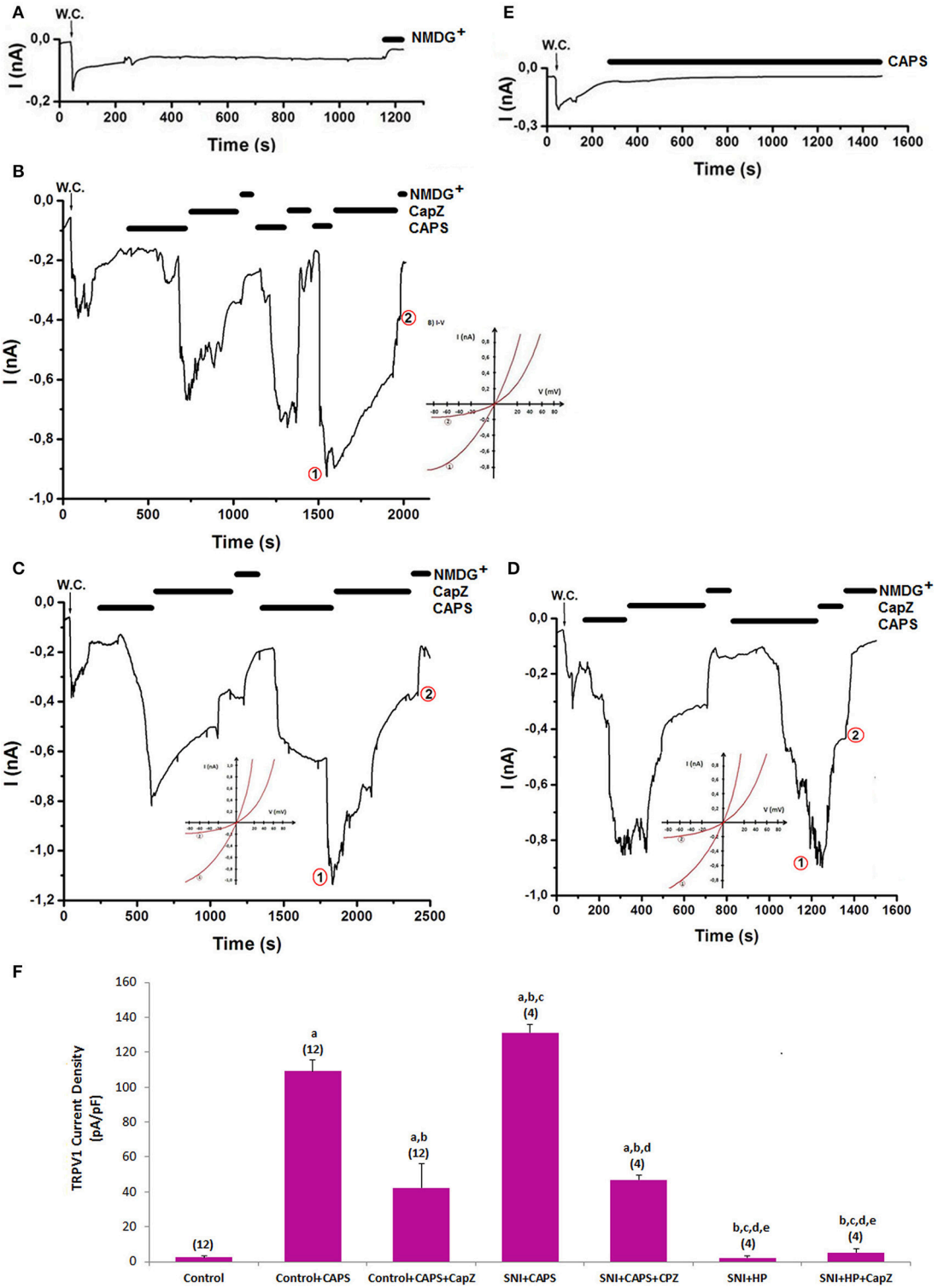


FIGURE 5 | Effects of HP on TRPV1 channel activation in dorsal root ganglion neuron (DRGN) of control and SNI-induced rat. The TRPV1 currents in DRGN were stimulated by extracelular capsaicin (CAPS and 0.01 mM in patch chamber) but they were inhibited by extracellular CaPZ (0.1 mM) in the patch-chamber. W.C.: Whole cell. Control (without SCI induction and stimulation): Original recordings from control neuron **(A)**. **(B)**. Control+CAPS group (without SCI induction). **(C)**. SNI group (with SCI induction). **(D)**. SCI+HP group: The rats received HP after SCI induction. **(E)**. HP group: The rats received HP without SCI induction. **(F)**. TRPV1 channel current densities in the DRGN. The numbers in parentheses indicated n numbers of groups were indicated by numbers in parentheses. ($^{a}p \leq 0.001$ vs. control. $^{b}p \leq 0.001$ vs. control+CAPS group. $^{c}p \leq 0.001$ vs. control+CAPS+CapZ group. $^{d}p \leq 0.001$ vs. SNI+ADPR group. $^{e}p \leq 0.001$ vs. SNI+ADPR+ACA group).

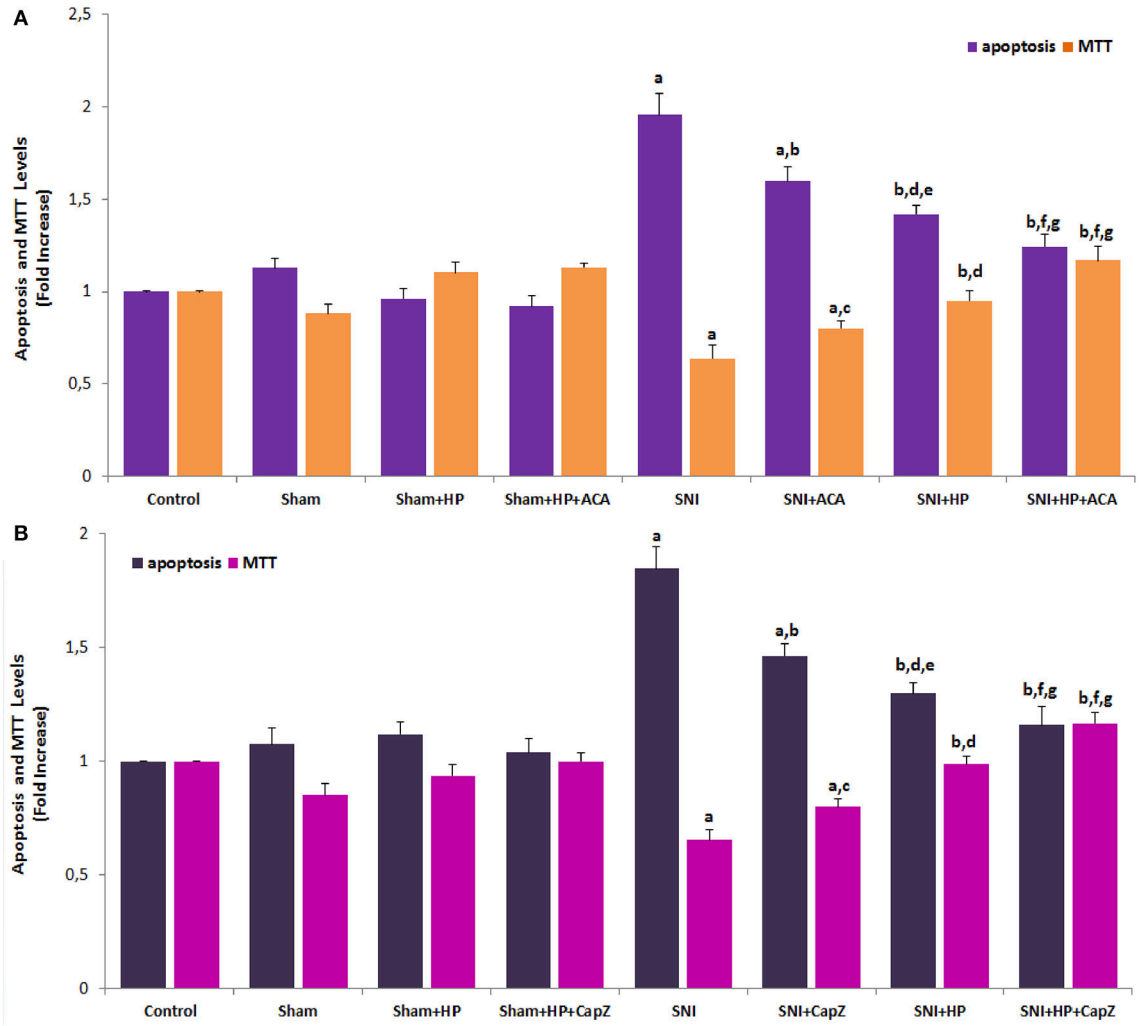


FIGURE 6 | Effects of *Hypericum perforatum* (HP) on the apoptosis and cell viability (MTT) levels through TRPM2 (A) and TRPV1 **(B)** in sciatic nerve of SNI-induced rats (mean ± SD and $n = 3$). Apoptosis level was measured by using a commercial kit. Values expressed as fold increase (experimental/control). These neurons were dissected from control, SNI and treated animals. The animals were received HP via gastric gavage. The neurons in TRPM2 and TRPV1 experiments were stimulated with cumene hydroperoxide (CPx and 0.1 mM) capsaicin (CAPS and 0.01 mM) although they were inhibited by ACA (0.025 mM) and CapZ (0.1 mM), respectively. ($^{a}p \leq 0.001$ and $^{e}p \leq 0.05$ vs. control, sham, sham+HP, sham+HP+ACA and sham+HP+CapZ groups. $^{b}p \leq 0.001$ and $^{c}p \leq 0.05$ vs. SNI group. $^{d}p \leq 0.05$ and $^{f}p \leq 0.001$ vs. SNI+ACA and SNI+CapZ groups. $^{g}p \leq 0.05$ vs. SNI+HP group).

no current in the absence of CAPS (**Figure 5A**). The current densities of DRGNs were significantly higher in the SNI+CAPS group than in control ($p \leq 0.001$) and control+CAPS ($p \leq 0.05$) groups although the densities were significantly ($p \leq 0.001$) lower in control+CAPS+CapZ and SNI+CAPS+CapZ groups as compared to in the SNI groups (**Figures 5E,F**). The densities were decreased in the neuron by HP treatment and they were low in SNI+HP and SNI+HP+CapZ groups ($p \leq 0.001$). These results indicate that CAPS and ROS overload the Ca^{2+} entry through TRPV1 channel activation. However, the SNI-induced TRPV1 currents through modulation of oxidative stress were decreased by the antioxidant HP treatments.

Effect of HP on the Apoptosis and Cell Viability (MTT) Values in the SNI-Induced Sciatic Nerve and DRGNs

Involvements of TRPM2 and TRPV1 on the apoptosis and MTT in the sciatic nerve and DRGN are shown in **Figures 6A,B**, respectively. Apoptosis levels were markedly ($p \leq 0.001$) measured high in the SNI group, although MTT-values were significantly ($p \leq 0.001$) lower in the SNI group. However, the apoptosis levels were markedly decreased in ACA ($p \leq 0.05$), CapZ ($p \leq 0.05$), and HP ($p \leq 0.001$) treated groups although MTT-values were ($p \leq 0.05$ and $p \leq 0.001$) increased by the treatments.

Effect of HP on the Caspase Activities, Intracellular ROS Production and JC-1 Level in the Sciatic Nerve of Control, SNI and HP Groups

Caspase activity analyses were performed caspase 3 and 9 substrate in the plate reader. The caspase 3 and 9 activities were markedly ($p \leq 0.05$) increased in sciatic nerve and DRGNs (data are not shown) of SNI groups through TRPM2 (**Figure 7A**) and TRPV1 (**Figure 7C**) activations. However, the caspase activities were markedly ($p \leq 0.05$) decreased in the neurons through inhibition of TRPM2 and TRPV1 channels by HP with/without ACA and CapZ treatments.

Induction of SCI in rats induced a mitochondrial membrane depolarization as detected by the increase in the mitochondrial-specific voltage-sensitive dye JC-1 fluorescence ratio. The JC-1-value in the sciatic nerve (**Figures 7B,D**) and DRGNs (unpublished data) was significantly ($p \leq 0.05$) higher in the SNI group than in the control and HP groups although its value was significantly ($p \leq 0.01$) lower in the SNI+ACA, SNI+CapZ, SNI+HP, SNI+HP+ACA, and SNI+HP+CapZ groups than in the SNI group only.

Previous studies demonstrated that DRGNs produced intracellular ROS under nerve injuries through TRPM2 and TRPV1 channel activations (Ding et al., 2016; Özdemir et al., 2016). To determine whether HP, as an antioxidant plant extract, can cause redundant ROS accumulation in cytosol of sciatic nerve and DRGNs, we investigated intracellular ROS levels through TRPM2 and TRPV1 channel activations in SNI-induced and HP-treated sciatic nerve and DRGNs. The SNI-induced increase of intracellular ROS level in SNI group was also decreased in the SNI groups by HP, ACA and CapZ treatment ($p \leq 0.05$). The results implied that HP treatments might decrease the levels of SNI-induced mitochondrial ROS in the sciatic nerve and DRGNs by inhibiting TRPM2 and TRPV1. The JC-1 and ROS levels were further decreased in SNI+ACA ($p \leq 0.05$ and ($p \leq 0.001$) and SNI+HP+ACA ($p \leq 0.05$ and ($p \leq 0.001$) groups as compared to SNI and SNI+HP groups. Therefore, involvement of TRPM2 channel inhibition on the JC-1 and ROS in the sciatic nerve was more significant than in inhibition of TRPV1 channels due to antioxidant properties of HP.

Effect of HP on PARP-1, Caspase 3, and 9 Expression Levels in Sciatic Nerve, DRGN, Skin, and Muscle of the SCI-Induced Rats

Caspase 3 is synthesized as an inactive pro-enzyme that is processed in cells undergoing apoptosis by self-proteolysis and/cleavage by other caspase activation, including caspase 9. The

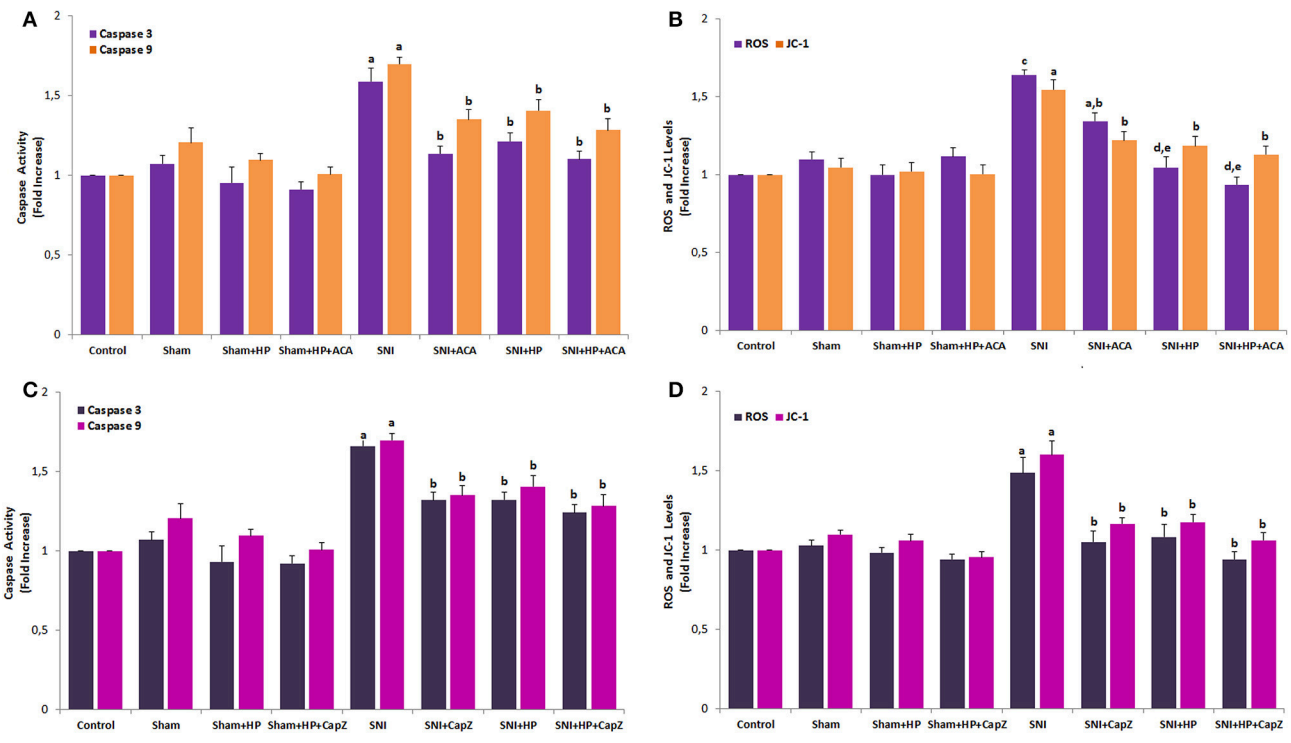


FIGURE 7 | Effects of *Hypericum perforatum* (HP) on the intracellular ROS production and cell mitochondrial membrane depolarization (JC-1) levels through TRPM2 (A,C) and TRPV1 **(B,D)** in sciatic nerve of SNI-induced rats (mean ± SD and $n = 3$). Values expressed as fold increase (experimental/control). Sciatic neurons were dissected from control, SNI and HP treated animals. The neurons in TRPM2 and TRPV1 experiments were stimulated with cumene hydroperoxide (CPx and 0.1 mM) capsaicin (CAPS and 0.01 mM) although they were inhibited by ACA (0.025 mM) and CapZ (0.1 mM), respectively. ($^{a}p \leq 0.05$ and $^{c}p \leq 0.001$ vs. control, sham, sham+HP, sham+HP+ACA and sham+HP+CapZ groups. $^{b}p \leq 0.05$ and $^{d}p \leq 0.001$ vs. SNI group. $^{e}p \leq 0.05$ vs. SNI+ACA group).

caspase 9 is activated by the active caspase 3 (Carrasco et al., 2015). Caspase 9 induces death signals by triggering other types of caspase activation. Active caspase 3 and 9 expression levels act main role the progress of apoptosis in neuronal injury (Özdemir et al., 2016). In the current study, Caspase 3 and 9 expression levels in the sciatic nerve (**Figure 8A**), DRGN (**Figure 8B**), skin (**Figure 8D**), and muscle (**Figure 8E**) were markedly ($p \leq 0.05$) higher in SNI group than in control. However, the caspase expression levels in the four samples were decreased by the HP treatments and their expression levels in the sciatic nerve ($p \leq 0.05$), DRGN ($p \leq 0.05$), skin ($p \leq 0.001$), and muscle ($p \leq 0.05$) were markedly lower in SNI+HP and sham+HP groups than in SNI group only.

PARP-1 acts main role in DNA repair (Nazıroğlu, 2007) and its expression level is increased in neurodegenerative diseases such as SCI and SNI but it expression level was decreased in SNI and DRGN by antioxidants (Wu et al., 2009; Yin et al., 2015). PARP-1 is also acted a source for many apoptotic proteases, including caspase 3 (Citron et al., 2000). In the current study, we analyzed PARP-1 expression levels in the sciatic nerve (**Figure 8C**), DRGN

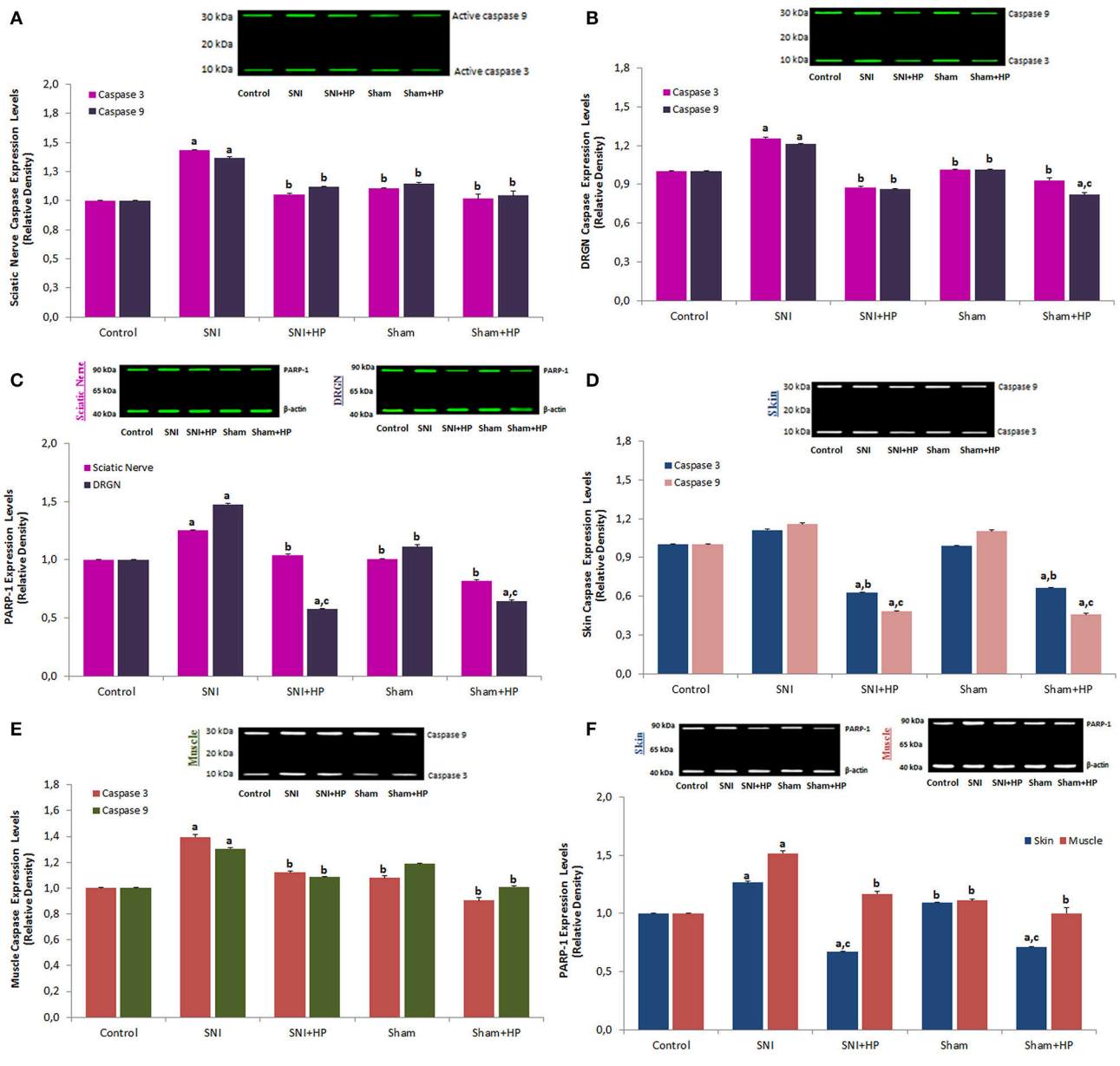


FIGURE 8 | Effects of *Hypericum perforatum* (HP) on the PARP-1, caspase 3 and 9 expression levels in sciatic nerve (A,C), DRGN **(B,C)**, skin **(D,F)** and muscle (Musculus piriformis) **(E,F)** and skin **(F)** of rats with SNI (mean ± SD and $n = 3$). Anti-β-actin was used as an internal control for the concentration of PARP1, caspase 3 and 9. Values expressed as fold increase (experimental/control). Sciatic nerve DRGN neurons were dissected from control, SNI and HP treated animals. ($^{a}p \leq 0.05$ vs. control, sham, sham+HP groups. $^{b}p \leq 0.05$ and $^{c}p \leq 0.001$ vs. SNI group).

(**Figure 8C**), skin and muscle (**Figure 8F**). PARP-1 expression levels in the sciatic nerve, DRGN, skin and muscle were markedly ($p \leq 0.05$) higher in SNI group as compared to control. However, the PARP-1 levels in the sciatic nerve ($p \leq 0.05$), DRGN ($p \leq 0.001$), skin ($p \leq 0.001$), and muscle ($p \leq 0.05$) were markedly lower in SNI+HP and sham+HP groups as compared to SNI group only.

DISCUSSION

The current results implied that HP treatments might decrease the levels of SNI-induced $[Ca^{2+}]_i$ accumulation, mitochondrial ROS, apoptosis levels, and PARP-1, caspase 3, 9 activities and expressions in the sciatic nerve and DRGNs by inhibiting TRPM2 and TRPV1. To our knowledge, this is the first evidence for a function of SNI pathophysiological process implicating the sciatic nerve and DRGN and, in particular, peripheral pain, and neurodegenerative diseases.

Recent reports indicate that functional TRPM2 and TRPV1 are expressed in the sciatic nerve and DRGNs (Isami et al., 2013; Pecze et al., 2013; Akpınar et al., 2016), the present literature findings suggest that TRPM2 and TRPV1 act role in acute mechanical nociceptive pain Ca^{2+} signaling. Considerable evidence indicated that TRPM2 and TRPV1 are activated and potentiated by excessive intracellular ROS production (Susankova et al., 2006; Ding et al., 2016). Activation of TRPM2 and TRPV1 enhanced $[Ca^{2+}]_i$ accumulation due to their permeability to Ca^{2+} (Pecze et al., 2013, 2016; Nazıroğlu et al., 2014a) which were involved in several physiological and pathological processes such as neuronal viability, apoptosis, and neuronal recovering signaling. The SCI-induced oxidative stress status evokes TRPM2 and TRPV1 channels to activation and triggers higher amounts of Ca^{2+} entry to the cell cytosol (Özdemir et al., 2016). HP is strong antioxidant because it contains several flavonoid antioxidants (Kusari et al., 2009; Stojanović et al., 2013). As source of these antioxidants, HP acts important role in etiology of neurodegenerative diseases such as SNI and SNI (Kusari et al., 2009; Stojanović et al., 2013). Recent studies have observed perturbations of Ca^{2+} homeostasis through TRPM2 and TRPV1 activations caused by excessive levels of mitochondrial oxidative stress in the neurons from experimental animals with nerve injury (Nazıroğlu et al., 2014a; Xiang et al., 2016). Induction of SNI elevates oxidative stress levels in neurons (Rogoz et al., 2015) and consequence of excessive Ca^{2+} influx, apoptosis exists by activation of cation channels (Özdemir et al., 2016). In the current study, we observed SNI-induced $[Ca^{2+}]_i$ accumulation and increased current densities through TRPM2 (ADPR and CPx) and TRPV1 (CAPS) channel activators caused by excessive levels of mitochondrial oxidative stress, although their levels were decreased by antioxidant property of HP.

We found also that the level of Ca^{2+} influx through the inhibition of TRPM2 and TRPV1 channels decreased by the HP treatment. It is well-known that intracellular Ca^{2+} signaling with/without oxidative stress acts an important role in pathophysiological functions of pain. Increases in Ca^{2+} concentration may conduce to the membrane mitochondrial depolarization (Bejarano et al., 2009; Espino et al., 2010), activation of ADPR pyrophosphatase that will enhance the TRPM2 channel potency, and activation of a variety of intracellular enzymes such as PARP-1 and caspase (Perraud et al., 2001; Hara et al., 2002; Nazıroğlu and Lückhoff, 2008). Previous studies have shown that the intracellular Ca^{2+} influx into sciatic nerve and DRGNs neurons through increased activity sensitization of TRPM2 and TRPV1 channels acted a main role in mechanical hypersensitivity and pain associated with nerve injury (Haraguchi et al., 2012; Nazıroğlu, 2012, 2015; Rogoz et al., 2015) although the hypersensitivity and pain are decreased by inhibition of calcium channels through HP treatment (Uchida et al., 2008; Nazıroğlu et al., 2014a; Özdemir et al., 2016). Hence, we provided the novel finding that HP treatment potently decreased SNI-induced overload intracellular Ca^{2+} entry by modulation of TRPM2 and TRPV1 channel activations.

The impairment of neuronal membrane permeability causes overload Ca^{2+} influx into cytosol and it leads to excessive production of ROS in the neurons (Kumar et al., 2014; Demirdaş et al., 2017). Increased $[Ca^{2+}]_i$ concentration through activation of TRPM2 and TRPV1 causes disruption of the Ca^{2+} contents of intermembrane space through mitochondrial permeability transition activation in the mitochondria (Pecze et al., 2013). The dysfunction of mitochondria triggers generation of endogenous ROS. Caspases, a group of enzymes are activated by overload $[Ca^{2+}]_i$ concentration and excessive ROS products that found cleavage (inactive) caspases before the neurons undergo apoptosis (Citron et al., 2000). However, taken together the excessive ROS production and Ca^{2+} impairment of the neurological cells have revealed that a key role in the pathogenesis of neurodegenerative diseases such as SNI and SCI (Gupta et al., 2014; Özdemir et al., 2016). Antioxidants through inhibition of TRPM2 and TRPV1 regulate the mitochondrial and apoptotic imbalance and help to normal neuronal functions (Nazıroğlu, 2012). In the current study, the apoptosis, caspase 3, caspase 9, PARP-1, JC-1, and intracellular ROS-values were increased in the sciatic nerve and DRGNs by SNI induction although their values were decreased in the neurons by HP, TRPM2 (ACA), and TRPV1 (CapZ) blockers. Similarly, apoptosis, ROS, JC-1, caspase 3 and 9 values through inhibition of TRPM2 in human phagocytic cells were decreased by HP incubations (Nazıroğlu et al., 2014b,c). The modulator role of HP on TRPM2 channels and oxidative stress in DRGN of rats was indicated by an experimental rat study (Nazıroğlu et al., 2014a). More recently, the HP extract has been reported to efficiently attenuate oxidative stress, apoptosis and Ca^{2+} entry through modulation of TRPM2 and TRPV1 channels in DRGN of SCI-induced rats (Özdemir et al., 2016). Current results supported results of the reports on HP treatment in the human phagocytic cells and rat DRGNs (Nazıroğlu et al., 2014a,b,c; Özdemir et al., 2016).

TRPC6 channel is belonging to the superfamily of TRP. It was reported that hyperforin caused intracellular Ca^{2+} elevations through TRP canonical 6 (TRPC6) in PC12 cells (Leuner et al., 2007) although other effects of hyperforin are described which might also participate in its pharmacological actions. For example, hyperforin attenuates voltage- and chemical-gated

Ca^{2+} conductances in isolated hippocampal neurons and cerebellar Purkinje neurons (Chatterjee et al., 1999; Fisunov et al., 2000). At the cellular level, the hyperforin induced mitochondrial membrane depolarization through releasing zinc and calcium ions from these intracellular organelles (Tu et al., 2010). Contrary, depletion of intracellular Ca^{2+} stores with the SERCA pump inhibitor (thapsigargin) did not affect hyperforin-induced $[Ca^{2+}]_i$ transients although hyperforin increased Ca^{2+} entry through TRPC6 channel activation in primary hippocampal neurons (Leuner et al., 2013). Decrease of indomethacin-induced Ca^{2+} mobilization, cytotoxicity, apoptosis, and caspase activation in Caco-2 cell line was reported by quercetin as a component of HP (Carrasco-Pozo et al., 2012). Hyperforin also induced Ca^{2+} transients in dissociated primary cultures of embryonic cortical neurons through channels displaying TRPC6-like properties (Tu et al., 2009). Recently it was reported that that hyperforin induces TRPC6-independent hydrogen ion currents in HEK-293 cells, cortical microglia, chromaffin cells, and lipid bilayers (Sell et al., 2014). No association between, hyperforin-induced apoptosis, TRPC6 activation and oxidative stress in neonatal pig glomerular mesangial cell was reported (Soni and Adebiyi, 2016). Contrary, cerebral ischemia-induced rat cortical neuron TRPC6 degradation, oxidative stress and apoptosis were reduced at 24 h of cerebral ischemia by hyperforin treatment (Lin et al., 2013). According to the conflicting results, the mechanisms of hyperforin on TRPC6 are not fully understood and its effect on the channel seems cell specific and different from antioxidant effect on TRPM2 and TRPV1 in sciatic nerve and DRGN.

In summary, our study provided for the first time that apoptotic pathway, overload Ca^{2+} entry, and mitochondrial ROS production through increased activation of TRPM2 and TRPV1 were increased in sciatic nerve and DRGNs of SNI-induced rats. We identified that SNI-induced sensitization of TRPM2 and TRPV1 activity to induce apoptosis and oxidative stress in the neurons was decreased through modulation of the channels by HP treatment. Inhibition of the channels through HP treatment was probably mediated by direct inhibiting ROS to decrease channel gating. Therefore, current results provide that HP acts a neuronal modulator role against ROS-induced apoptosis Ca^{2+} mobilization through inhibition of TRPM2 and TRPV1 channels in sciatic nerve and DRGNs. This finding is of particular significance and may provide an explanation for the SNI-induced neuronal survival and peripheral pain reduce properties of HP. TRPM2 and TRPV1 channels may become an important pharmacological target in the treatment of SNI-induced apoptosis and pain.

ETHICS STATEMENT

The study was approved by the Local Experimental Animal Ethical Committee of Suleyman Demirel University (SDU) (Protocol number: HADYEK-07-2015). The study was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the European Community's Council Directives (86/609/EEC). All experiments were carried out in accordance with the approved guidelines.

AUTHOR CONTRIBUTIONS

MN and FU formulated the present hypothesis and MN was responsible for writing the report. FU was responsible for induction of SNI. BÇ was responsible for sciatic nerve, DRGN isolation and cytosolic Ca^{2+} release analyses. Graphical abstract figure was produced by BÇ.

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TNF-α Increases Production of Reactive Oxygen Species through Cdk5 Activation in Nociceptive Neurons

Rodrigo Sandoval[1], Pablo Lazcano[1], Franco Ferrari[1], Nicolás Pinto-Pardo[1,2], Christian González-Billault[3,4,5] and Elías Utreras[1]**

[1] *Laboratory of Molecular and Cellular Mechanisms of Pain, Department of Biology, Faculty of Science, Universidad de Chile, Santiago, Chile,* [2] *Doctorate in Biomedicine, Universidad de los Andes, Santiago, Chile,* [3] *Laboratory of Cellular and Neuronal Dynamics, Department of Biology, Faculty of Science, Universidad de Chile, Santiago, Chile,* [4] *Center for Geroscience, Brain Health and Metabolism (GERO), Santiago, Chile,* [5] *The Buck Institute for Research on Aging, Novato, CA, United States*

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***Correspondence:**
Christian González-Billault
chrgonza@uchile.cl
Elías Utreras
elias.utreras@uchile.cl

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The participation of reactive oxygen species (ROS) generated by NOX1 and NOX2/NADPH oxidase has been documented during inflammatory pain. However, the molecular mechanism involved in their activation is not fully understood. We reported earlier a key role of Cyclin-dependent kinase 5 (Cdk5) during inflammatory pain. In particular, we demonstrated that TNF-α increased p35 expression, a Cdk5 activator, causing Cdk5-mediated TRPV1 phosphorylation followed by an increment in Ca^{2+} influx in nociceptive neurons and increased pain sensation. Here we evaluated if Cdk5 activation mediated by p35 transfection in HEK293 cells or by TNF-α treatment in primary culture of nociceptive neurons could increase ROS production. By immunofluorescence we detected the expression of catalytic subunit (Nox1 and Nox2) and their cytosolic regulators (NOXO1 and $p47^{phox}$) of NOX1 and NOX2/NADPH oxidase complexes, and their co-localization with Cdk5/p35 in HEK293 cells and in nociceptive neurons. By using a hydrogen peroxide sensor, we detected a significant increase of ROS production in p35 transfected HEK293 cells as compared with control cells. This effect was significantly blocked by VAS2870 (NADPH oxidase inhibitor) or by roscovitine (Cdk5 activity inhibitor). Also by using another ROS probe named DCFH-DA, we found a significant increase of ROS production in nociceptive neurons treated with TNF-α and this effect was also blocked by VAS2870 or by roscovitine treatment. Interestingly, TNF-α increased immunodetection of p35 protein and NOX1 and NOX2/NADPH oxidase complexes in primary culture of trigeminal ganglia neurons. Finally, the cytosolic regulator NOXO1 was significantly translocated to plasma membrane after TNF-α treatment and roscovitine blocked this effect. Altogether these results suggest that Cdk5 activation is implicated in the ROS production by NOX1 and NOX2/NADPH oxidase complexes during inflammatory pain.

Keywords: Cdk5, NOX, TNF-α, pain signaling, ROS

INTRODUCTION

During tissue damage, several inflammatory mediators as tumor necrosis factor-α (TNF-α) are locally released, activating signaling pathways in sensory neurons that increase peripheral sensitization and pain signaling (Khan et al., 2008; Sessle, 2011; Rozas et al., 2016). Activated protein kinases such as PKC, PKA, and Cdk5 are involved in peripheral sensitization through phosphorylation of several ion channels expressed on nociceptive neurons (Basbaum et al., 2009; Rozas et al., 2016; Coddou et al., 2017). Cdk5 is an essential kinase in brain development and function (Dhariwala and Rajadhyaksha, 2008; Utreras et al., 2009a; Contreras-Vallejos et al., 2012). Interestingly, our group reported earlier that Cdk5 plays a crucial role during inflammatory pain signaling (Utreras et al., 2009a, 2011; Prochazkova et al., 2013; Rozas et al., 2016; Coddou et al., 2017). Cdk5 is a proline-directed serine/threonine kinase, mostly active in post-mitotic neurons, where its specific activators p35 and p39 are mainly expressed (Lew et al., 1994; Dhariwala and Rajadhyaksha, 2008; Utreras et al., 2009c). We reported also that cytokines such as TNF-α or TGF-β1 up-regulate p35 expression and Cdk5 kinase activity with a subsequent phosphorylation of transient receptor potential vaniloid 1 (TRPV1) and purinergic P2X2a receptor (P2X2aR), which are important receptors channels involved during inflammatory pain signaling (Utreras et al., 2009b, 2011, 2012, 2013; Prochazkova et al., 2013; Rozas et al., 2016; Coddou et al., 2017).

On the other hand, reactive oxygen species (ROS) represent important players during inflammation (Mittal et al., 2014). There is growing evidence supporting ROS as molecules that contribute to pain hypersensitivity (Kallenborn-Gerhardt et al., 2013). Interestingly, antioxidant therapy has been used to overcome painful effects developed in inflammatory pain models (Khattab, 2006; Lauro et al., 2016; Wu et al., 2017). There are several enzymatic systems that generate ROS such as lipoxygenases, xanthine oxidases, cyclooxygenases, cytochrome P450 monooxygenases, nitric oxide synthases, and NADPH oxidases (NOX) (Holmström and Finkel, 2014; Bórquez et al., 2016; Wilson et al., 2017). NOX enzymes belong to the NOX family that is composed by seven members (NOX1-5 and Duox 1-2). Interestingly, Nox1, Nox2, and Nox4 catalytic enzymes have been associated with pain signaling (Kallenborn-Gerhardt et al., 2012, 2013, 2014; Geis et al., 2017). NOX enzymes reside at the plasma membrane in tight association with the integral membrane protein $p22^{phox}$. When NOX complex becomes active, it generates ROS by a catalytic transfer of electrons from NADPH to O_2 to form superoxide anion and hydrogen peroxide (Sumimoto et al., 2005). Nox1 activation occurs by PKC-mediated phosphorylation of the cytosolic subunit Nox organizer 1 (NOXO1) allowing NOXO1-$p22^{phox}$ interaction (Debbabi et al., 2013). Similarly, activation of Nox2 ($Gp91^{phox}$) needs the recruitment of the regulatory cytosolic subunits $p47^{phox}$, $p67^{phox}$, and Rac1 (El-Benna et al., 2008). Multiple serine phosphorylation of $p47^{phox}$ by PKC is a key step that induces association with $p22^{phox}$ at the plasma membrane and activation of the complex (Fontayne et al., 2002; Meijles et al., 2014). In the present work we describe a new role of Cdk5 in the modulation of redox balance in nociceptive neurons. Thus, activation of Cdk5 in HEK293 cells and primary sensory neurons was related to increased ROS production. Interestingly, this effect was blocked by inhibition of NOX complex or Cdk5 kinase activity, suggesting a molecular link between Cdk5 and NOX-mediated ROS production. Implications of these findings could address additional roles of Cdk5 in pain signaling.

MATERIALS AND METHODS

Transfection of HEK293 Cells

HEK293 cells (ATCC#CRL-1573) were grown in Dulbecco Modified Eagle Medium (DMEM) containing 10% of fetal bovine serum (FBS) and penicillin/streptomycin (100 mg/mL) (Invitrogen, Carlsbad CA). HEK293 cells were transiently co-transfected with mouse CMV-p35 (Coddou et al., 2017) and HyPer constructs (Evrogen, Moscow, Russia) by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad CA) and treated with roscovitine (30 μM, Sigma-Aldrich, Saint Louis, MO) or VAS2870 (1 μM, Calbiochem, CA USA) during 24 h and 1 h, respectively.

Primary Culture of Mouse Nociceptive Neurons

Nociceptive neurons were cultured as described previously (Coddou et al., 2017). Briefly, trigeminal ganglia (TG) and dorsal root ganglia (DRG) were dissected out from 7 to 10 month-old C57/FVB mice and incubated with collagenase XI (0.66 mg/mL) and dispase II (3 mg/mL, Sigma-Aldrich, Saint Louis, MO) in an INCmix solution (NaCl 155 mM; K_2HPO_4 1.5 mM; HEPES 10 mM; glucose 5 mM; at pH 7.4). Enzymatic digestion was performed for 45 min at 37°C in 5% CO_2, and consecutively treated with DNase I (100 μg/mL, Roche Diagnostic, Indianapolis, IN) during 10 min at 37°C. TG/DRG cell suspensions were separated over discontinuous 28–12.5% Percoll gradients (GE Healthcare). Isolated cells were cultured in minimum essential media (MEM) supplemented with 10% FBS, penicillin/streptomycin (100 mg/mL), and MEM-Vit (Invitrogen, Carlsbad CA). Cells were plated on 12-mm poly-L-lysine-coated glass coverslips and cultured for 2 days *in vitro* (2DIV). To evaluate the involvement of Cdk5 activation by TNF-α and NOX signaling, TG and DRG primary cultures (2DIV) were treated with TNF-α (25–50 ng/mL, Sigma-Aldrich, Saint Louis, MO) in the presence or absence of roscovitine (20 μM) or VAS2870 (1 μM) during 24 h and 1 h, respectively. Animal experiments were conducted in accordance with the principles and procedures of the Ethics Committee of the Biology Department, Faculty of Sciences, Universidad de Chile, Santiago, Chile.

Immunofluorescence Assays

HEK293 cells transfected with p35 or primary cultures of TG and DRG neurons were washed with warm PBS for 5 min and fixed with a 4% PFA-4% sucrose solution in PBS at 37°C for 20 min. Cells were washed and permeabilized for 5 min with 0.2% Triton X-100-PBS solution. After washout with PBS, cells were blocked with a 5% BSA solution in PBS at room temperature for 1 h. Primary antibodies were used at following concentrations:

anti-Cdk5 mouse DC17 (1:100), anti-p35 rabbit C19 (1:100), anti-Nox1 goat sc-292094 (1:100), anti-NOXO1 rabbit sc-5821 (1:100), anti-NOXA1 rabbit sc-160597-R (1:100), anti-p47^{phox} rabbit sc-14015 (1:100), anti-MAP1B goat N-19 (1:200), anti-p35 goat A-18 (1:100) (from Santa Cruz Biotechnology); anti-gp91^{phox} mouse ab109366 (1:100), and anti-p22^{phox} rabbit ab75941 (1:100) (from Abcam); anti-βIII tubulin mouse clone G7121 (1:1000) (from Promega); p35 rabbit C64B10 (1:100) (from Cell Signaling Technology, Denver, USA). All primary antibodies were diluted in 1% BSA solution and incubated overnight at 4°C. The coverslips were washed with PBS and then incubated with corresponding Alexa Fluor-conjugated secondary antibodies. We used the following secondary antibodies:-Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 #A21202-Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 #A21206-Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 546 #A10040-Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 546 #A10036-Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 633 #A21082 (Molecular Probes, Life Technologies, Grand Island, NY) in combination with Dapi (Thermo Fisher Scientific) for 1 h at room temperature. Finally, coverslips were washed with PBS and mounted on a slide with FluorSave (Calbiochem). Images were acquired using confocal microscopy (LSM 710 Meta Model, Carl Zeiss Microscopy) and processed with the LSM Image Browser (Carl Zeiss Microscopy) software.

Immunofluorescence Quantification Analysis

As an estimation of protein amount in individual neurons, the fluorescence intensity was quantified as reported earlier (McCloy et al., 2014). Confocal images (40X) acquired from immunofluorescences were processed by using ImageJ 1.46r software (NIH, Bethesda, MD) and individual ROIs were assigned to each neuron and integrated density was obtained for each fluorescent emission.

Hydrogen Peroxide Measurement in Transfected HEK293 Cells

We evaluated hydrogen peroxide content in HEK293 cells by using HyPer sensor as previously reported (Belousov et al., 2006). HEK293 cells co-transfected with HyPer and CMV-p35 plasmids during 24 h were fixed in 4% paraformaldehyde/4% sucrose solution in PBS. Then, cells were excited at 488 and 405 nm and emission was collected at 505–530 nm in a confocal microscopy (LSM 710 Meta Model, Carl Zeiss Microscopy). Fluorescence emission from excitation at 488 nm was divided by fluorescence emission at 405 nm excitation (488:405) as a measure of the hydrogen peroxide content (Belousov et al., 2006).

Neuronal ROS Measurement

To evaluate intracellular ROS levels, primary cultures of mouse TG and DRG neurons were incubated simultaneously with CellTracker^{TM} Orange dye (CMTMR 1 μM, Thermo Fisher Scientific) and 2′,7′-Dichlorofluorescin diacetate (DCFH-DA 1 μM, Sigma) for 20 min at 37°C similarly to previously reported (Wilson et al., 2015). DCFH-DA detects intracellular oxidative species by increasing fluorescence emission after oxidation (Lebel and Bondy, 1990). Sensory neurons were fixed and mounted to measure the fluorescence by excitation at 488 nm and emission acquisition at 505–530 nm. Fluorescence from CMTMR dye incorporated in neurons was used to normalize DCFH-DA emission, and was acquired by excitation at 543 nm and emission acquisition at 548–679 nm.

Translocation to Plasma Membrane Analysis

To estimate plasma membrane translocation of NOX cytosolic subunits (NOXO1 and p47^{phox}), we drew a line that crosses the 2D surface of each neuron from end to end (**Figure 6A**). Gray value was obtained along the drawn line to estimate the amount of protein in zones near and far of plasma membrane (peripheral and centric areas, respectively). Mean gray value of centric areas were calculated as the mean of all gray values comprising the medial portion of the drawn line (30% of total line). Peripheral areas were defined as the first 4 μm from the periphery to the center in the drawn line. Mean gray value was calculated in this short section alike centric areas. Finally, the mean gray value of peripheral area (two per neuron) was normalized to corresponding centric area mean gray value (**Figure 6A**).

Western Blot Analysis

Protein extracts from HEK293 cells transfected with p35 were obtained in T-PER buffer (Pierce, Rockford, IL) with Complete Mini protease inhibitor cocktail tablets and PhosSTOP phosphatase inhibitor cocktail tablets (Roche Diagnostic, Indianapolis, IN). Protein extracts were resolved in 12% SDS-PAGE gels and transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA). Membranes were soaked in blocking buffer (5% non-fat dry milk in Tris-Buffered Saline (TBS) with 0.05% Tween-20 (TBS-T)) for 1 h at room temperature, and then incubated overnight at 4°C, with primary antibody diluted in 1% non-fat dry milk blocking buffer. The membranes were washed in TBS-T and incubated for 1 h at room temperature with the secondary antibodies diluted in 1% non-fat dry milk blocking buffer. Immunoreactivity was detected by using Super-Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). Western blots were performed by using anti-p35 rabbit C19 (1:250) and anti-Cdk5 mouse DC17 (1:500) from Santa Cruz Biotechniology; anti-α-tubulin mouse (1:10.000) from Sigma. We used secondary antibodies anti-mouse and anti-rabbit coupled to Horseradish Peroxidase from Santa Cruz Biothecnology. The optical densities of the bands were quantified using an image analysis system with ImageJ 1.46r software (NIH, Bethesda, MD).

Statistical Analysis

All experiments were performed a minimum of three times. All graphs show the mean ± *SD*. Statistical evaluation was performed with GraphPad Prism software, version 6.1 (GraphPad, San Diego, CA). Significant differences between experiments were assessed by an unpaired *t*-test or a one-way analysis of variance with a Bonferroni's multiple comparison test, where α was set to 0.05.

RESULTS

Endogenous Expression of NOX Complexes in HEK293 Cells

To establish Cdk5 participation in NOX1 and NOX2-dependent ROS production, we analyzed the immunolocalization of NOX1 and NOX2/NADPH oxidase complexes in HEK293 cells transfected with p35 that endogenously express Cdk5 (**Figure 1**). By immunofluorescence we detected co-localization of catalytic (Nox1 and Nox2) and cytosolic subunits (NOXO1 and $p47^{phox}$) with p35 protein. We observed endogenous expression of Nox1, Nox2, NOXO1 and $p47^{phox}$ in plasma membrane and in cytoplasmic regions of HEK293 cells (**Figures 1A–D**, green label). We also found that HEK293 cells expressed other members of the catalytic NOX core such as $p22^{phox}$ and NOXA1 (data not shown). Most important, we immunodetected p35 in some transfected cells co-localizing with NOX1 and NOX2 complexes (**Figures 1A–D**, red label). Additionally, we detected endogenous Cdk5 and transfected p35 proteins by Western blot from HEK293 cells in the presence or absence of roscovitine (**Figure 2A**). We also confirmed that HEK293 cells transfected with p35 have increased Cdk5 kinase activity (data not shown), similarly as previously reported (Zheng et al., 2002).

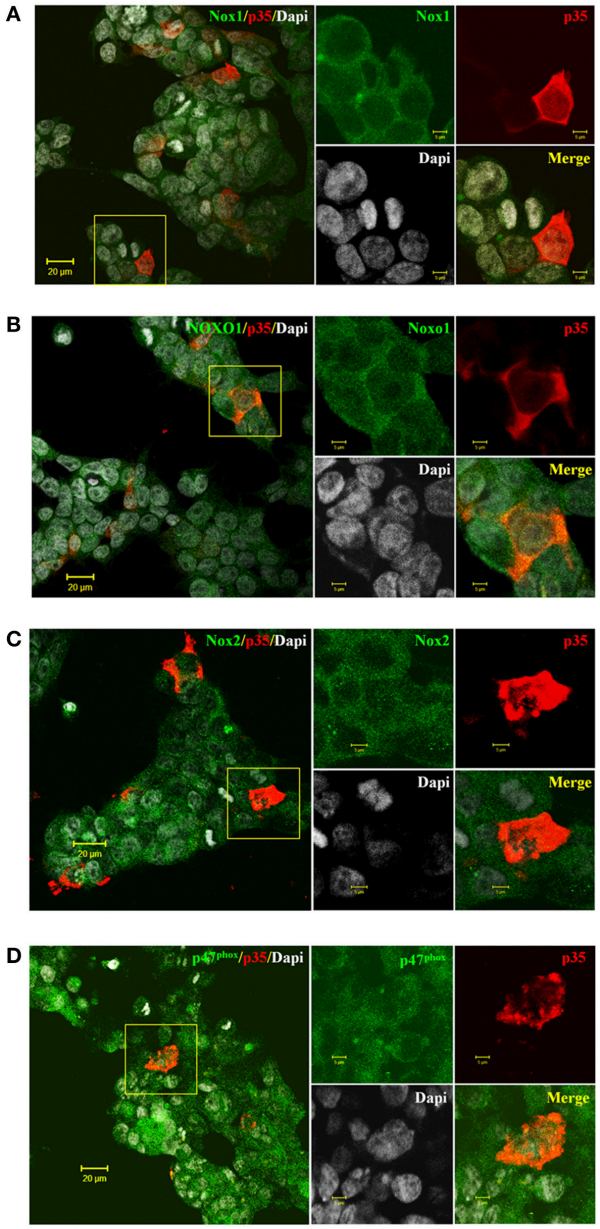

FIGURE 1 | Endogenous expression of Nox1/NOXO1 and Nox2/$p47^{phox}$ in HEK293 cells transfected with p35 plasmid**. (A,C)** Representative immunofluorescences showing catalytic subunits Nox1 and Nox2 (in green, respectively) co-localizing with p35 (in red) in transfected HEK293 cells. **(B,D)** Representative immunofluorescences of regulatory subunits NOXO1 and $p47^{phox}$ (in green, respectively) co-localizing with p35 (in red) in transfected HEK293 cells. Inset for each figure showing staining by separate in larger magnification. DAPI for nuclear staining (gray). Scale bars are 20 and 5 µm insets.

Increased Cdk5-Dependent ROS Production in HEK293 Cells

To evaluate if ROS production is affected by increased Cdk5 activity in transfected HEK293 cells, we used the genetically encoded biosensor HyPer which detects intracellular production of hydrogen peroxide (Lukyanov and Belousov, 2014; Wilson et al., 2015). Firstly, by immunofluorescence we determined p35 expression in HEK293 cells co-transfected with p35 and HyPer plasmids (**Figure 2B**). Then, we generated a HyPer map (480/405 nm ratio) for each treatment condition of HEK293 cells. In HEK293 cells overexpressing p35 we found a significant increase of hydrogen peroxide content and this effect was significantly reverted by roscovitine (**Figures 2B,C**). Interestingly, Cdk5-dependent ROS production was also reverted by NOX inhibitor, VAS2870 (**Figures 2B,C**), suggesting that NOX complexes represent a source for the elevated ROS observed in this model. The basal hydrogen peroxide production was not affected by roscovitine treatment and only slightly reduced by VAS2870 treatment (**Figures 2B,C**). These results suggest that production of ROS is dependent, in part, on Cdk5 activity in HEK293 cells expressing active NOX1 and NOX2 complexes.

Endogenous Expression of NOX1 and NOX2 Complexes and Cdk5/p35 in Primary Culture of TG Neurons

Our previous data suggests that Cdk5 is involved in ROS production mediated by the NOX1 and NOX2 complex in HEK293 cells. Importantly, the production of ROS (Kallenborn-Gerhardt et al., 2013) and also Cdk5 kinase (Utreras et al., 2009a) are active participants during pain signaling. Therefore, we evaluated the co-distribution of Cdk5/p35 with members of NOX1 and NOX2 complexes in sensory neurons. The NOX family members NOX1 and NOX2 have been associated with pain processes (Kallenborn-Gerhardt et al., 2013), consequently we studied their distribution in primary culture of mouse TG neurons. By immunofluorescence, we identified that NOX

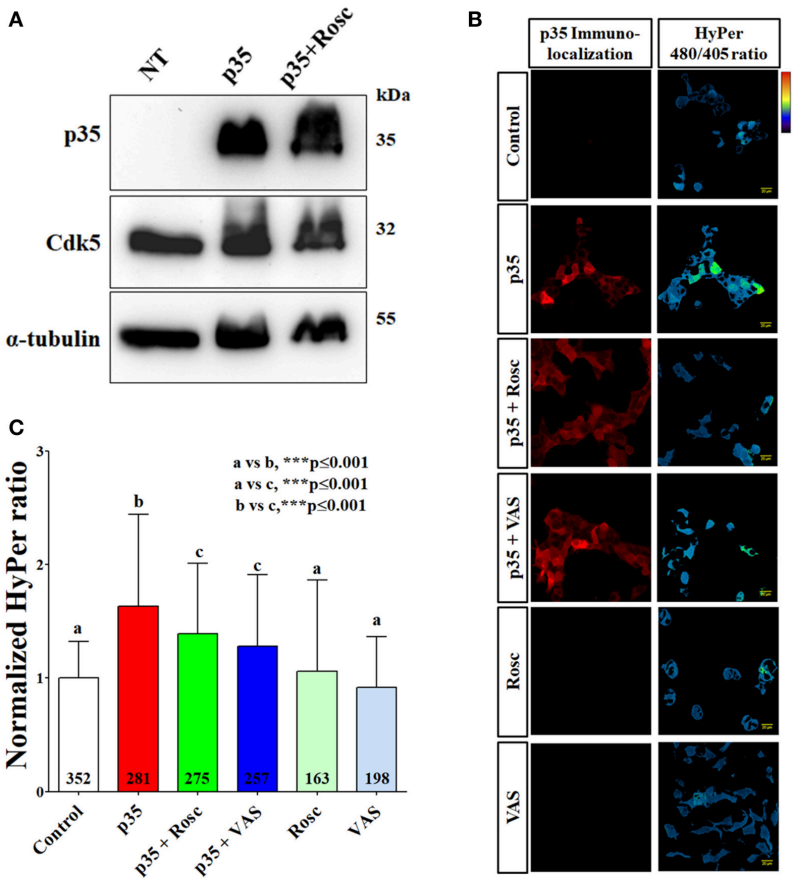

FIGURE 2 | Increased Cdk5 kinase activity in HEK293 cells transfected with p35 produced elevated hydrogen peroxide content measured by HyPer probe. **(A)** Western blot analysis against p35, Cdk5, and α-tubulin from HEK293 cells untransfected (UT), transfected with p35 (p35), and transfected with p35 and treated with roscovitine (30 μM) (p35 + Rosc) during 24 h. **(B)** Representative immunofluorescences of p35 (left) and HyPer 480/405 ratio (right) from HEK293 cells transfected with p35 and HyPer, treated with roscovitine or VAS2870. Thermal scale: increased hydrogen peroxide content toward red. **(C)** Normalized HyPer 480/405 ratio in same conditions of **(B)**. Scale bars are 20 μm. The bar graphs represent mean ± SD of $n = 4–7$ different cell cultures. Number of cells analyzed is showed inside each column. Statistical differences correspond to a one-way ANOVA with a Bonferroni's multiple comparison test.

members of both complexes are highly expressed in neurons of all sizes (small, medium, and large neurons), respect to non-neuronal cells (**Figure 3**, negative cells for βIII-tubulin). Catalytic (Nox1 and Nox2) and cytoplasmic (NOXO1 and $p47^{phox}$) subunits were mostly found in somata and in neurites, with the exception of NOXO1, which was equally identified in both neuronal regions including large processes (**Figures 3A–H**). We also observed endogenous Cdk5 and p35 expression in TG neurons and others cells co-localizing with both NOX1/NOX2 complexes (**Figures 3A–H**), suggesting that Cdk5-dependent ROS production is likely to occur in trigeminal ganglia neurons.

TNF-α Increased ROS Production in Primary Culture of TG and DRG Neurons

Previously, we reported that TNF-α increases Cdk5 kinase activity by transcriptional up-regulation of p35 in cell lines and in TG and DRG neurons (Utreras et al., 2009b; Rozas et al., 2016). To evaluate the association between TNF-α-mediated Cdk5 activation and NOX-dependent ROS production in nociceptive neurons, we performed primary cultures of mouse TG and DRG neurons treated with TNF-α and we further measured ROS production by using DCFH-DA probe (Lebel and Bondy, 1990; Wilson et al., 2015) (**Figures 4A,B**). We found that TNF-α treatment significantly increased the intracellular ROS content. In contrast, this effect was completely blocked by roscovitine, suggesting the involvement of Cdk5 in ROS production in sensory neurons (**Figures 4A–D**). Interestingly, we found that the source of ROS content in TG and DRG primary culture was NOX-dependent, since VAS2870 also blocked the TNF-α-mediated ROS production (**Figures 4A–D**). Basal ROS production was not affected by roscovitine treatment and only slightly reduced by VAS2870 in DRG neurons. These data are in agreement with our previous results obtained in HEK293 cells, suggesting that Cdk5 contributes to ROS balance in nociceptive neurons. However, the molecular mechanism involving Cdk5 activity in ROS production through NOX complexes is not clear.

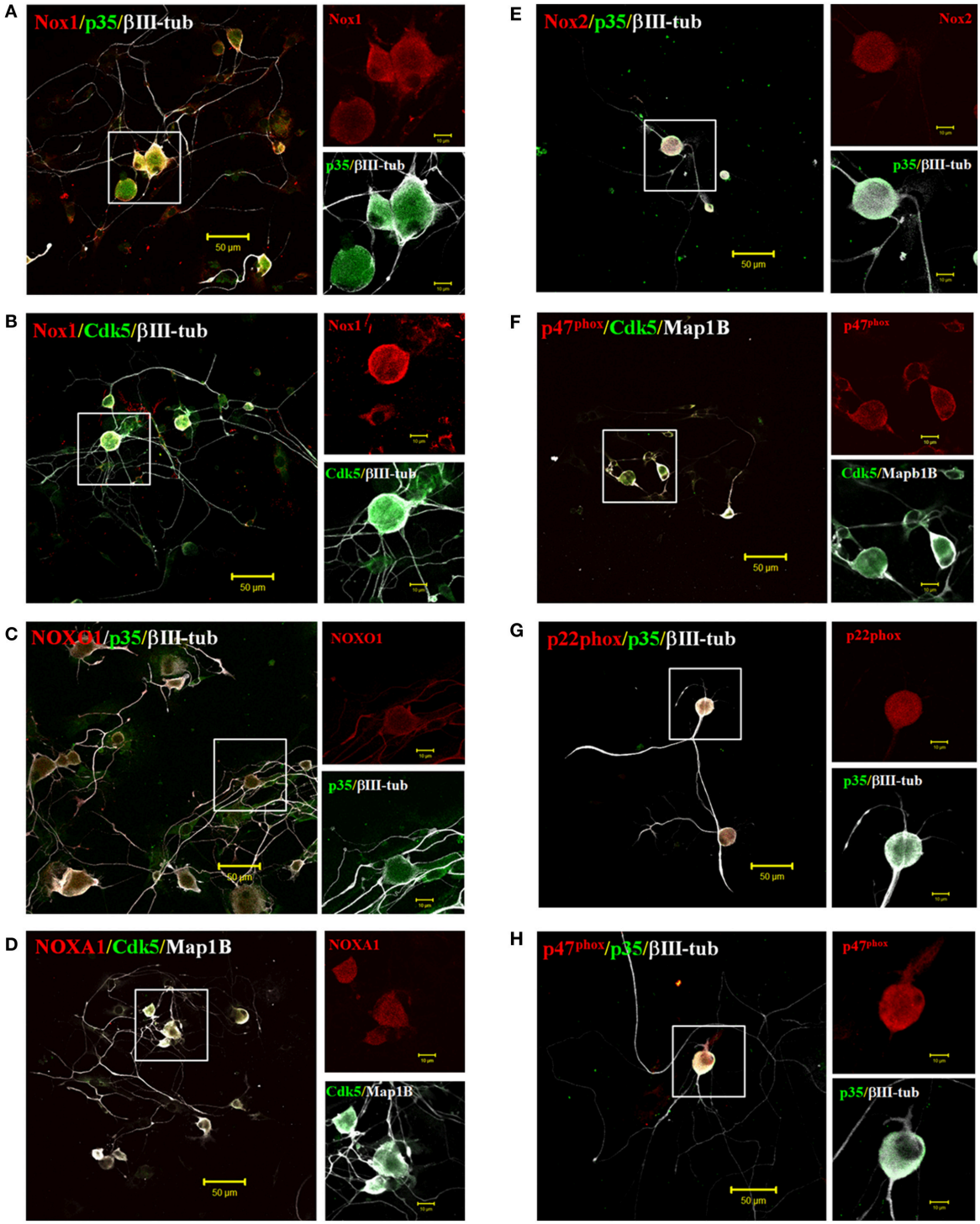

FIGURE 3 | Endogenous expression of NOX1 and NOX2 complexes with Cdk5/p35 in primary culture of trigeminal ganglia neurons. Representative immunofluorescences of catalytic subunit Nox1 **(A,B)** and its regulatory subunit NOXO1 **(C)** and NOXA1 **(D)** in red, co-localizing with Cdk5 and p35 (in green) expressed endogenously in primary culture of trigeminal ganglia neurons. Representative immunofluorescences of catalytic subunit Nox2 **(E)** and its regulatory subunits $p47^{phox}$ **(F,H)** and $p22^{phox}$ **(G)** in red, co-localizing with Cdk5 and p35 (in green) expressed endogenously in primary culture of trigeminal ganglia neurons. βIII-tubulin or MAP1B is a marker of neurons (gray). Scale bars are 50 or 10 μm (insets).

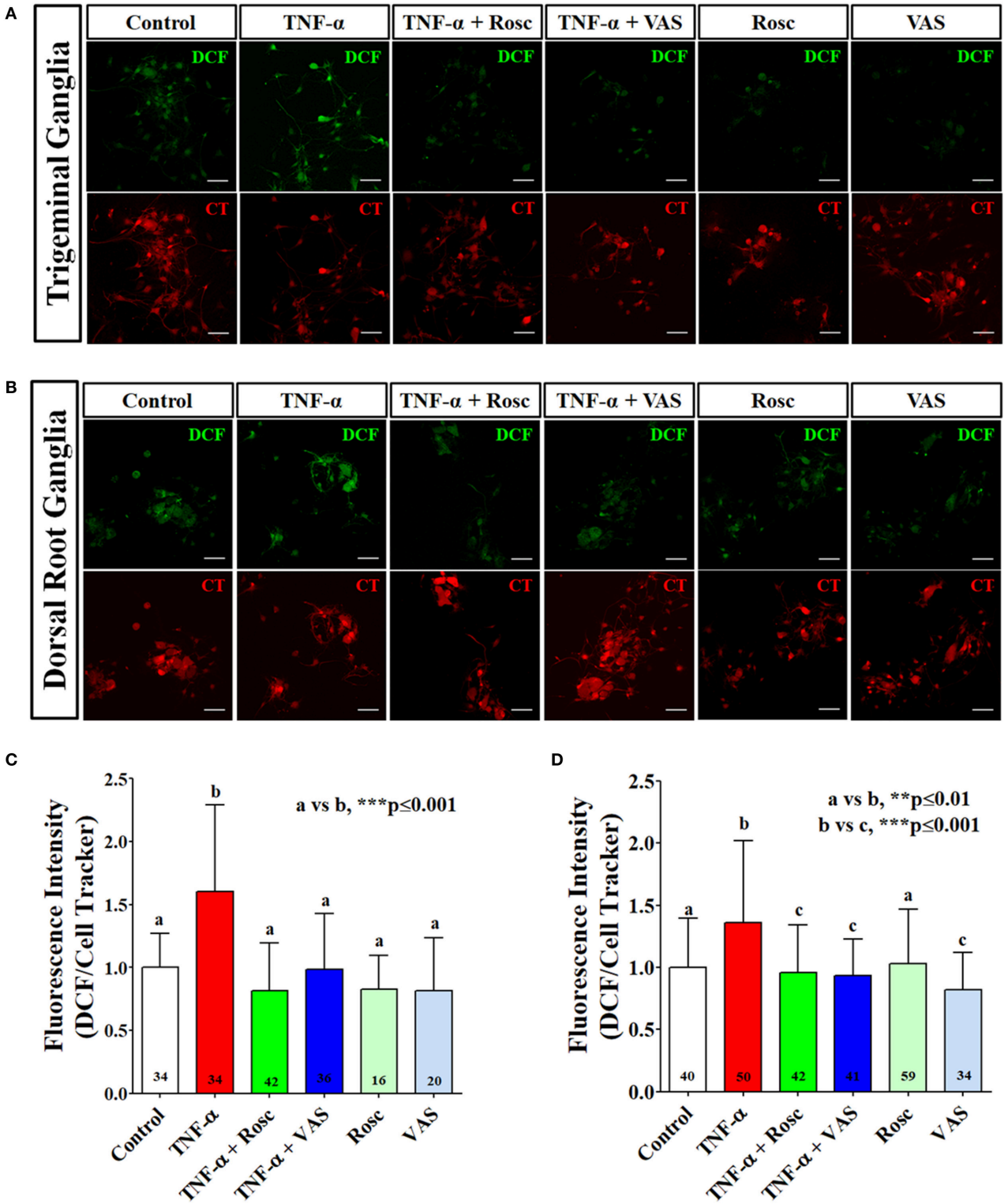

FIGURE 4 | TNF-α increases ROS production in primary culture of mouse TG and DRG neurons**.** Representative images of primary cultures loaded with DCFH-DA (DCF) and CMTMR (CT) probes from TG **(A)** and DRG **(B)** neurons treated with TNF-α alone or with roscovitine or VAS2870. **(C,D)** Quantification of fluorescence intensity of DCF normalized by CT under each corresponding treatment in TG neurons **(C)** or DRG neurons **(D)**. Scale bars are 20 μm. The bar graphs represent mean ± *SD* of $n = 3$ different experiments. Statistical differences correspond to a one-way ANOVA with a a Bonferroni's multiple comparison test. Number of analyzed neurons for each treatment is inside the corresponding bar.

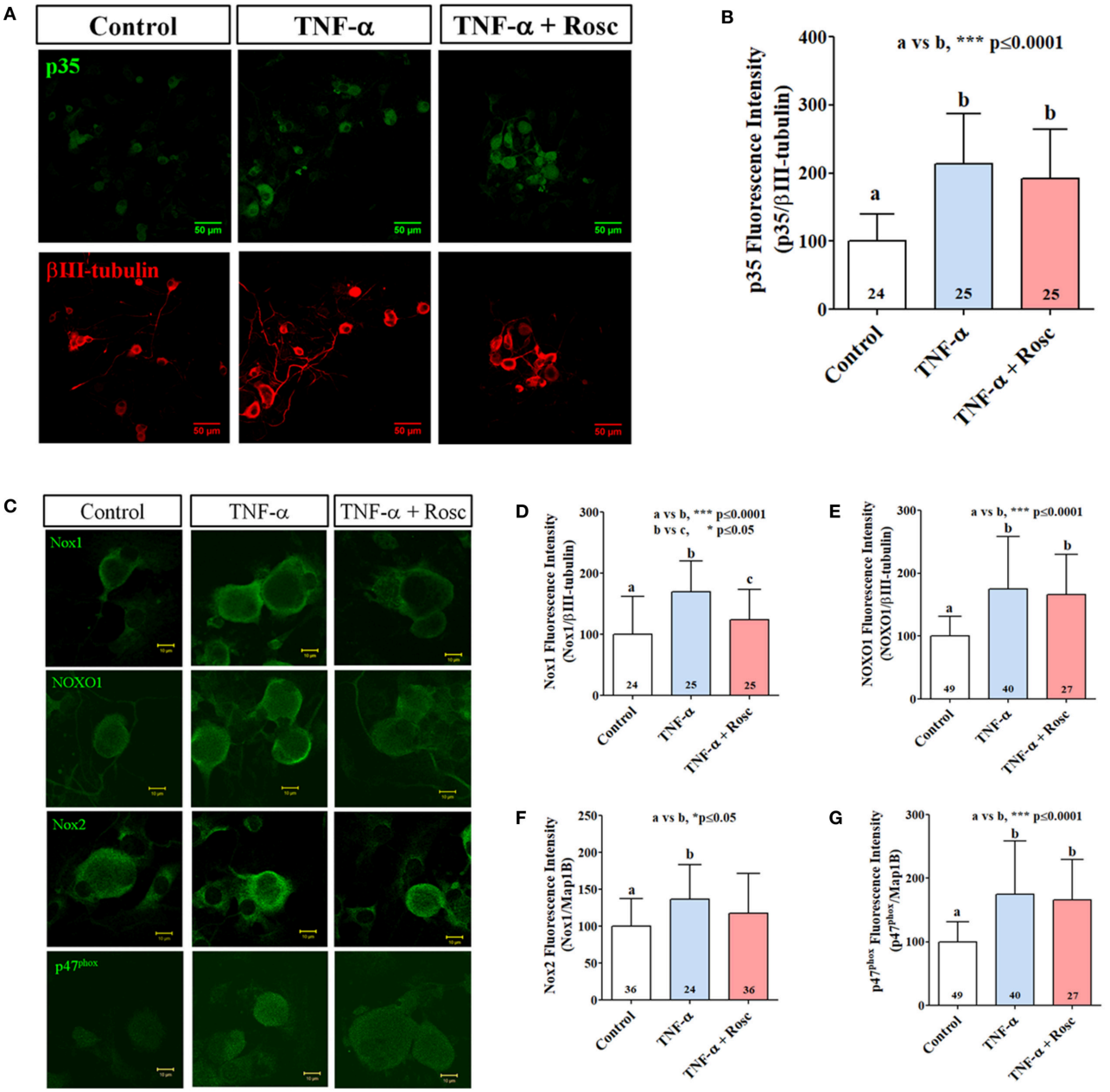

FIGURE 5 | TNF-α treatment increases p35, Nox1 and Nox2 expression in primary culture of mouse TG neurons. **(A)** Representative immunolocalization of p35 (green) and βIII-tubulin (red) from TG neurons treated with TNF-α alone or with roscovitine. Scale bars are 50 μm. **(B)** Quantification of p35 fluorescence intensity of images in **(A)**. **(C)** Representative images of Nox1, NOXO1, Nox2, and p47^{phox} endogenous expression in TG neurons treated with TNF-α alone or with roscovitine. Scale bars are 10 μm. **(D–G)** Quantification of Nox1 **(D)**, NOXO1 **(E)**, Nox2 **(F)**, and p47^{phox} **(G)** fluorescence intensity in TG neurons of images in **(C)**. The bar graphs represent mean ± *SD*. Statistical differences correspond to a one-way ANOVA with a Bonferroni's multiple comparison test. Number of analyzed neurons is inside each corresponding bar.

TNF-α Increased Expression of NOX1 and NOX2 Members in Primary Culture of Mouse TG Neurons

Because TNF-α increased ROS production in nociceptive neurons, presumably by NOX/NADPH oxidase increased activation, we analyzed the catalytic and cytoplasmic subunits of NOX1 and NOX2 complexes in primary TG cultures treated with TNF-α by immunofluorescence. First, we confirmed that TNF-α increased p35 immunodetection in primary culture of TG neurons (**Figures 5A,B**) similarly as previously reported (Rozas et al., 2016). We also found that TNF-α treatment increased immunodetection of Nox1, NOXO1, Nox2, and p47^{phox} in TG neurons. Interestingly, roscovitine treatment significantly

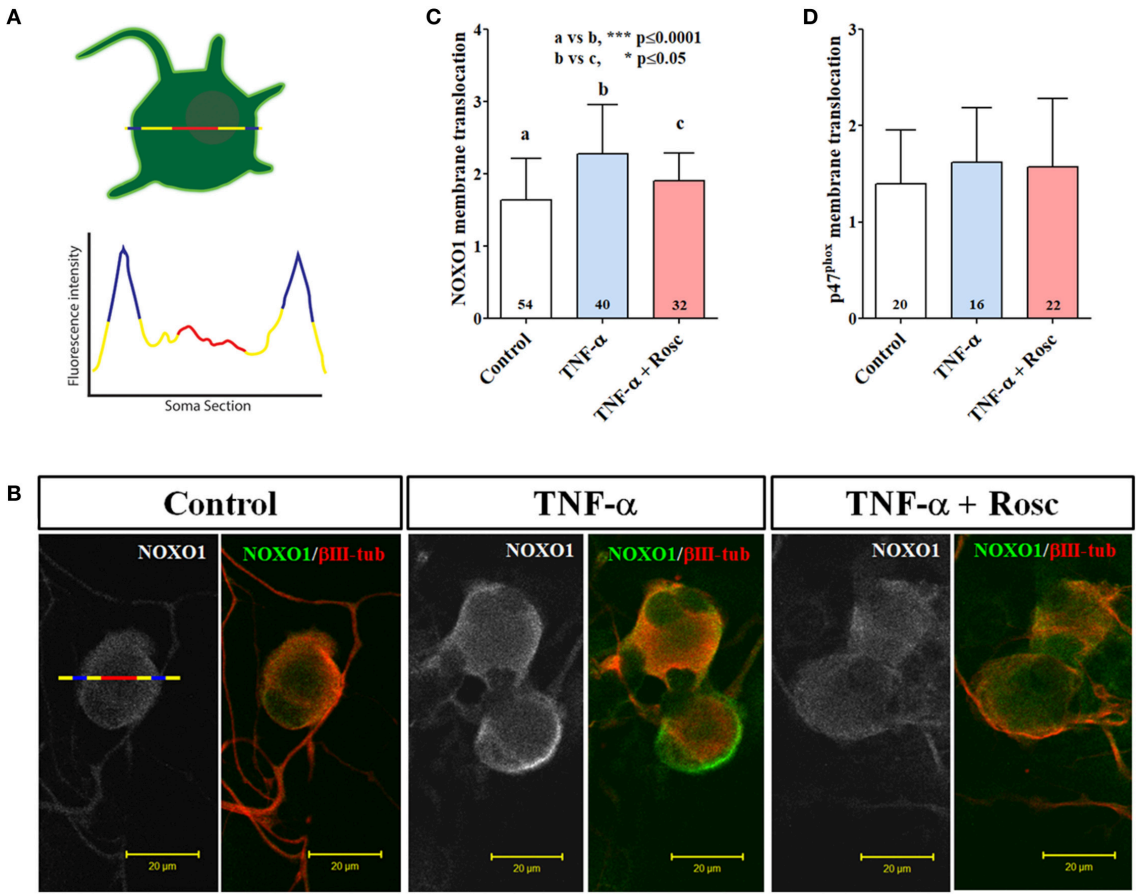

FIGURE 6 | TNF-α increases NOXO1 translocation to plasma membrane in primary culture of mouse TG neurons. **(A)** Upper panel: Descriptive sketch for measurement of plasma membrane translocation of cytosolic NOX subunits. Line represents the measurement of plasma membrane protein translocation indicating a peripheral area (blue section) followed for a centric area (red section). Lower panel: Descriptive plot profile showing measurement of cytosolic NOX along 2D neuron surface. **(B)** Representative immunofluorescences of NOXO1 (white/green) and βIII-tubulin (red) from TG neurons treated with TNF-α (50 ng/ml) alone or with roscovitine (20 mM) during 24 h. Scale bars are 20 µm. **(C,D)** Measurement of NOXO1 **(B)** and $p47^{phox}$ **(D)** translocation to plasma membrane in 2DIV primary culture of TG neurons treated with TNF-α alone or with roscovitine. Plasma membrane translocation was measured from fluorescence intensity into plasma membrane (peripheral area) of TG neurons, normalized against fluorescence intensity in the cytosolic compartment (centric area). The bar graphs represent mean ± *SD*. Statistical differences correspond to a one-way ANOVA with a Bonferroni's multiple comparison test. Number of analyzed neurons is inside each corresponding bar.

blocked TNF-α effect in the immunodetection of Nox1 in cultured TG neurons. On the other hand, immunodetection of NOXO1, Nox2, and $p47^{phox}$ was not affected by roscovitine treatment (**Figures 5C–G**).

TNF-α Increased NOXO1 Translocation to Plasma Membrane in Primary Culture of Mouse TG Neurons

Our results showed a significantly increased NOX-dependent ROS production in nociceptive neurons directed by TNF-α signaling and in part by Cdk5 activation. However, an important aspect in NOX complex activation is the recruitment of cytoplasmic subunits to plasma membrane (Debbabi et al., 2013). Therefore, we analyzed the plasma membrane translocation (**Figures 6A,B**) of cytoplasmic subunits NOXO1 and $p47^{phox}$ after TNF-α treatment by using confocal images (**Figure 5C**). These analyses revealed that NOXO1 translocation to plasma membrane was enhanced upon TNF-α treatment (**Figure 6C**). In contrast, we did not find a clear change in its distribution toward peripheral regions for $p47^{phox}$ (**Figure 6D**). Interestingly, roscovitine treatment reverted TNF-α effect on NOXO1 plasma membrane translocation (**Figures 6B,C**), which suggests a role of Cdk5 in the recruitment of NOXO1 to peripheral compartments in primary cultured nociceptive neurons.

DISCUSSION

In the present work we established for the first time an association between Cdk5 activation and ROS production directed by NOX1 and NOX2 complexes suggesting an important role during inflammatory pain (**Figure 7**). First, in HEK293 cells transfected with p35, a heterologous expression system, the activation of Cdk5 promotes hydrogen peroxide production that was reverted by pharmacological inhibition of NOX complex or

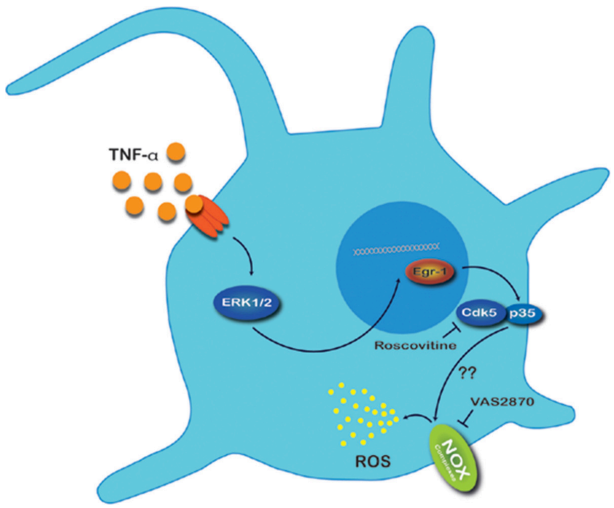

FIGURE 7 | Proposed mechanism for ROS production in nociceptive neurons exposed to TNF-α. Under an inflammatory state, primary sensory neurons are exposed to a pro-inflammatory cytokines soup. TNF-α binds to TNF-α receptor and triggers the MAPK/ERK1/2 pathway. ERK1/2 translocate to nucleus promoting the expression of the transcription factor Early growth response protein 1 (Egr1) which consecutively binds to the p35 promoter causing an increase of p35 protein expression. Activation of Cdk5 by p35 leads to phosphorylation of diverse substrates located in nociceptive neurons; we suggest that this event favors NOX complex association and consequently increases ROS production in these neurons. As ROS represent signaling molecules in pain hypersensitivity, we speculate that this redox imbalance could modify functions of other proteins involved in signal transmission of nociceptive neurons.

by inhibition of Cdk5 activity. Second, in primary culture of mouse nociceptive neurons, an endogenous expression system, TNF-α treatment increased ROS production and this effect was reverted by inhibition of Cdk5 or NOX complex. Third, TNF-α treatment increased the expression of catalytic (Nox1 and Nox2) and cytosolic (NOXO1 and $p47^{phox}$) members of NOX1 and NOX2 complexes in TG neurons. Moreover, TNF-α treatment induced NOXO1 plasma membrane translocation and roscovitine blocked this effect. Altogether these results demonstrate that Cdk5 contributes to ROS production mediated by NOX1 and NOX2 activation and suggest its involvement during inflammatory pain.

Few years ago, our group established the participation of Cdk5 in pain signaling (Pareek et al., 2006; Utreras et al., 2009a) principally after initiation of an inflammatory response (Utreras et al., 2009b, 2011, 2012; Rozas et al., 2016). In particular, Cdk5 phosphorylates many substrates important in pain such as TRPV1 (Pareek et al., 2007; Jendryke et al., 2016; Rozas et al., 2016), P2X2aR (Coddou et al., 2017), KIF13B (Xing et al., 2012), delta opioid receptors (Xie et al., 2009), among others. Cdk5 phosphorylates TRPV1 in Thr407 decreasing its activation threshold (Jendryke et al., 2016; Rozas et al., 2016). Similarly, Cdk5 phosphorylates purinergic receptor P2X2aR in Thr372 slowing desensitization of the channel (Coddou et al., 2017). Interestingly, both TRPV1 and P2X2aR are ion channels related with hypersensitization during inflammatory pain (Linley et al., 2010). On the other hand, ROS molecules play an important role during inflammatory and neuropathic pain (Kallenborn-Gerhardt et al., 2013), however, the link between ROS production and Cdk5 activation has not yet been addressed until now.

Our results showed that direct activation of Cdk5 by p35 overexpression significantly increased intracellular ROS production in HEK293 cells. Since VAS2870 treatment significantly decreases this redox imbalance, NOX complex raises as a good candidate of ROS source in HEK293 cells. Several members of NOX1 and NOX2 complexes were immunodetected in HEK293 cells, which supports the participation of these enzymes in ROS production mediated by Cdk5 activation. In addition, we detected a NOX-dependent basal production of hydrogen peroxide by HyPer sensor in these cells, because NOX inhibition with VAS2870 significantly decreased ROS production in untransfected cells. Since VAS2870 treatment did not revert Cdk5-mediated ROS levels totally, it could be explained by the activation of other sources of ROS induced by p35 overexpression. In addition, ROS production was not completely abolished in HEK293 cells transfected with p35 and treated with roscovitine, probably because Cdk5 activation by p35 overexpression overcomes the inhibition capacity of roscovitine and a fraction of Cdk5 remained active. Interestingly, this effect was not observed in primary culture of TG neurons where p35 levels were considerably smaller as compared with our heterologous expression system. Moreover, higher concentration of roscovitine could generate a toxic effect on the cells or favor non-specific inhibition of other biological pathways (Bach et al., 2005; Li et al., 2008).

We reported earlier that overexpression of TNF-α in nociceptive neurons increases p35 expression and Cdk5 activity, with a subsequent TRPV1 phosphorylation and an increment in pain signaling (Rozas et al., 2016). Here, we evaluated whether increased Cdk5 activity mediated by TNF-α enhances ROS production in nociceptive neurons. Our results showed that TNF-α treatment significantly increased ROS production in DRG and TG neurons approximately in 50% as compared with control neurons. Similar to HEK293 cells, both roscovitine and VAS2870 treatment reverted ROS production induced by TNF-α, which supports a molecular link between Cdk5 and NOX complexes function, and establishes a potential contribution of Cdk5 activation in the redox balance of nociceptive neurons. Most important, participation of NOX complexes in pain signaling has been already reported (Ibi et al., 2008; Kim et al., 2010; Im et al., 2012b; Kallenborn-Gerhardt et al., 2012; Lim et al., 2013; Kallenborn-Gerhardt et al., 2014). However, only few reports are linked to pro-inflammatory activation (Ibi et al., 2008; Lim et al., 2013). On the other hand, there is evidence that pro-inflammatory cytokines like TNF-α modulate NOX-dependent ROS production in different types of cells (Chen et al., 2008; Lin et al., 2015a; Blaser et al., 2016) including neurons (Barth et al., 2009, 2012; Kuhn, 2014). In addition, interleukin 1β and interleukin 6 have been involved in ROS production mediated by NOX enzymes in different cellular models (So et al., 2007; Kim et al., 2010; Pang et al., 2012; Kuhn, 2014). Our data suggests that increased ROS production mediated by TNF-α treatment could be explained by a higher expression of NOX1 and NOX2 members in nociceptive neurons similarly to

previous reports (Kim et al., 2007, 2010; Blaser et al., 2016). However, NOX4 represents an important source of ROS in DRG neurons contributing to pain hypersensitization (Im et al., 2012a; Kallenborn-Gerhardt et al., 2012; Ding et al., 2017), moreover regulating TRPV1 activity (Lin et al., 2015b; Ding et al., 2016). Therefore, this study cannot exclude the possibility that NOX4 participates in Cdk5-mediated ROS production.

NOX cytosolic members NOXO1 and $p47^{phox}$ can undergo post-translational modification by phosphorylation dependent of PKC (Fontayne et al., 2002; Debbabi et al., 2013) inducing NOX activation and ROS production. Interestingly, we found a minimal consensus sequence (Ser/Thr-Pro) for Cdk5 phosphorylation in mouse $p47^{phox}$ protein (Ser215 and Thr356) and a full consensus sequence for Cdk5 (Ser/Thr(Pro)X(Lis/His/Arg) (Bórquez et al., 2013) in NOXO1 protein (Ser3). Therefore, we postulate that Cdk5 upon activation could phosphorylate cytosolic subunits of NOX1 and NOX2, promoting activation and ROS production, although further experiments are needed to demonstrate such a novel regulation.

Considering this scenario, we think that Cdk5-mediated ROS production in nociceptive neurons could contribute to enhancing pain signaling by an additional mechanism. Interestingly, several receptors expressed on the surface of TG and DRG neurons are susceptible to activity modulation by cysteine oxidation, such as TRPV1, transient receptor potential ankyrin 1 (TRPA1), N-methyl-d-aspartate (NMDA) receptors, and T-type Ca^{2+} channels, among others (Gamper and Ooi, 2015). However additional experiments are needed to demonstrate the real impact of Cdk5-mediated ROS production in the nociceptive circuitry including the central nervous system. In summary, taken together our results suggest that Cdk5 activation may be implicated in the ROS production by NOX1 and NOX2 complexes during inflammatory pain and this relationship could address additional roles to Cdk5 in pain signaling.

AUTHOR CONTRIBUTIONS

RS, CG-B, and EU designed the project. RS, PL, FF, NP, and EU performed the experiments. RS, PL, FF, CG-B, and EU analyzed the data. RS, CG-B, and EU wrote the manuscript. EU supervised the experiments and finalized the manuscript.

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frontiers in Physiology

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Pathophysiological Role of Transient Receptor Potential Ankyrin 1 in a Mouse Long-Lasting Cystitis Model Induced by an Intravesical Injection of Hydrogen Peroxide

Shohei Oyama[1], Koji Dogishi[1], Mizuki Kodera[1], Masashi Kakae[1], Kazuki Nagayasu[1], Hisashi Shirakawa[1], Takayuki Nakagawa[2] and Shuji Kaneko[1]*

[1] Department of Molecular Pharmacology, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan,
[2] Department of Clinical Pharmacology and Therapeutics, Kyoto University Hospital, Kyoto, Japan

Chronic inflammatory bladder disorders, such as interstitial cystitis/bladder pain syndrome, are associated with poor quality of life. The exact pathological processes remain unclear, but accumulating evidence suggests that reactive oxidative species (ROS) are involved in urinary bladder disorders. Transient receptor potential ankyrin 1 (TRPA1), the most sensitive TRP channel to ROS, was shown to be responsible for urinary bladder abnormalities and hyperalgesia in an acute cystitis model. However, the roles of TRPA1 in chronic inflammatory bladder are not fully understood. We previously established a novel mouse cystitis model induced by intravesical injection of hydrogen peroxide (H_2O_2), resulting in long-lasting frequent urination, bladder inflammation, pain-related behavior, and histopathological changes. In the present study, we investigated the pathophysiological role of TRPA1 in the H_2O_2-induced long-lasting cystitis mouse model. Under anesthesia, 1.5% H_2O_2 solution was introduced transurethrally into the bladder of female wild-type (WT) and TRPA1-knockout mice and maintained for 30 min. This increased the number of voids in WT mice at 1 and 7 days after injection, but reduced the number in TRPA1-knockout mice at 1 day but not 7 days after injection. Spontaneous locomotor activities (increase in freezing time and decrease in distance moved) were reduced at 3 h after injection in WT mice, whereas the spontaneous visceral pain-related behaviors were attenuated in TRPA1-knockout mice. Furthermore, upregulation of *c-fos* mRNA in the spinal cord at 1 day after injection was observed in WT but not TRPA1-knockout mice. However, there was no difference in histopathological changes in the urinary bladder, such as edematous thickening in the submucosa, between WT and TRPA1-knockout mice at 1 or 7 days after injection. Finally, *Trpa1* mRNA levels in the L5-S1 dorsal root ganglion were not altered, but levels in the urinary bladder were drastically increased at 1 and 7 days after injection. Taken together, these results suggest that TRPA1 contributes to acute bladder hyperactivity such as frequent urination and bladder pain, but does not appear to play a major role in the pathological processes of long-lasting cystitis.

Keywords: TRPA1, cystitis, hydrogen peroxide, mouse model, chronic inflammatory bladder, gene knockout

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Mustafa Naziroglu,
Süleyman Demirel University, Turkey

Reviewed by:
Felipe Simon,
Universidad Andrés Bello, Chile
Marcelo Catalan,
Arturo Prat University, Chile

***Correspondence:**
Takayuki Nakagawa
tknakaga@kuhp.kyoto-u.ac.jp

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Oyama S, Dogishi K, Kodera M, Kakae M, Nagayasu K, Shirakawa H, Nakagawa T and Kaneko S (2017) Pathophysiological Role of Transient Receptor Potential Ankyrin 1 in a Mouse Long-Lasting Cystitis Model Induced by an Intravesical Injection of Hydrogen Peroxide. Front. Physiol. 8:877. doi: 10.3389/fphys.2017.00877

INTRODUCTION

Lower urinary tract symptoms, such as urinary frequency, urgency, nocturia, and abdominal visceral pain, lead to impaired quality of life. These symptoms are features of chronic inflammatory bladder disorders including interstitial cystitis/bladder pain syndrome (IC/BPS). Many hypotheses about the pathogenesis of chronic cystitis have been proposed, such as urothelial dysfunction, inflammation, neural hyperactivity, an autoimmune response, and toxic urinary agents (Homma et al., 2016). However, the exact pathological processes involved remain unclear.

Accumulating evidence suggests that reactive oxidative species (ROS) contribute to bladder disorders. Excess ROS are a feature of various bladder pathological conditions including bladder outlet obstruction (Lin et al., 2005), bladder ischemia/reperfusion (Yu et al., 2004), and bladder inflammation models (Chien et al., 2003). Furthermore, ROS are abundantly produced by inflammatory cells such as macrophages, neutrophils, and mast cells when they infiltrate an inflamed bladder (Brooks et al., 1999; Winterbourn, 2002; Chien et al., 2003; Ndengele et al., 2005). ROS induce bladder hyperactivity by activating capsaicin-sensitive C-fiber afferent pathways (Masuda et al., 2008; Nicholas et al., 2017). In cyclophosphamide- or ifosfamide-induced acute cystitis animal models, the metabolite acrolein enters the urothelium and causes bladder inflammation, which is prevented by ROS scavengers or antioxidants (Yildirim et al., 2004; Batista et al., 2007; Song et al., 2014). In addition, a human study demonstrate that the serum total antioxidant capacity in IC/BPS patients is lower than that in healthy controls (Ener et al., 2015). Thus, it is likely that ROS play a critical role in the etiology and/or pathology of chronic cystitis.

Transient receptor potential ankyrin 1 (TRPA1), a non-selective cation channel, is highly expressed in a subset of nociceptive C-fibers where it acts as a polymodal nociceptor (Wu et al., 2010). TRPA1 is activated by various irritants and oxidative stimuli including ROS, and contributes to nociceptive and inflammatory pain generation (Jordt et al., 2004; Bautista et al., 2006; Andersson et al., 2008; Sawada et al., 2008). In the lower urinary tract, TRPA1 is expressed in the urothelium or detrusor of the urinary bladder in addition to the C-fibers (Du et al., 2008; Streng et al., 2008). This is because intravenous administration of a TRPA1 antagonist does not alter the voiding function, while intravesical infusion of a TRPA1 agonist increases the micturition frequency (Streng et al., 2008; Minagawa et al., 2014), indicating that TRPA1 does not play a major role in bladder function under physiological conditions. By contrast, in a cyclophosphamide-induced cystitis model, bladder hyperalgesia, and voiding frequency are caused by activation of TRPA1 (Meotti et al., 2013; DeBerry et al., 2014). Moreover, human studies reveal that *Trpa1* mRNA levels in the urinary bladder are markedly elevated in patients with IC/BPS (Homma et al., 2013) and bladder outlet obstruction (Du et al., 2008). Thus, it is likely that ROS-sensitive TRPA1 may play a key role in the pathogenesis or pathology of chronic cystitis, although this is not fully understood at present.

We previously established a novel long-lasting cystitis mouse model by intravesical injection of hydrogen peroxide (H_2O_2) (Homan et al., 2013). The H_2O_2-induced long-lasting cystitis model is characterized by long-lasting frequent urination, bladder inflammation, pain-related behavior, and histopathological changes (Homan et al., 2013; Dogishi et al., 2015). In the present study, we investigated the pathophysiological roles of TRPA1 in the H_2O_2-induced long-lasting cystitis model using TRPA1-knockout (KO) mice.

MATERIALS AND METHODS

Animals

All experiments were performed according to the ethical guidelines recommended by the Kyoto University Animal Research Committee. The protocol was approved by the Kyoto University Animal Research Committee (permit number: 2015–40, 2016–40). *Trpa1*$^{+/+}$ (wild-type; WT) and *Trpa1*$^{-/-}$ (TRPA1-KO) mice lines were bred from heterozygous mice with a C57BL/6 × 129 S1 background that were obtained from Jackson Laboratory (Bar Harbor, ME). Mouse lines were backcrossed to C57BL/6 J mice for at least 10 generations, and genotyped by genomic PCR using primers 5′-tcatctgggcaacaatgtcacctgct-3′ and 5′-tcctgcaagggtgattgcgttgtcta-3′. Female WT and TRPA1-KO mice aged between 5 and 6 weeks old were used, while female C57BL/6 J mice of the same age were purchased from Japan SLC (Shizuoka, Japan) and used in some experiments. All mice were housed under constant ambient temperature (24 ± 1°C) and humidity (55 ± 20%), with an alternating 12 h light/dark cycle (lights came on automatically at 8:00 a.m.). Food and water were freely available.

H_2O_2-Induced Cystitis Model

The H_2O_2-induced cystitis model was generated as previously reported (Homan et al., 2013). Briefly, under 2–3% isoflurane (Pfizer, NY) anesthesia, a polyethylene tube (PE-10; Clay-Adams, Parsippany, NJ) was introduced into the bladder transurethrally and the lower abdomen was pressed gently to withdraw urine. Next, 50 μL of 1.5% H_2O_2 solution (Wako Pure Chemical Industries, Osaka, Japan) in sterile saline was introduced into the bladder through the catheter. The H_2O_2 solution was drained from the bladder after 30 min by pressing the lower abdomen.

Measurement of the Number of Voids and Spontaneous Locomotor Activities

Mice were kept in an individual plastic cage (10 × 20 × 30 cm: width × length × height) lined with filter paper (Advantec Chromatography Paper No. 50; Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and allowed to acclimate for 30 min before experiments. After replacing the filter paper, the mouse was videotaped for 15 min, and the number of voids was quantified from the videotape by counting urine spots on the filter paper. Subsequently, freezing time and move distance were analyzed using the ANY-maze video tracking system (Stoelting Co., Wood Dale, IL).

Histological Examination

Mice were deeply anesthetized with 64.8 mg/kg sodium pentobarbital (Kyoritsu Seiyaku Co., Tokyo, Japan) and perfused

transcardially with potassium-free phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Bladders were removed, postfixed overnight in 4% paraformaldehyde, and embedded in paraffin (Sakura Finetek Japan, Tokyo, Japan). Paraffin-embedded tissues were cut into 5 μm sections and stained with hematoxylin and eosin (HE) using standard procedures. Histopathological examination was performed with a light microscope (BX-53F; OLYMPUS, Tokyo, Japan).

Real-Time RT-PCR

Anesthetized mice were perfused transcardially with PBS and bladders, the L5-S1 dorsal root ganglion (DRG), and the L5-S1 spinal cord were removed, flash-frozen in liquid nitrogen, and stored at −80°C until use. Total RNA was isolated from tissues with ISOGEN reagent (Nippon Gene, Tokyo, Japan), and cDNAs were synthesized with a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Real-time quantitative PCR was performed using the StepOne real-time PCR system (Life Technologies, Carlsbad, CA) with 20 μL reactions containing 1 μg of total RNA and the THUNDERBIRD SYBR qPCR Mix (Toyobo). Oligonucleotide primer pairs for 18S rRNA (5′-GCAATTATTCCCCATGAACG-3′ and 5′-GGCCTCACTAAACCATCCAA-3′), *Trpa1* (5′-TGA GATCGACCGGAGT-3′ and 3′-TGCTGAAGGCATCTTG-5′), *c-fos* (5′-CCGAAGGGAACGGAAT-3′ and 3′-TGCAACGCA GACTTCT-5′), glutathione peroxidase 1 (GPx1; 5′-GTTTCC CGTGCAATCAGTTC-3′ and 3′-CAGGTCGGACGTACTTGA GG-5′), and catalase (5′-GCGGATTCCTGAGAGAGTGG-3′ and 3′-TGTGGAGAATCGAACGGCAA-5′) were used. The results for each gene were normalized relative to 18S rRNA levels measured in parallel in each sample.

Statistical Analysis

Data are expressed as means ± S.E.M. Statistical analysis was performed with the GraphPad Prism 6 program (GraphPad Software, La Jolla, CA). Unpaired *t*-tests or Mann-Whitney *U*-tests were used to determine mRNA expression levels. The number of voids, freezing time, and distance moved were analyzed with two-way ANOVA, followed by the Tukey *post-hoc* test. In all cases, statistical significance was defined by a p-value < 0.05.

RESULTS

Effect of TRPA1 Deletion on the Number of Voids in H_2O_2-Induced Cystitis Mice

The number of voids was measured in WT and TRPA1-KO mice at 1 and 7 days after intravesical injection of saline (controls) or H_2O_2. Consistent with our previous report (Homan et al., 2013), an intravesical injection of 1.5% H_2O_2 significantly increased the number of voids 1 day after injection [$F_{(1, 69)} = 71.82$, $p < 0.001$]. This increase was significantly suppressed in TRPA1-KO mice [$F_{(1, 69)} = 12.30$, $p < 0.001$]. Both H_2O_2-injected WT and TRPA1-KO groups exhibited a significant increase in the number of voids compared with saline-injected WT and TRPA1-KO groups, respectively, and the number of voids in H_2O_2-injected TRPA1-KO mice was significantly lower than in H_2O_2-injected WT mice (**Figure 1A**). At 7 days after injection, the number of voids was significantly increased in H_2O_2-injected groups [$F_{(1, 67)} = 16.31$, $p < 0.001$], with significant increases observed in both WT and TRPA1-KO mice. However, there was no significant difference between WT and TRPA1-KO H_2O_2-injected groups [$F_{(1, 67)} = 0.1099$, $p = 0.7413$; **Figure 1B**].

Effect of TRPA1 Deletion on Visceral Pain-Related Behaviors in H_2O_2-Induced Cystitis Mice

Reduced spontaneous locomotor activity in rodents is considered evidence of visceral pain-related behavior, as previously reported

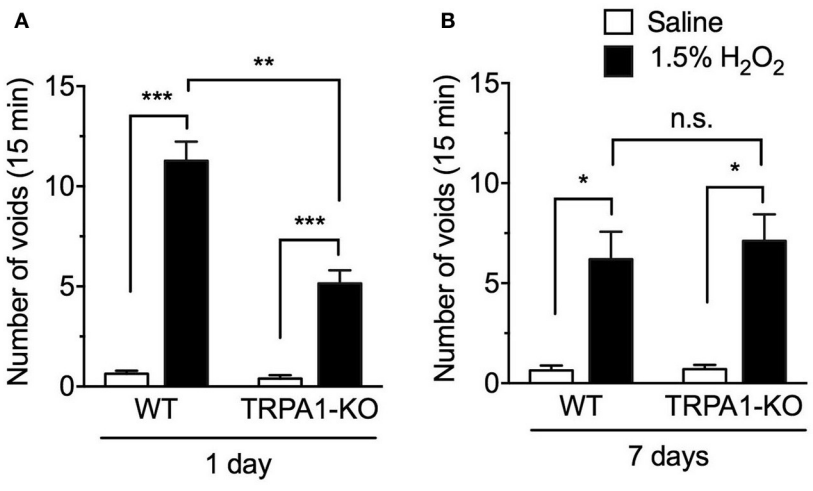

FIGURE 1 | Number of voids in H_2O_2-injected wild-type (WT) and TRPA1-KO mice. WT or TRPA1-KO mice were injected intravesically with saline or 1.5% H_2O_2. At 1 day **(A)** and 7 days **(B)** after injection, the number of voids was counted in a 15 min period. Values are means ± S.E.M. for each group of 10–27 mice. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (n.s., not significant).

in a cyclophosphamide-induced cystitis mouse model (Miki et al., 2011). We previously reported a decrease in spontaneous locomotor behavior at only 3 h after intravesical H_2O_2 injection (Dogishi et al., 2015). In the present study, to examine the effect of TRPA1-KO on visceral pain-related behavior, spontaneous locomotor activities including freezing time and distance moved were analyzed over a 15 min period in freely moving WT and TRPA1-KO mice at 3 h after H_2O_2 injection. Intravesical H_2O_2 injection significantly increased freezing time [$F_{(1, 39)} = 8.323$, $p < 0.01$] and reduced the distance moved [$F_{(1, 39)} = 4.717$, $p < 0.05$] in WT but not in TRPA1-KO mice. TRPA1 deficiency significantly reduced freezing time [$F_{(1, 39)} = 11.23$, $p < 0.01$]. The freezing time in WT mice was significantly increased in the H_2O_2-injected group compared with the saline-injected control group, but a significant increase was not observed in H_2O_2-injected TRPA1-KO mice. Furthermore, the freezing time in H_2O_2-injected TRPA1-KO mice was significantly shorter than that in H_2O_2-injected WT mice (**Figure 2A**).

Similarly, H_2O_2-injected WT mice displayed a significant decrease in the distance moved compared with saline-injected WT mice. In TRPA1-KO mice, no significant difference was observed between saline- and H_2O_2-injected groups, and moving distance in the H_2O_2-injected group was increased compared with H_2O_2-injected WT mice, but not significantly (**Figure 2B**).

Effect of TRPA1 Deletion on Upregulation of *c-fos* mRNA in the Spinal Cord of H_2O_2-Induced Cystitis Mice

Activation of bladder sensory neurons responsible for bladder hyperactivity and pain-related behaviors is correlated with the induction of *c-fos* mRNA expression, an immediate early gene, in the spinal cord (Avelino et al., 1999; Dinis et al., 2004). To determine whether TRPA1 deletion affects neuronal activity in the spinal cord caused by H_2O_2-induced cystitis, *c-fos* mRNA levels in the L5-S1 spinal cord, the area of termination of most bladder afferents (Nadelhaft and Booth, 1984), were examined 1 day after intravesical saline or H_2O_2 injection. In WT mice, H_2O_2 injection caused a significant upregulation in the relative expression of *c-fos* mRNA compared with the saline-injected control group. By contrast, in TRPA1-KO mice, there was no significant difference between saline- and H_2O_2-injected groups (**Figure 3**).

Effect of TRPA1 Deletion on Histopathological Changes in the Bladder of H_2O_2-Induced Cystitis Mice

Cystitis induced by intravesical H_2O_2 injection was histopathologically examined by HE staining of the bladder of WT and TRPA1-KO mice. In H_2O_2-injected mice, severe edematous thickening in the submucosa was observed compared with the saline-injected control group at 1 day after injection, which was partially alleviated by 7 days after injection in both WT and TRPA1-KO mice. There was no difference in histopathological changes between WT and TRPA1-KO mice (**Figure 4**).

Expression of *Trpa1* mRNA in the Bladder and DRG of H_2O_2-Induced Cystitis Mice

The effects of H_2O_2 injection on *Trpa1* mRNA levels in the urinary bladder and L5-S1 DRG were examined. Intravesical H_2O_2 injection drastically elevated the relative expression levels of *Trpa1* mRNA in the bladder on day 1 and 7. By contrast, there were no differences in the expression levels of *Trpa1* mRNA in the L5-S1 DRG between saline- and H_2O_2-injected groups at 1 and 7 days after injection (**Figure 5**).

DISCUSSION

In the present study, using an intravesical H_2O_2-induced long-lasting cystitis mouse model (Homan et al., 2013), we showed that TRPA1 is involved in initial bladder hyperactivity, but apparently not in the pathological processes involved in long-lasting cystitis,

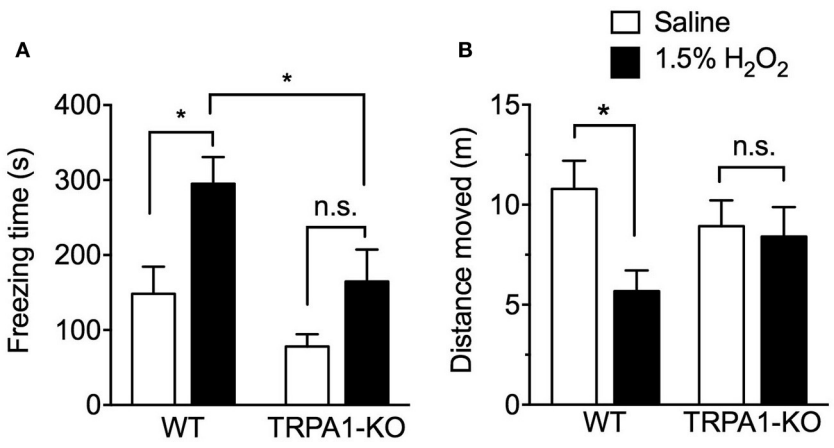

FIGURE 2 | Spontaneous locomotor activities in H_2O_2-injected WT and TRPA1-KO mice. WT or TRPA1-KO mice were injected intravesically with saline or 1.5% H_2O_2. At 3 h after injection, freezing time **(A)** and distance moved **(B)** were measured in a 15 min period. Values are means ± S.E.M. for each group of 10–13 mice. *$p < 0.05$ (n.s., not significant).

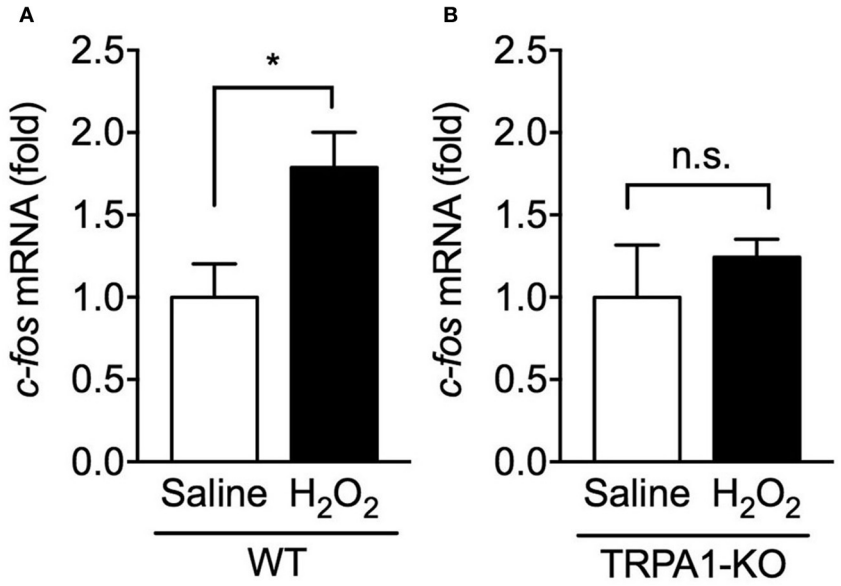

FIGURE 3 | Expression levels of *c-fos* mRNA in the spinal cord of H_2O_2-injected WT and TRPA1-KO mice. WT **(A)** or TRPA1-KO **(B)** mice were injected intravesically with saline or 1.5% H_2O_2. At 1 day after injection, the L5-S1 spinal cord was removed, and *c-fos* mRNA levels were measured by real-time RT-PCR. The values were normalized against 18S rRNA mRNA levels and presented relative to those of saline-injected mice (set as 1). Values are means ± S.E.M. for each group of 5–14 mice. $^*p < 0.05$ (n.s., not significant).

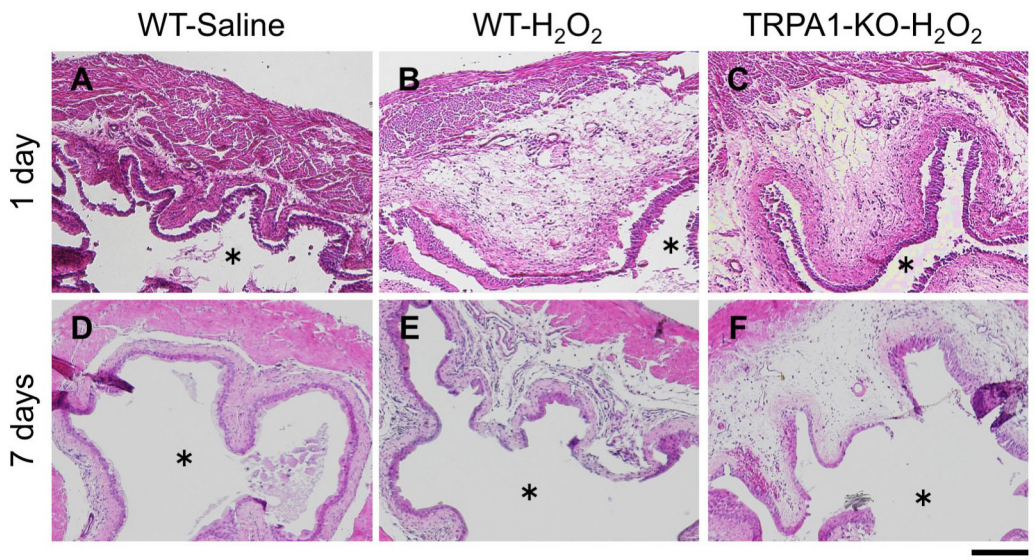

FIGURE 4 | Histopathological examination of the bladders of H_2O_2-injected WT and TRPA1-KO mice. WT **(A,B,D,E)** or TRPA1-KO **(C,F)** mice were injected intravesically with saline **(A,D)** or 1.5% H_2O_2 **(B,C,E,F).** At 1 day **(A–C)** and 7 days **(D–F)** after injection, bladders were removed and fixed, and tissue sections (5 μm) were stained with hematoxylin and eosin. Asterisks indicate the bladder lumen. Scale bar = 200 μm.

since (1) TRPA1 deletion reduced the initial increase in the number of voids and the decrease in spontaneous locomotor behaviors, which were accompanied by a reduction in *c-fos* mRNA upregulation in the spinal cord; (2) TRPA1 deletion had no effect on the delayed frequent urination; (3) TRPA1 deletion had no effect on histopathological changes in the urinary bladder at 1 or 7 days after injection. Furthermore, we found that *Trpa1* mRNA levels in the urinary bladder were drastically increased at 1 and 7 days after H_2O_2 injection, but levels were not altered in the DRG.

We confirmed that an intravesical injection of H_2O_2 produced long-lasting frequent urination, visceral pain-related behaviors,

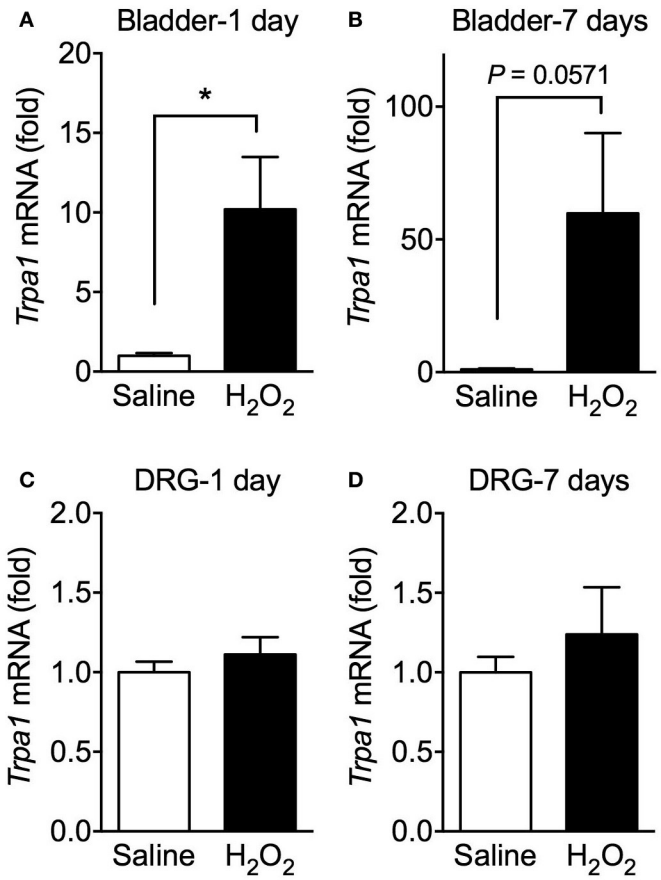

FIGURE 5 | Expression levels of *Trpa1* mRNA in the urinary bladder and DRG of H_2O_2-injected mice**.** WT mice were injected intravesically with saline or 1.5% H_2O_2. At 1 day **(A,C)** or 7 days **(B,D)** after injection, bladders and the L5-S1 bilateral DRG were removed and *Trpa1* mRNA levels in the bladder **(A,B)** and DRG **(C,D)** were measured by real-time RT-PCR. The values were normalized against 18S rRNA mRNA levels and presented relative to those of the saline-injected group (set as 1). Values are means ± S.E.M. for each group of 3–6 mice. $^*p < 0.05$.

and bladder inflammation, as previously reported (Homan et al., 2013; Dogishi et al., 2015). Since H_2O_2 can activate TRPA1 (Andersson et al., 2008; Sawada et al., 2008), it is conceivable that H_2O_2 injected intravesically could directly stimulate TRPA1 in the bladder, leading to the generation of long-lasting cystitis. However, the present findings showed no apparent differences in histopathological changes in the urinary bladder between WT and TRPA1-KO mice injected with H_2O_2. Thus, direct stimulation of bladder TRPA1 by exogenous H_2O_2 appears not to play a major role in the induction of cystitis. Since the H_2O_2 solution was immediately drained from the bladder at 30 min after injection, and because H_2O_2 remaining in the bladder was rapidly degraded, H_2O_2-induced cystitis appears to be caused by non-selective insults to the bladder wall, probably by lipid peroxidation, protein oxidation, and DNA damage, as we discussed previously (Homan et al., 2013). In addition, we confirmed that TRPA1 deletion had no effects on the mRNA expression levels of antioxidant enzymes, GPx1, and catalase (Supplementary Figure 1).

The present behavioral experiments revealed that initial bladder hyperactivity, including frequent urination and visceral pain-related behaviors, was mediated, at least in part, through TRPA1 activation. In the lower urinary tract, sensations in the urinary bladder are conveyed to the spinal cord through primary sensory afferent neurons consisting of two types of fibers; myelinated (Aδ) and unmyelinated (C). It is well-known that C-fibers respond to noxious stimuli, while Aδ-fibers respond to bladder filling under physiological conditions (Fowler et al., 2008). Several pieces of evidence suggest that intravesical resiniferatoxin- or capsaicin-induced desensitization of C-fibers results in an increased bladder capacity and reduced bladder pain perception through inactivation of spinal cord neurons in an animal model of acute cystitis (Dinis et al., 2004; Saitoh et al., 2009). Taken together with the present results showing the loss of *c-fos* mRNA upregulation in the spinal cord of H_2O_2-injected TRPA1-KO mice, this suggests that activation of C-fibers through TRPA1 stimulation enhances the activity of spinal cord neurons, resulting in frequent urination and abdominal visceral pain during the initial phase of long-lasting cystitis.

Acute damage to bladder urothelial cells induced by exogenous H_2O_2 injection causes hyperpermeability of the urothelial barrier (Homan et al., 2013). Thus, the submucosa is exposed to irritants in the urine, which may activate TRPA1 expression on the bladder terminal of C-fibers. Alternatively, H_2O_2-induced acute inflammation of the bladder may be accompanied by bladder vascular hyperpermeability and infiltration of abundant inflammatory cells, including neutrophils, into the submucosa (Homan et al., 2013; Dogishi et al., 2017). Several lines of evidence suggest that ROS produced from infiltrated inflammatory cells contribute to bladder hyperactivity (Chien et al., 2003; Masuda et al., 2008). Consequently, it is conceivable that excessive ROS produced from infiltrated inflammatory cells in the submucosa may activate TRPA1. Under severe initial bladder inflammation, it is possible that TRPA1 may be sensitized to ROS by various inflammatory mediators (Gouin et al., 2017).

By contrast, TRPA1 appears not to play a major role during the latter stages of long-lasting cystitis. At 7 days after intravesical H_2O_2 injection, frequent urination was partially alleviated, although it still persisted, and the decrease in spontaneous locomotor behaviors ceased, as reported previously (Homan et al., 2013; Dogishi et al., 2015). The observed severe edematous thickening of the submucosa was partially alleviated by 7 days after injection. Furthermore, we previously reported that the urothelial damage and hyperpermeability are recovered within several days, while bladder inflammation, such as accumulation of inflammatory cells and increased expression of inflammatory cytokines, persisted (Homan et al., 2013). Under such long-lasting inflammatory bladder conditions, excessive ROS production and/or sensitization of TRPA1 to ROS in the bladder may be recovered. We previously reported that bladder tissue remodeling, such as hyperplasia of the urothelium, vascularization, and fibrosis, is induced in the late phase of long-lasting cystitis (Homan et al., 2013; Dogishi et al., 2017). In addition to hyperactivity of bladder sensory neurons induced by long-lasting inflammation, bladder structural changes may affect the micturition function, leading to frequent urination. However, it is difficult to perform cystometric analysis in the present mouse cystitis model, although we could measure intercontraction interval and intravesical pressure in intravesical H_2O_2-induced rat cystitis model (Dogishi et al., 2017). Such technical problems by using genetically-modified mice limit to analyze the urodynamics in the mouse cystitis model. Further detailed investigations including cystometry will be needed to elucidate the roles of TRPA1 in the long-lasting bladder hyperactivity.

Recent evidence suggests that activation of TRPA1 may cause and/or enhance neurogenic inflammation (Gouin et al., 2017). However, the present results suggest that TRPA1 is not responsible for the occurrence and maintenance of bladder inflammation. Consistently, a TRPA1 antagonist attenuates visceral nociception in an ifosfamide-induced cystitis model, although ifosfamide-induced bladder inflammation is not suppressed (Pereira et al., 2013).

In the lower urinary tract, TRPA1 is expressed in both C-fibers and the bladder epithelium (Streng et al., 2008; Wu et al., 2010). This raises the question of which sites expressing TRPA1 are associated with initial bladder hyperactivity. In the present study, we found that *Trpa1* mRNA levels were drastically upregulated in the urinary bladder from the initial to the late phases, but not in the L5-S1 DRG. Consistent with these findings, upregulation of *Trpa1* mRNA was reported in the urinary bladder of patients with bladder outlet obstruction or IC/BPS (Du et al., 2008; Homma et al., 2013), suggesting that upregulation of TRPA1 expression in the urinary bladder may be pathologically correlated with bladder disorders. Given these expression changes, it is possible that TRPA1 expressed in the urinary bladder, rather than in the DRG, may be responsible for initial bladder hyperactivity. However, this interpretation may be a hasty judgement because the involvement of TRPA1 was observed only during the initial phase, but not in the late phase, although upregulation of *Trpa1* mRNA persisted until at least 7 days after injection. Under inflammatory conditions, the sensitivity of TRPA1 in the DRG is reportedly enhanced without changes in expression levels, and this allegedly contributes to hyperalgesia (Zhou et al., 2013). Thus, functional sensitization of TRPA1 expressed in the DRG may contribute to initial bladder hyperactivity, including frequent urination and visceral pain-related behaviors. Further investigation is therefore required to identify the sites of TRPA1 expression responsible for the pathology of long-lasting cystitis.

In conclusion, the present study revealed that TRPA1 contributes to initial bladder hyperactivity, affecting the frequency of urination and abdominal visceral pain, but it does not appear to play a major role in the pathology of long-lasting cystitis. Therapeutic strategies targeting TRPA1 may be effective for minimizing bladder hyperactivity in acute cystitis, but its usefulness for chronic cystitis may be limited.

AUTHOR CONTRIBUTIONS

SO, TN, and SK designed the project. SO, KD, MKo and MKa performed the experiments. SO, KD, MKo, HS, KN, and TN analyzed the data. SO and TN wrote the manuscript. SK supervised the experiments and finalized the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2017.00877/full#supplementary-material

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REVIEW
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Molecular Surgery Concept from Bench to Bedside: A Focus on TRPV1+ Pain-Sensing Neurons

László Pecze[1], Béla Viskolcz[2] and Zoltán Oláh[2,3]*

[1] Unit of Anatomy, Department of Medicine, University of Fribourg, Fribourg, Switzerland, [2] Institute of Chemistry, Faculty of Materials Science and Engineering, University of Miskolc, Miskolc, Hungary, [3] Acheuron Ltd., Szeged, Hungary

"Molecular neurosurgery" is emerging as a new medical concept, and is the combination of two partners: (i) a molecular neurosurgery agent, and (ii) the cognate receptor whose activation results in the selective elimination of a specific subset of neurons in which this receptor is endogenously expressed. In general, a molecular surgery agent is a selective and potent ligand, and the target is a specific cell type whose elimination is desired through the molecular surgery procedure. These target cells have the highest innate sensitivity to the molecular surgery agent usually due to the highest receptor density being in their plasma membrane. The interaction between the ligand and its receptor evokes an overactivity of the receptor. If the receptor is a ligand-activated non-selective cation channel, the overactivity of receptor leads to excess Ca^{2+} and Na^{+} influx into the cell and finally cell death. One of the best known examples of such an interaction is the effect of ultrapotent vanilloids on TRPV1-expressing pain-sensing neurons. One intrathecal resiniferatoxin (RTX) dose allows for the receptor-mediated removal of TRPV1+ neurons from the peripheral nervous system. The TRPV1 receptor-mediated ion influx induces necrotic processes, but only in pain-sensing neurons, and usually within an hour. Besides that, target-specific apoptotic processes are also induced. Thus, as a nano-surgery scalpel, RTX removes the neurons responsible for generating pain and inflammation from the peripheral nervous system providing an option in clinical management for the treatment of morphine-insensitive pain conditions. In the future, the molecular surgery concept can also be exploited in cancer research for selectively targeting the specific tumor cell.

Keywords: TRPV1, vanilloids, capsaicin, resiniferatoxin, sensory neurons, necrosis, apoptosis

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****Correspondence:***
Laszlo Pecze
laszlo.pecze@unifr.ch

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THE CONCEPT OF MOLECULAR SURGERY AND RELATED TECHNOLOGIES

Our goal with this review is to summarize the basics behind "molecular surgery," a new concept of biomedical technology, which have prototyped with the vanilloid receptor type 1 (TRPV1) target. Currently, resiniferatoxin (RTX) is the number 1 drug candidate to implement the "molecular neurosurgery" technology at cellular levels. To demonstrate the safety and efficacy of the molecular

Abbreviations: RTX, resiniferatoxin; CAP, capsaicin; TG, trigeminal ganglia; DRG, dorsal root ganglia; FDA, Food and Drug Administration; *NIH,* National Institutes of Health; PAM, positive allosteric modulator; CGRP, Calcitonin Gene Related Peptide; SP, Subtance P; B2B, bench-to-bedside.

neurosurgery, Dr. Michael J. Iadarola and Dr. Zoltan Olah have initiated a bench-to-bedside (B2B) project in 2000 that has recently entered Phase II (Brown D. C. et al., 2015). Headed by clinicians, the bedside team has already recruited more than 30 cancer pain patients in the clinical trial, which represents a novel and unique treatment option in clinical management of morphine-insensitive cancer pain (Brown D. C. et al., 2015).

The current clinical protocol prescribes a single dose of intrathecal RTX, to treat pain with an agonist of TRPV1 channel. TRPV1 was molecularly identified in 1997 (Caterina et al., 1997). TRPV1 belongs to the diverse transient receptor potential (TRP) family of non-selective cation channels (Benemei et al., 2015). The human TRP superfamily is composed of 27 members which are grouped into six subfamilies based on their amino acid sequence homology: canonical (C), vanilloid (V), melastatin (M), polycystin (P), mucolipin (ML), and ankyrin (A). They all share the common feature of six transmembrane domains and permeability to cations (Montell, 2005).

TRPV1 is dominantly expressed at the source of the inflammatory pain signals (Patapoutian et al., 2009), thus agonist-mediated removal of specific TRPV1+ inflammatory pain-sensing neurons, or "acheurons," as we call them, can manage even severe chronic pain situations. The detailed mechanism, as it has been revealed, is the RTX-induced cytotoxicity, which exploits the high specificity and affinity of this exovanilloid to TRPV1. This induces a subsequent flux of Ca^{2+} and Na^{+} ions into the acheurons (Olah et al., 2001; Karai et al., 2004; Pecze et al., 2013).

One of the preferred goals of the clinical trial, started in 2008 in the National Institutes of Health (NIH, Bethesda, Maryland), is to manage morphine-insensitive, and subsequently unbearable pain cases that are currently an unmet medical need. The trial also will provide evidence on better end-of-life quality and palliative care of cancer patients and deliver the proof of concept of molecular neurosurgery: only TRPV1+ acheurons, a verified subset of sensory neurons, can be removed by RTX-induced cytotoxicity. The technology is amenable due to the fact that it is supported by a number of experiments carried out in various mammals, from rodents to primates (Olah et al., 2001; Karai et al., 2004; Brown et al., 2005; Tender et al., 2005; Brown D. C. et al., 2015; Brown, 2016). The methods and kits for the selective ablation of pain-sensing neurons have been patented (Iadarola et al., 2012).

Although there are drug leads acting as TRPV1 inhibitors in different stages of R&D pipelines at a number big pharma companies (Kaneko and Szallasi, 2014), currently, there is no vanilloid drug on the market other than capsaicin (CAP). In contrast to RTX, however, CAP is not an optimal compound to target TRPV1 and implement the molecular neurosurgery technology. First of all, ours and others' experiments validated that CAP is less potent as an agonist of TRPV1 than RTX (Szallasi and Blumberg, 1999). In general, one can say that RTX acts in a low nanomolar range on TRPV1, while CAP acts in a low micromolar range, but the exact EC50 values vary between assays (Szallasi and Blumberg, 1999; Olah et al., 2001). As many pharmaceuticals may lose their specificity at higher doses, TRPV1-independent cytotoxic effects have been reported at concentrations above 10 micromolar for RTX and above several hundred micromolar for CAP, tested on Sf9 insect cells that do not have the TRPV1 gene (Pecze et al., 2008).

The vanilloid-binding site of TRPV1 is mapped to a protein region embedded in the lipid membrane, which justifies the use of the more lipophilic RTX. Thus, currently RTX is the vanilloid with the highest affinity and efficacy. We have also determined that CAP, due to its lower affinity and quicker dissociation from the receptor, is an inappropriate drug for implementation of the molecular neurosurgery and unable to deliver robust agonist-induced cytotoxicity within minutes, as noted with RTX, even *in vivo* (Olah et al., 2001; Karai et al., 2004; Brown et al., 2005; Tender et al., 2005).

EXTENDED USE OF THE AGONIST-INDUCED CYTOTOXIC MECHANISM FOR PAIN MANAGEMENT

TRPV1 channels are highly expressed on C- and Aδ-type sensory neurons. The cell bodies of somatic sensory afferent fibers lie in the dorsal root ganglia (DRG) and trigeminal ganglia (TG). TRPV1 can be stimulated by (i) endovanilloids, produced naturally by peripheral tissues in response to injury, (ii) heat source of moderately high temperature (42–49°C), and (iii) extracellular acidosis (pH ∼6.0; Caterina et al., 1997; Tominaga et al., 1998). Endovanilloids are defined as endogenous ligands of TRPV1 (van der Stelt and Di Marzo, 2004). Three different classes of endogenous lipids have been found recently that can activate TRPV1, and these are unsaturated N-acyldopamines, lipoxygenase products of arachidonic acid and linolenic acid, and the endocannabinoid anandamide (van der Stelt and Di Marzo, 2004). These compounds are produced at the site of inflammation. Endogenous TRPV1 ligands have different pharmacological properties (e.g., affinity, potency, metabolic rate, etc.) compared to naturally occurring exogenous agonists such as CAP or RTX, and consequently endogenous ligands have different physiological functions. As an example, endogenous agonists are involved in the generation of chronic pain, while exogenous agonists are capable of alleviating chronic pain (Carnevale and Rohacs, 2016). Potent vanilloids such as CAP or RTX can be administered in a different manner for the removal of TRPV1+ neurons. Routes of administration include (I) topical epicutaneous (application onto the skin), (II) intraarticular, (III) intrathecal (IV) intraganglionic, and (V) systemic intraperitoneal.

I. Topical CAP has been used for medicinal puposes for centuries, mainly to treat toothache. Creams containing CAP, generally in the range of 0.025–0.1% by weight, are now available in many countries, and often do not require a prescription, for the management of neuropathic and musculoskeletal pain. CAP creams have shown analgesic benefits in postherapeutic neuralgia, painful polyneuropathies including diabetic and HIV-related neuropathy, and postmastectomy/surgical neuropathic syndromes (Jorge et al., 2010). The CAP 8% patch is approved by FDA (U.S.Food and Drug Administration)

for postherapeutic neuralgia. Epidermal nerve fiber density in skin areas exposed to the high-concentration CAP patch (8%) was clearly lower 1 week after a single 60-min application as compared with control biopsies, but at 24 weeks, epidermal nerve fiber density appears similar to the control (Kennedy et al., 2010). Topical RTX administration was studied for treatment of ophthalmic pain. In rat cornea, a single application of RTX dose-dependently eliminated the CAP-induced eye-wiping response for 3–5 days (Bates et al., 2010). This analgesic effect was fully reversible (Bates et al., 2010). The distant surviving neuronal body generates new C-, and Aδ-afferents, which repairs inflammatory pain sensation (Donnerer, 2003; Kennedy et al., 2010). Thus, the analgesic effect of topical vanilloid administration is reversible.

II. In the sinovial fluid of joints, RTX can inactivate the C-, and Aδ-nerve pain signaling, but only temporarily.These nerves recover in a month due to fiber regeneration, guided by the unaffected axons in the nerve (Neubert et al., 2003; Kissin E. Y. et al., 2005). This process should be much faster and more complete after intra-articular than sciatic nerve RTX administration, due to the shorter part of the ablated nerve fibers in the first case. These experimented approaches will provide the possibility for a reversible interruption of nerve fiber signaling without their complete and irreversible destruction (Neubert et al., 2003; Kissin E. Y. et al., 2005; Kissin I. et al., 2005).

III. In contrast to topical administration, other routes of application generate irreversible changes, because these treatments eliminate not only the periferal axons, but also the body of the TRPV1-positive sensory neurons. Complete removal of the pain sensing neurons are a treatment option for chronic, uncurable pain conditions, such as cancer pain. RTX can be equally potent as an ablative agent of C-fiber and Aδ-afferents in patients with non-terminal stage of diseases coupled with neuropathic complication, including chronic phantom pain and type 2 diebetes (Karai et al., 2004; Brown et al., 2005; Mannes et al., 2005; Neubert et al., 2005; Tender et al., 2005). We and others have collected the necessary evidence for the extended use of the agonist-induced cytotoxic mechanism to carry out molecular neurosurgery. Due to the extreme specificity of RTX to TRPV1, the cytotoxicity does not affect other sensory modalities, such as super-heat, cold, light touch, noxious mechano-, and proprioceptive sensations. The RTX-assisted neurosurgery neither impairs bystander motor neurons nor influences consciousness (Olah et al., 2001; Karai et al., 2004; Brown et al., 2005; Tender et al., 2005; Patapoutian et al., 2009). The safety and efficacy of RTX have successfully been validated even in dogs and monkeys (Olah et al., 2001; Karai et al., 2004; Brown et al., 2005; Tender et al., 2005; Gunthorpe and Szallasi, 2008).

IV. In contrast to intrathecal administration, intraganglionic injection may need an advanced imaging technology R&D process, in order to fully support robotic anatomical guidance (i.e., needle placement by computer tomography). With this technology at hand, we can treat severe focal pain syndromes right at the source, and remove acheurons from a sub-domain of DRG or TG branches. One of the well-defined medical needs in trigeminal neuralgia requires CT-guided needle placement to inject RTX with an intra-nerve anatomic precision (Brown J. D. et al., 2015).

V. Prior to transgenic TRPV1 knockout models, systemic chemo-denervation was employed with potent vanilloids (Jancso et al., 1967; Szolcsanyi et al., 1990) to study animal behavior with altered pain sensations. Respiratory depression represents the limiting factor in rats for acute and systemic administration of CAP or RTX (Szallasi et al., 1989). The therapeutic window for RTX is wider than for CAP. RTX administered at a dosage of 50 μg/kg body weight effectively removed all pain-sensing neurons in young adult mice (Pecze et al., 2009). Mice survived the procedure and lived to an old age in the animal facility. They remained fertile, however they were not able to adapt to heat stress (Pecze et al., 2009). If subjected to elevated ambient temperature (38°C), RTX-treated rats showed a steady rise in body temperature, ultimately leading to collapse, in contrast to control animals, which did not show these behaviors (Szallasi and Blumberg, 1989).

In conclusion, RTX usually provides a better pharmacological profile than CAP. CAP is effective in micromolar concentrations and ranked approximately three factors of magnitude less potent as a vanilloid agonist than RTX. Although RTX is more potent, paradoxically it evokes less pain feeling than CAP, because the initial activation of the pain pathway *via* TRPV1 is immediately cut by the ionic influx-induced fragmentations of pain-sensing C- and Aδ-fibers. Even an intrathecally administered 1 μg/kg dose of RTX can rapidly eradicate the inflammatory pain signaling by a robust TRPV1-amplified cytotoxicity without significant side effect, tested in patient dogs suffering either cancer or osteoporosis pain (Brown et al., 2005). At the site of application the action potential conductivity of the acheuron's membranes is blocked in seconds, either when RTX is given proximally (i.e., intrathecal, intraganglionic) or distally (i.e., transdermal) to the neuronal body. Thus, only a small dosage of noxious stimuli can reach the central nervous system (Caudle et al., 2003; Neubert et al., 2003; Karai et al., 2004). In the veterinary practice, RTX-treatments of severe cancer and osteoporotic patient dogs have demonstrated initial, short-lived physiological changes, but then the blood pressure and cardiac parameters went back to the normal range. The benefit of longterm and permanent elimination of unbearable inflammatory pain justifies the effective ablation of acheurons (Karai et al., 2004; Tender et al., 2005).

EMPIRICAL USAGE OF MOLECULAR SURGERY

Nowadays, rational and mechanism-based applications of technologies based on specific molecular surgery agents can replace previous empiric practices. The surface of the human body, externally, while the gastrointestinal system, internally, are continuously exposed to pungent compounds such as

CAP, gingerol, piperine, allyl-isothiocyanate (i.e., chili pepper, ginger, black pepper, and mustard, respectively), and other phytochemicals from spices for hundreds or thousands of years (Iwasaki et al., 2008; Masamoto et al., 2009). Likewise, either by chance or conscious use, RTX had been applied in human since the ancient times. Tribal witchdoctors in Africa often administered the latex from different Euphorbia species to the wound after removal of a tooth to diminished pain and inflammation. Empirically, a number of pungent vanilloids supplement our daily meals. Various Hungarian dishes are made exclusively with hot peppers, so the human body is frequently exposed to vanilloids. In Asia as well, different pungent bioactive ingredients are used in their daily diet and target either the TRPV1 or TRPA1 channels. Thus, many of the agents mentioned in this review are administered to humans, either by frequent consumption or empiric application, first as folk medicine, then in medical practice (Sterner and Szallasi, 1999; Szallasi and Blumberg, 1999). Epidemiologic evaluations and natural uses have demonstrated that these bioactive phytochemicals can save medical expenses and prolong lifespan. A large cohort study revealed that consumption of spicy food is inversely correlated with the mortality caused by cancer, ischemic heart diseases and respiratory diseases (Lv et al., 2015).

THE ROLE OF ADVANCED IMAGING TOOLS

For the exploration of the function of the TRPV1 channel, confocal fluorescence microscopy was vastly instrumental. Firstly, confocal microscopy was used for the imaging of dynamics in intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) (**Figure 1**). Moreover, by tagging of TRPV1 channel with fluorescent proteins, the mechanism of cell death became visually trackable in real-time (**Figure 2** and Supplementary Video; Olah et al., 2001). Both N- and C-terminally tagged TRPV1 proteins results in fully functional TRPV1 channels. Cells ectopically expressing the TRPV1 receptor show necrotic bleb formation upon CAP stimulation (Pecze et al., 2013). Bleb formation is dependent on both Ca^{2+} and Na^{+} influx (Pecze et al., 2016b). Bleb formation can be so intensive that the cell blows up, until finally the loss of the plasma membrane integrity leads to necrotic cell death (Pecze et al., 2016b). Besides this, cell organelles such as the mitochondria and endoplasmic reticulum also show fragmentization within 1 h (Olah et al., 2001). High resolution confocal images has helped in the figuring out of the molecular neurosurgery mechanism and in distinguishing the difference between the efficacy of CAP and RTX (Olah et al., 2001). Cells ectopically expressing fluorescently tagged TRPV1 channels were voltage-clamped and 10 μM CAP induced large inward currents similar to that of 125 pM RTX. Capsazepine, a competitive TRPV1 antagonist, attenuated the vanilloid-induced currents (Olah et al., 2001; Liu et al., 2003; Marshall et al., 2003).

Fusion of TRPV1 with rationally chosen fluorescent protein allows for co-localization studies which exploit the fluorescence resonance energy transfer (FRET) phenomenon (Hellwig et al., 2005; Zagotta et al., 2016). The optical sectioning capabilities of confocal fluorescence microscopes followed by 3D reconstruction revealed the innervation pattern of the epithelium of guinea-pig trachea by TRPV1 immunoreactive axons (Watanabe et al., 2005). Confocal images showed the loss of TRPV1-immunoreactive DRG neurons and afferent terminals in the spinal cord after RTX treatment (Chen and Pan, 2006).

Ectopically expressed fusion proteins allowed for the determination of the sub-cellular distribution of TRPV1 receptors. It became clear that in addition to the plasma membrane (PM), where previously, TRPV1 was expected to mechanistically localize ($TRPV1_{PM}$), it was also found and noted to operate in the endoplasmic reticulum ($TRPV1_{ER}$), as well (Olah et al., 2001; Karai et al., 2004). The endoplasmic reticulum (ER) is the major intracellular storage of Ca^{2+} ions and like

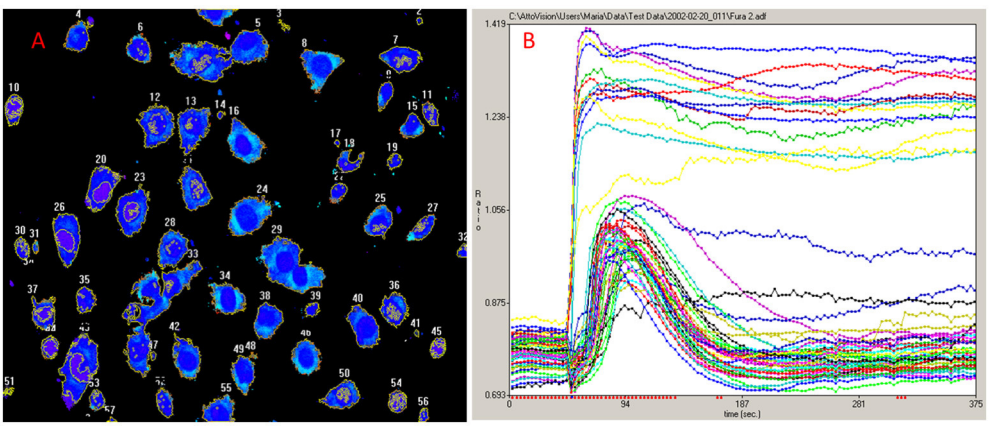

FIGURE 1 | Ca^{2+} imaging. **(A)** NIH-3T3 cells ectopically expressing TRPV1 were loaded with the cytosolic Ca^{2+} indicator Fluo-4-AM. **(B)** Minute scales imaging of $[Ca^{2+}]_i$ reveals two populations in NIH-3T3^{TRPV1} cell line. A set of cells responds to 1 μM CAP with non-declining increase of $[Ca^{2+}]_i$ and dies very soon while the other population survives the initial necrotic-phase in which the $[Ca^{2+}]_i$ transitions back to closed to its resting levels. This later option rarely occurs with RTX, as RTX is several thousandfold more potent than CAP in several assays (Szallasi and Blumberg, 1999). The original experiment was published in 2004 (Karai et al., 2004).

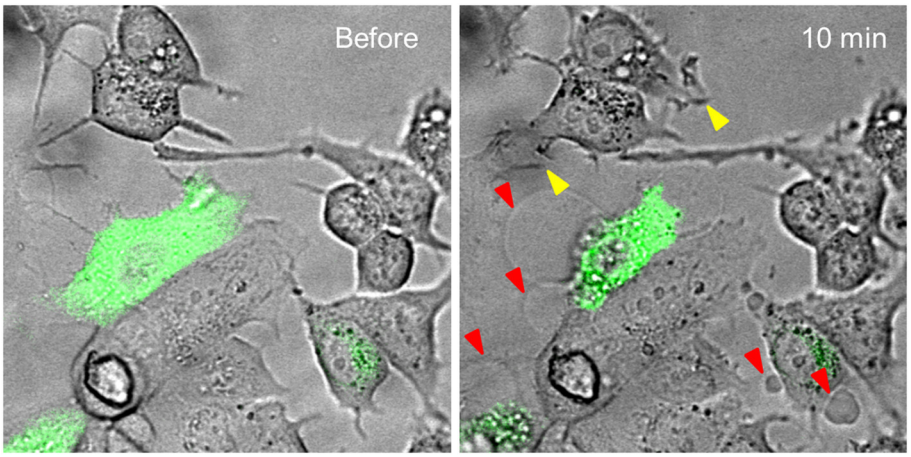

FIGURE 2 | Time-lapse analysis of vanilloid evoked structural changes in MCF7 breast cancer cell line. MCF7 cells were transiently transfected with TRPV1-GFP construct. These show green fluorescence. Cells were treated with 50 μM CAP. TRPV1-GFP expressing cells, and only these cells, blow blebs during a 10-min period (red arrowheads). MCF7 cells also endogenously express low levels of TRPV1 channels. This level is not enough to induce bleb formation, but some MCF7 cells produce invadopodium, an ameboid structure promoting cancer cell invasion, in response to CAP treatment (yellow arrowheads). The original experiment was published in 2016 (Pecze et al., 2016b).

$TRPV1_{PM}$, the $TRPV1_{ER}$ receptor is also gated by vanilloids and contributes to the agonist-induced cytotoxicity. The potency of less lipophilic CAP on $TRPV1_{ER}$, is likely hampered by its slow membrane penetration and distribution to deeper cellular compartments (Lazar et al., 2006).

MECHANISM OF ACTION

In physiological conditions, TRPV1 is activated by the endogeneous ligand produced at the site of inflammation or tissue injury. TRPV1 is a non-selective cation channel with a higher permeability for divalent cations, such as Ca^{2+} (permeability ratio P_{Ca}/P_{Na} is around ∼10; Gees et al., 2010). However, both Ca^{2+} and Na^{+} influxes through the TRPV1 channel play a role in the transmission of nociceptive signals from the periphery toward the central nervous system. Besides this, activation of TRPV1 causes cell depolarization. Sensory neurons as excitable cells express voltage-operated ion channels. Activation of TRPV1 channels triggers the gating of those channels. The firing pattern of neuronal cells is modulated by conductance changes *via* TRP channel activation or inhibition (Gees et al., 2010). It is worth noting that TRPV1 produces an analog Ca^{2+} signal i.e., the amount of Ca^{2+} ions passing through the channel is proportional to the stimulus intensity. The activated inositol phospholipid pathway acts as an amplifier and frequency-based modulator on Ca^{2+} signals produced by TRPV1. The frequency of the intracellular Ca^{2+} oscillations are related to the strength of TRPV1 stimulation (Pecze et al., 2016a). Since exogenous TRPV1 ligands (CAP and RTX) have different pharmacological properties such as higher affinity and potency compared to endogenous agonists, they consequently induce an over-activity of the TRPV1 receptor. Thus, RTX and CAP induce a prolonged increase in $[Ca^{2+}]_i$, but only in sensory neurons expressing TRPV1 while not in other cells (Olah et al., 2001; Karai et al., 2004).

DESENSITIZATION VS. DELETION OF A CELL/NEURON

Desensitization is the phenomenon in which a receptor's responsiveness decreases after continued or repeated stimulation with an agonist. Prolonged or repeated applications of CAP causes persistent desensitization of TRPV1 in an electrophysiology-based experiment (Touska et al., 2011). Although this effect can also contribute to pain insensitivity after vanilloid treatment to some extent, our experiments show that sensory neurons or axons were absent in the treated region. Thus, in contrast to desensitization, an alternative mechanism of potent vanilloids has been proposed; complete removal of TRPV1-specific nociceptive neurons is the cause of the long-lasting/permanent inflammatory pain-free state (Olah et al., 2001; Caudle et al., 2003; Karai et al., 2004) and these findings were later confirmed by others (Chen and Pan, 2006; Kennedy et al., 2010; Kun et al., 2012).

However, controversy regarding nerve fiber degeneration vs. long-lasting desensitization without neuronal degeneration still exists in terms of the explanation for the mechanism of potent vanilloid agonism. Prior to transgenic TRPV1 knockout models, chemo-denervation was employed with potent vanilloids (Jancso et al., 1967; Szolcsanyi et al., 1990) to study animal behavior without pain sensation. Although the inflammatory pain-free state that either CAP or RTX treatment produced was unusually long lasting (Szallasi et al., 1989; Szallasi and Blumberg, 1992), more than minutes or hours, the early explanations of the analgesic actions of vanilloids suggested a desensitization of nerve terminals (Szolcsanyi et al., 1975). Thus, the literature still uses long-lasting desensitization as an explanation.

NECROTIC VS. APOPTOTIC PROCESSES

It was determined in a number of experiments that RTX (i.e., 1 μg/kg) produces analgesia by robust Ca^{2+}-mediated cytotoxicity, if applied (i) intradermally, lasting for several days to a month, or (ii) intrathecally and intraganglionically, permanent for a lifetime, removing the entire TRPV1+ neuron (Szabo et al., 1999; Karai et al., 2004).

The cellular and molecular mechanisms underlying the vanilloid-induced neural loss are still unresolved. Evidence for CAP-induced neuronal cell death by apoptosis with caspase activation has been reported (Shin et al., 2003; Jin et al., 2005), while some studies state that it is an apoptosis-like, but caspase-independent process (Movsesyan et al., 2004; Davies et al., 2010). Still other studies doubt the process to be apoptotic at all in nature (Olah et al., 2001; Caudle et al., 2003). It is very likely that both apoptosis and necrosis might play a role in TRPV1-mediated toxicity, depending on the strength of the activation and moreover on the experimental protocol. The Ca^{2+} ionophore ionomycin acts by creating Ca^{2+}-permeable pores in cell membranes. Analagous to TRPV1-related cytotoxicity, it can induce either apoptosis or necrosis in cultured cortical neurons (Gwag et al., 1999).

The MCF7 breast cancer cell line, although it expresses endogeneously low levels of TRPV1 receptors, cannot be subjected to necrotic-like procecesses by administration of CAP or RTX, but shows the typical structural changes when TRPV1 is ectopically overexpressed (**Figure 2**). Interestingly, in MCF7 cells, the mere overexpression of GFP-tagged TRPV1 channels decreased cell viability (Pecze et al., 2016b). We observed that mainly apoptotic processes were activated, but mitotic arrest in $MCF7^{GFP\text{-}TRPV1}$ cells was also detected. The absence of mitosis in the surviving $MCF7^{GFP\text{-}TRPV1}$ cells subsequently did not allow for the establishment of stable $MCF7^{GFP\text{-}TRPV1}$ clones, although we had been successful in establishing cell clones permanently expressing ectopic TRPV1 proteins using non-tumor-derived cell lines such as HaCaT, a spontaneously immortalized keratinocyte cell line from adult human skin (Pecze et al., 2008), or NIH-3T3 cells, a spontaneously immortalized mouse embryo fibroblast cell line (Olah et al., 2007). Moreover, prolonged treatment of non-transfected MCF7 cells with CAP induces apoptotic processes due to increased oxidative stress (Kosar et al., 2016). The supposed mechanisms of necrotic and apoptoic processes are summarized in **Figure 3**.

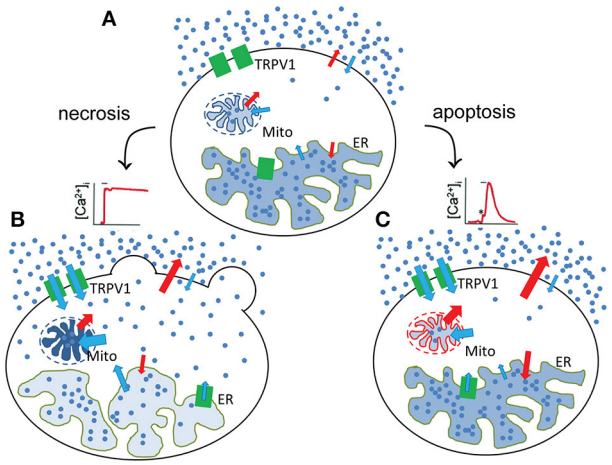

FIGURE 3 | Necrotic and apoptotic processes after TRPV1 stimulation. **(A)** In the unstimulated state, the resting $[Ca^{2+}]_i$ is the result of the low rate of influx and efflux across the plasma, ER- and mitochondria membranes. Red and blue arrows indicates the energy-requiring and energy-independent fluxes, respectively. After stimulation, two types of Ca^{2+} response can be observable leading to necrotic **(B)** and potentially apoptotic processes **(C)**. **(B)** During the necrotic processes TRPV1 activation results in a sustained increase in $[Ca^{2+}]_i$. After that, Ca^{2+} ions are accumulated in the mitochondria (Pecze et al., 2016b) but released from the endoplasmatic reticulum. These processes lead to the fragmentation of these organelles (Olah et al., 2001). Blebs appears at the plasmamembrane due to the cell volume increase. **(C)** During the apoptotic processes TRPV1 activation does not result in a sustained increase in $[Ca^{2+}]_i$, but rather to a transient Ca^{2+} signal mainly due to the depletion of the ER Ca^{2+} stores. In this situation, Ca^{2+} extruding systems is still able to create an equilibrium between the Ca^{2+} influx and Ca^{2+} efflux reverting $[Ca^{2+}]_i$ close to its basal levels before stimulation. However, this new equilibrium requires elevated energy consumption. Mitochondria therefore produce more energy, but during their normal operation they also produce reactive oxygene species (ROS; Michael Murphy, 2009). ROS production was significantly increased in cultured DRG neurons after bath application of CAP (1 μM) or RTX (200 nM) compared with the untreated neurons (Ma et al., 2009). This can induce oxidative stress and apoptosis Fleury et al., 2002.

SAFETY AND EFFICACY OF MOLECULAR SURGERY AGENTS IN HUMANS

Resistance to vanilloids provides additional safety and efficacy to the molecular surgery technology. We and others have noted that TRPV1 and its mRNA are detected in a broader spectra of cells rather than only from DRG or TG origins. Paradoxically, the occurrence of TRPV1 does not necessarily mean that the cell can automatically be deleted by the vanilloid-mediated molecular surgery. Vanilloid binding cannot be mechanistically linked either to channel opening or to permanent elevation in $[Ca^{2+}]_i$. This issue has been addressed in studies of human keratinocytes (Pecze et al., 2008; Kun et al., 2012).

One potential explanation for vanilloid resistance is that TRPV1 subunits need to form a homotetramer channel, a quaternary structure required for maximal vanilloid sensitivity and channel activity (Kedei et al., 2001; Garcia-Sanz et al., 2004; Moiseenkova-Bell et al., 2008). Any obstacles that hamper quaternary structure formation of the pore from the subunits may reduce the cell's sensitivity to vanilloids. Mounting evidence shows that TRPV1 is capable of heteromerization with other TRP channel homologs upon co-expression (Fischer et al., 2014). Major determinants of TRPV1 oligomerization have recently been localized to the C-terminal ^{684}Glu-^{721}Arg, the so-called association domain (Garcia-Sanz et al., 2004). Recombinant association domains form stable multimers, however, association domain-deleted TRPV1 is monomeric and blocks self-assembly of wild-type subunits in functional homotetramers. Evolutionarily conserved, homologous, but not identical, association domains,

however, may allow for the combinatorial assembly of different TRP channels that are gated by distinct ligands other than vanilloids.

Besides this, our experiments indicate that TRPV1 channel density on the plasma membrane is an important factor for the cell's sensitivity to vanilloids (Pecze et al., 2016b). Cells expressing lower density of the TRPV1 channel are evidently more resistant to TRPV1-mediated cytotoxicity. Unfortuntely, although tumor cells express higher levels of TRPV1 than normal epithelial cells, they still do not have enough receptors to perform tumor-targeted TRPV1-mediated necrotic-type eradication (Pecze et al., 2016b). Nevertheless, several experiments (Diaz-Laviada and Rodriguez-Henche, 2014) as well as a case report (Jankovic et al., 2010) suggest that vanilloids have anti-cancer activity. The origin of the anticancer effects of vanilloids is not completely solved and it needs further examination. Whether it is TRPV1-mediated or a TRPV1-independent effect is still in question (Diaz-Laviada and Rodriguez-Henche, 2014). To make things more complicated, pain sensing neurons innervate the tumor mass and communicate with the tumors (Li et al., 2013). Systemic removal of TRPV1+ neurons in mice increased the number of metastasis of breast cancers (Erin et al., 2004).

PROOF OF THE EFFICACY OF MOLECULAR NEUROSURGERY FOR THE TREATMENT OF TYPE II DIABETES AND URINARY DISFUNCTIONS

The proof of the applicability of molecular surgery reveals a second use of RTX in type II diabetes as an anti-neuropathic treatment agent (Gram et al., 2005; Moesgaard et al., 2005). The system that regulates insulin secretion from beta-cells in the islet of Langerhans has a vanilloid-sensitive inhibitory component. Calcitonin Gene Related Peptid (CGRP)-expressing TRPV1+ primary sensory fibers innervate the islets. The CGRP-containing primary sensory neurons are targets of the RTX-mediated molecular neurosurgery. Elimination of vanilloid-sensitive primary afferents by vanilloids before the development of hyperglycemia prevents the increase of plasma glucose levels and coincides with enhanced insulin secretion and a loss of CGRP-expressing islet-innervating fibers. These data indicate that CGRP-containing fibers in the islets are sensitive to molecular neurosurgery, and that elimination of these fibers contributes to the prevention of the deterioration of glucose homeostasis through increased insulin secretion in genetically obese rats (Gram et al., 2005, 2007; Moesgaard et al., 2005).

Vanilloid-sensitive C- and Aδ-afferents are present in the human bladder's urothelium and are involved in the micturition reflex. Although, intravesically administered RTX most likely acts analogous to CAP, its better pharmacodynamic profile allows for an increase in bladder volume and a higher threshold for the micturition reflex (Payne et al., 2005; Raisinghani et al., 2005). This improvement coincides with a disappearance of CGRP and Substance P (SP) immunoreactive fibers, selective biomarkers of afferents of TRPV1+ neurons. Thus, the loss of CGRP and SP peptide immunoreactivity, consistent either with agonist-mediated depletion of neurotransmitters, or deletion of these fibers by vanilloid-mediated Ca^{2+}-cytotoxicity via molecular neurosurgery.

Overactive bladder syndrome, a common type of micturition disorder, can lead to the loss of bladder control, which is then known as urge incontinence. First CAP (Szallasi et al., 1993; De Ridder et al., 1997), then RTX (Lazzeri et al., 1997), were tried as experimental drugs to inactivate incontinency in the clinical settings (**Figure 4**). It has been long known that these reflexes in the bladder are mediated by C- and Aδ-fiber afferents of nociceptive neurons located in the sacral DRGs. The Afferon Inc., in the late 90's, patented a method of treating neurogenic urinary dysfunction with RTX (Cruz and Agersborg, 2014), and has enrolled patients affected with urge incontinence due to various neurological diseases. The Afferon was admitted into phase II clinical trials. Currently, Eli Lilly and Company has exclusive worldwide license rights for the commercial use of RTX for the treatment of bladder disease or function. Unfortunately, in contrast to these practices (Guo et al., 2013; Foster and Lake, 2014), RTX is still not a registered drug.

Nevertheless, the effectiveness of intravesical RTX treatment strongly varies from study to study. The reasons for these inconsistencies in the clinical outcome might be manifold: too dilute samples of vanilloids were used, different origins of the

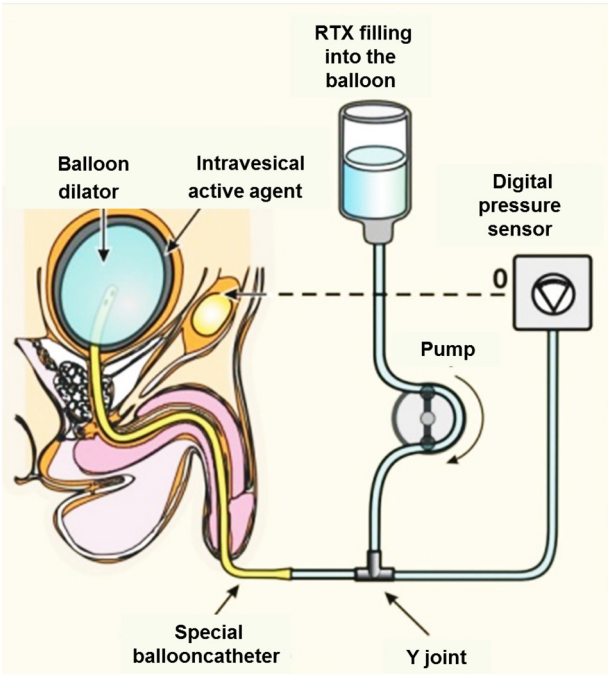

FIGURE 4 | Topical intravesical medication of RTX *via* transurethral instillation promises several advantages over oral systemic CAP therapy. Intravesically administered RTX penetrates the vesical mucosa and submucosa by diffusion and binds to TRPV1+ nerve endings. The suggested "balloon dilator" method benefits from the increased surface of urothelium due to the thinning of the bladder mucosa.

latent disease resulting in urinary disfunction, or the simple lack of a significant effect of RTX in a specific type of urinary problem. We would like to pay attention to the fact that strong adsorption of RTX and CAP molecules into the tubes of the application devices might also occur. These technical problems can cause a huge variance in the clinical outcomes. Animal experiments will be required to obtain the appropriate material for these tubes. By using a water-soluble formulation of vanilloids (Appendino et al., 2010) the adsorption of vanilloid molecules onto the tubes might also be avoidable. The loss of the TRPV1+ nerve endings in the urothelium can serve as a marker for the successful intravesical instillation.

OTHER AGONIST-ACTIVATED CA^{2+}-CHANNELS AS TARGETS OF NOVEL MOLECULAR SURGERY AGENTS

The molecular mechanism of TRPV1-mediated cytotoxicity shows conspicuous similarity to glutamate-receptor mediated excitotoxicity, i.e., robust Na^+ and Ca^{2+} influx, cell swelling, mitochondrial Ca^{2+} loading, and production of reactive oxygen species (Dong et al., 2016). Ionotropic glutamate receptors, as glutamate is the main excitatory neurotransmitters in the central nervous system, play important role in production of excitatory postsynaptic potentials, neuronal migration, synapse formation, learning, and memory (Choi and Rothman, 1990; Dugan et al., 1995). Kainic acid, an agonist for kainate-class ionotropic glutamate receptors, is commonly injected into laboratory animal models to study the effects of experimental ablation. However, attempts to limit cell loss to specific hippocampal neurons have been met with mixed successes and failures (Jarrard, 2002).

The concept of moleculary surgery would be an ideal approach for cancer treatment. Tumors express a different composition of TRP channels than normal cells (Park et al., 2016). Finding a specific TRP target overexpressed only in the tumor cells and finding a potent agonist would provide an ideal pair for the tumor-specific eradication. Recently, it was found that kidney cancer cells can be efficiently and specifically targeted by (-)-Englerin A, a potent and selective activator of TRPC4 and TRPC5 channels (Akbulut et al., 2015). The renal cancer cell line A498, which is most sensitive for (-)-Englerin A, has a highest degree of expression of TRPC4 among the NCI60 cell lines (Akbulut et al., 2015). New derivatives of (-)-Englerin A have been synthetized in order to find effective drugs for the treatment of renal cell carcinoma. A patent application has been filed for this treatment (Echavarren et al., 2011). These new derivatives open a new way of the final goal of finding effective drugs for the treatment of renal cell carcinoma.

CONCLUSIONS AND PERSPECTIVES

Evidence-based and clinically-tried B2B-approaches, such as molecular neurosurgery prototyped with RTX and TRPV1 channel, can be extended for other applications. Here we put an emphasis to the analgesic use of the agonist-induced selective cytotoxic mechanism, however by analogy, a number of robust, biological cell deletion mechanisms may be used in the near future. For example, treatment of metastatic cancers might be amenable by using cancer-specific TRP targets.

Currently, RTX is the most powerful molecular surgery agent in Phase II clinical trials to manage cancer pain in humans. RTX has also been evaluated in severe inflammatory pain states and various neuropathies, as it only removes acheurons and preserves any other bystander cells, fibers, and nerve endings, with little or no side-effects. RTX works in conjunction with TRPV1, with a lack of any effect to cells which do not express TRPV1.

Combination treatment, however, may extend the utility of CAP or other weaker agonists enhancing the cytotoxic effect of vanilloids. A family of positive allosteric modulators (PAM) of TRPV1 only activates Na^+ and Ca^{2+} entry *via* the vanilloid receptor channel if a vanilloid (CAP, RTX, piperine, etc.) is already bound to the receptor (Roh et al., 2008). These compounds extend the molecular tools of molecular surgery. PAM further increases by 2–3-fold the maximal effect of vanilloids on the induction of Na^+/Ca^{2+}-uptake, producing little or no action when used alone (Roh et al., 2008; Kaszas et al., 2011; Lebovitz et al., 2012). Thus, weaker exovanilloids such as CAP and piperine, present in hot peppers and black peppers, or even weaker endovanilloids can serve as agents to fight against inflammation, pain, and neuropathies. Morevover, RTX, together with a PAM molecule, MRS1477, can provide innovative solutions to number of currently unmet medical needs. The exact mechanism of positive allosteric modulation and the domain specificity of the binding site is not enterely known. Therefore, a more detailed quantitative structure-activity relationship and the determination of the TRPV1-mediated cytotoxic capacity of PAMs must be examined by further studies.

AUTHOR CONTRIBUTIONS

LP, ZO wrote the review BV critically revised the work and approved its version to be submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00378/full#supplementary-material

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