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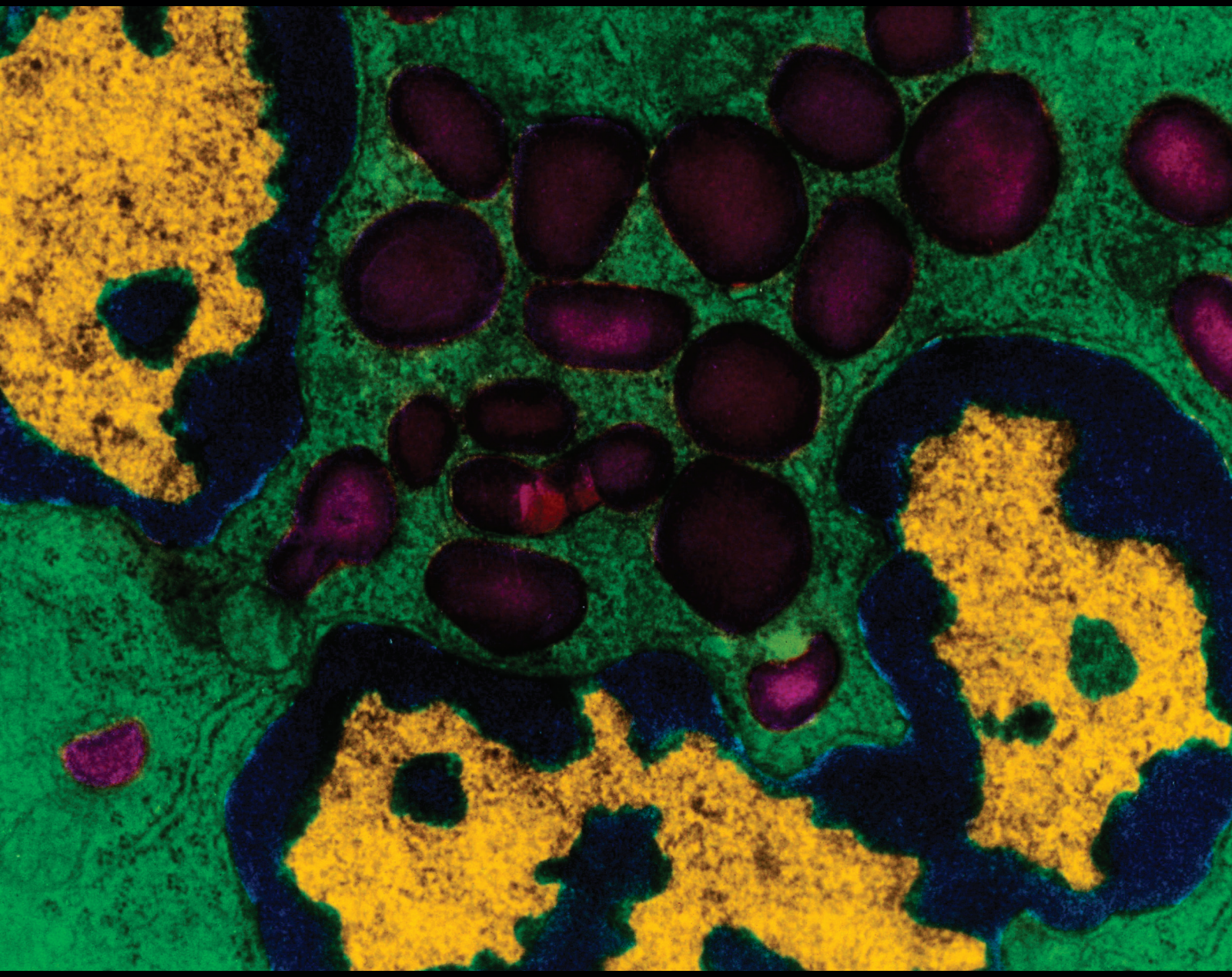
Ion Channels in Inflammatory Processes: What Is Known and What Is Next?

Guest Editors: Mauricio Retamal, Michael V. L. Bennett, Pablo Pelegrin, and Ricardo Fernandez



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Editorial Board

Contents

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Editorial

Ion Channels in Inflammatory Processes: What Is Known and What Is Next?

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Inflammation is the primary response of the immune system to infection or tissue injury. Ion channels in different immune cells are very important for inflammatory processes. In this context, connexin (Cx) and pannexin (Panx) cell-cell channels and unapposed hemichannels and P2 receptors play central roles in the initiation and/or progression of inflammation in different tissues. Thus, cellular responses during inflammation can be initiated and/or enhanced by the opening of Cx and/or Panx hemichannels, which in turn allow the release of ATP and other metabolites to the extracellular media. These can act as "danger" signals to further propagate the original inflammatory response. Extracellular ATP can activate important intracellular signaling pathways through the activation of P2 receptors. However, it is yet not completely clear how these channels become active in these processes, how they interact, and how their pharmacological modulation may have a potential advantageous or beneficial therapeutic effect. In this special issue, M. Leo et al. show that agonists of TNFR-1 and TNFR-2 increase voltage gated sodium currents in cultured DRG neurons. These authors suggest that the activation of voltage gated sodium channels increases excitation of sensory neurons and this might explain the sensitization associated with neuropathic and inflammatory pain. On the other hand, kidneys are affected by ischemia, endotoxemia, and diabetic nephropathy, where the concentrations of proinflammatory cytokines increase. K^+ channels play important roles in the function of renal tubule epithelial cells. Therefore, the effect of cytokines on K^+ channels could be associated with alterations of tubular transport or onset of renal cell injury. Thus, K. Nakamura et al. provide an exhaustive review about the effect of proinflammatory cytokines on K^+ channels present in the kidney. Ion channels are fundamental for normal lung function and their alteration may cause some lung diseases. Cl^- channels have particular relevance because they contribute to mucus synthesis, secretion, function, and mucociliary clearance. According to this idea, M. Sala-Rabanal et al. discuss the role of Cl^- channels, transporters, and regulators upon the progression of inflammatory diseases in lungs. Another type of channel that participates in lung function is the TRPA1 channel. A.-H. Lin et al. show that this channel is crucial for transducing ROS production in the extracellular space in Ca^{2+}-dependent signaling pathways. This phenomenon could be important in our understanding of the relationship between cigarette smoke and lung inflammation. Finally, S. S. Kim reviewed several light-associated techniques useful for the study of P2X channels, which are quite important to several steps of inflammatory responses.

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Review Article

Manipulation of P2X Receptor Activities by Light Stimulation

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P2X receptors are involved in amplification of inflammatory responses in peripheral nociceptive fibers and in mediating pain-related signals to the CNS. Control of P2X activation has significant importance in managing unwanted hypersensitive neuron responses. To overcome the limitations of chemical ligand treatment, optical stimulation methods of optogenetics and photoswitching achieve efficient control of P2X activation while allowing specificity at the target site and convenient stimulation by light illumination. There are many potential applications for photosensitive elements, such as improved uncaging methods, photoisomerizable ligands, photoswitches, and gold nanoparticles. Each technique has both advantages and downsides, and techniques are selected according to the purpose of the application. Technical advances not only provide novel approaches to manage inflammation or pain mediated by P2X receptors but also suggest a similar approach for controlling other ion channels.

1. Introduction

P2X receptors belong to the ATP-gated cation channel family, which has seven members [1–8]. The receptor structure consists of trimeric homo- or heteromers with two transmembrane domains (TM1 and TM2) [2–4]. P2X receptors have various roles in neuropathic pain [5, 6], synaptic transmission [2], cancer [7], neurodegenerative disorders [8], and inflammation [9–12]. Release of ATP during inflammation is characteristic at the site of injury. Activated inflammatory cells release a plethora of ATP extracellularly in an uncontrolled fashion from intracellular storage [13, 14]. In some cases, controlled release of ATP is observed through transmembrane proteins connexin or pannexin [13, 15]. ATP also functions as a neurotransmitter in primary afferent neurons by relaying pain information to the CNS [16]. Considering the distribution of P2X, its involvement in nociceptive sensation cannot be ruled out. P2X1 to P2X6 are expressed in sensory ganglia, especially in dorsal root ganglia (DRG) [17]. The dense expression of P2X3 in small diameter DRG and its coexpression with TRPV1 implicate P2X3 as a designated pain sensor [18, 19]. Thus, activation of P2X in inflammatory or noxious conditions provokes widespread responses in neuropathic pain. Nerve damage induces upregulation of P2X receptors, leading to hypersensitivity [20].

Considering the fundamental contribution of P2X receptors in pain and inflammation, receptor modulation has therapeutic significance. Traditionally, chemical compounds are applied to local tissues or systemic circulation to control ion channel activity. Oral intake, stable structure in ordinary conditions, and high efficacy at target sites are advantages of using chemical drugs. However, side effects resulting from systemic distribution and harmful metabolite production require improved approaches. Due to advances in the optogenetic field in the last decade, novel techniques have been introduced to control ion channel activity. The use of photoswitches or genetically modified receptors has increased specificity in controlling target channel activities with optimal light stimulation. In addition to optogenetics, the incorporation of novel materials into cells has enabled optimal control of intact receptors without genetic modifications. These techniques encompass important target channels or receptors involved in neuronal firing, inflammation, or incurable diseases. P2X receptors are targets of interest due to their important role in inflammation and nociceptive sensation. This review investigates past trials on control of

P2X receptors by light stimulation and explores cutting edge techniques with potential for therapeutic application.

2. Activation of P2X Receptors by Releasing ATP from Photosensitive Caging Compounds

In conventional neuronal stimulation, an electric stimulus is delivered through an electric probe to activate the group of neurons surrounding the probe. This direct electrical activation is easily applicable with a potential caveat of nonspecific activation of neuronal circuits. P2X receptors have been favored for optical stimulation due to their simple channel architecture, large extracellular ligand binding domain, and relatively rare presence in the CNS [21–24]. The initial application was with an uncaging method that tethered ATP to caging compounds as a protecting group. The first generation was 2-nitrobenzyl (NB) esters of ATP that were susceptible to photolysis by UV light [25, 26]. The side effect of NB photolysis, however, produced reactive by-product compounds that interfered with reliable interpretation of the response. Improved second-generation caging groups, such as 4,5-dimethoxy-2-nitrobenzyl (DMNB) and α-carboxy-2-nitrobenzyl (CNB), overcame these limitations with fewer by-products and stable kinetics [27]. Indeed, robust neuronal spikes were generated in P2X2 expressing neurons using a 1 s UV light pulse (355 nm) in the presence of DMNPE-ATP [28]. P2X2 activation kinetics fell into the millisecond range in a reproducible manner, suggesting applications in spatially restricted sites to invoke the intended neuronal activation. This scenario was realized in phototrigger experiments affecting the locomotor behavior of flies. Lima and Miesenböck expressed P2X2 in a small group of neurons of the giant fiber (GF) system through DMNPE-ATP injection in the CNS [29]. Brief UV illumination for 150–250 ms provoked characteristic escape movements mediated through giant fibers without desensitization in repeated photostimulation of 2.5 s intervals. The induction of dopaminergic signaling by photostimulation of P2X2 expressing dopaminergic neurons increased locomotor activities affecting the walking pattern of flies. Likewise, remote control of P2X activation by photostimulation not only changed the cellular response but also caused behavioral changes.

3. Control of Neuronal Activation by Photoisomerizable Molecules Transporting through P2X Receptor Pores

The optochemical genetic approach has downsides in therapeutic applications due to exogenous expression of effective receptors and systemic injection of caging compounds, which generates nonselective cellular responses. To circumvent these problems, photoisomerizable molecules were introduced through certain receptor pores. They then underwent conformational changes induced by light stimulation. This idea was originally developed to silence nociceptive neurons by delivering membrane-impermeable local anesthetic lidocaine, QX-314, intracellularly with the help of TRPV1 pore opening [30]. The attempt was successful in allowing QX-314 influx by TRPV1 activation, but structural irreversibility of the compound lasted for several hours, preventing therapeutic usage [31].

To overcome this limitation, Mourot et al. developed a novel photoswitch, quaternary ammonium-azobenzene-quaternary ammonium (QAQ). QAQ has versatile isomerizable characteristics and converts from the elongated *trans* form to bent *cis* form with 380 nm light [32]. Once introduced intracellularly, the *trans* form of QAQ blocks various voltage-gated Na^+, Ca^{2+}, and K^+ channels with the few exceptions of inward-rectifier (Kir), hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, *N*-methyl-D-aspartic acid (NMDA), and non-NMDA receptors. Light stimulation of 380 nm on QAQ embedded neurons recovered the silenced nociceptive neurons, restoring their firing rate and action potential amplitude. Light of 500 nm generated the opposite effect. The transition with light of specific wavelengths was evoked in an on and off fashion without altering basic neuronal properties such as resting membrane potential or firing threshold. QAQ can enter cells through large ionic pores, which limits the number of mediator channels or receptors. TRPV1 expressing cells showed sufficient QAQ loading in the presence of capsaicin [32]. P2X7 also proved its eligibility for photosensitization with ATP treatment [32, 33]. In heterologous expression of HEK 293 cells, P2X7 demonstrated photosensitization of Shaker K^+ channels by QAQ application in the presence of ATP. Endogenous voltage-gated K^+ channel activity was also prone to photocontrol through exogenously expressed P2X7 receptors in cultured rat hippocampal neurons.

This novel photoswitching technology is applicable in pain and inflammation control due to the immense role of TRPV1 and P2X7 in pathological processes. Because QAQ has selective entry without affecting nonnociceptive neurons, it provides an effective peripheral analgesic method. QAQ can enter any nociceptor location from the cell soma to the long axonal process and nerve endings through pores of mediating channels, which is impossible with conventional electrophysiology or drugs.

4. Optogating of P2X Receptors through Introduction of Photoswitches into the Subunits

With recent advances in optogenetic technology, remote control of neuronal activity enables selective activation of certain neuronal circuits, leading to local or systemic changes on demand. Among various applications, pharmacology takes advantage of photoswitchable ligands, which are tethered to genetically modified receptor sites. Photoisomerizable chemical ligands can convert geographical structures into more favorable forms by light stimulation with a specific wavelength, enabling binding to active sites of receptors. This method improved conventional chemical stimulation by increasing the specificity of target locations and instant stimulation.

Efforts have been made to enable optogating of P2X receptors. An azobenzene ammonium derivative was synthesized to create a maleimide ethylene azobenzene trimethyl ammonium derivative (MEA-TMA). This was photoisomerized from the long *trans*-isomer to the *cis*-isomer by applying a 365 nm light, a change that was reversed with 525 nm light [34]. MEA-TMA was affixed to a modified P2X receptor, P2X2-3T, in which outer transmembrane residues were substituted to cysteine [35]. Indeed, 365 nm light precisely opened the channel within a short temporal range, reaching maximal current in almost 1 second. The optogating of P2X2 consisted of two different mechanisms, an ATP dependent pore-blocking pathway and direct binding to the receptor without ATP. With ATP independent opening, a pore dilation effect was still observed with *N*-methyl-D-glucamine (NMDG) treatment [2, 36, 37].

A similar attempt using azobenzene isomerization was made for optical control of p2X2 and P2X3 receptors. In this experiment, *bis*(maleimido)azobenzene (BMA) was synthesized to bridge receptor subunits of TM2 through covalent linkage to cysteine residues [38]. BMA underwent conformational changes between the *cis* and *trans* forms at 360 and 440 nm illumination, respectively. P2X2 receptor P329C mutation with BMA treatment allowed an inward current with 440 nm in the absence of ATP. Cysteine substitution of P320C at an equivalent site of P2X3 also generated light-activated current, as with P2X2 activation. Homomeric expression of each channel and P2X2/3 heteromeric expression showed rapid photocontrol with opening at 440 nm and closing at 360 nm in HEK293 cells and PC12 cells. Photoisomerization showed similar receptor kinetics as ATP activation. This result suggests that gating rearrangement is the limiting factor of P2X activation, not agonist-binding steps.

5. Neuronal Activation Using Gold Particles to Target Intact P2X Receptors

Various methods have been suggested to achieve specific optical stimulation of neurons, as described above. P2X receptor activation photoswitch or photoisomerization offers custom designed activation tools. However, genetic modification of receptors is inevitable. Actual application in nongenetically modified animals, including humans, is hindered. To overcome this limitation, direct optical stimulation of intact neurons is proposed by applying infrared (IR) wavelength to neuronal membranes [39, 40]. IR wavelength delivers energy in the form of heat to cellular membranes, leading to upregulation of membrane capacitance. This elevated membrane capacitance results in depolarization and action potential generation.

To effectively deliver heat to cell membranes, gold nanorods (AuNRs) or spherical gold nanoparticles (AuNPs) are treated in cell culture media to absorb IR energy and convert it to heat. In this way, cultured neurons can evoke cellular activation by IR or near-infrared (NIR) excitation [41–43]. Bezanilla et al. elaborated upon this method by using channel specific antibodies and second antibodies conjugated with AuNPs to specifically activate neurons expressing target channels [44]. They initially tested dorsal root ganglion (DRG) neurons with Ts1-conjugated AuNPs. Light of 532 nm was sufficient to generate action potentials in DRG neurons, even with repetitive washout and multiple photostimulations. The next trial targeted endogenously expressed channels such as TRPV1 and P2X3. Overnight incubation of DRG neurons with each antibody following AuNP-secondary antibody treatment demonstrated the photosensitivity of DRG neurons to green light stimulation (532 nm). The amplitude of action potentials generated by light stimulation was almost equal to that of electrical stimulation. Long washout time of more than 20 min still preserved responsiveness to light, proving tight binding of primary antibodies. This method encourages clinical applications to control nociceptive neuronal activation in pain or inflammation. Unlike any other technique, this method does not require injection of a virus containing modified receptor DNA at the site of interest. Instead, simple treatment with AuNP-conjugated antibody and NIR illumination is sufficient to activate target neurons. Since the activation mechanism does not depend on temperature itself but rather on the rate of temperature change, less intense light stimulation can be applied to avoid tissue damage.

6. Conclusion

Growth in optogenetics has generated abundant opsin containing channels with ample wavelength ranges of light stimulation to activate or deactivate neurons. Channelrhodopsins and halorhodopsins are the most representative light-gated ion channels, and each intracellularly introduces cations and anions. Genetic expression of these photosensitive channels using viral vectors is helpful for controlling neuronal activities. However, introduction of foreign genes can cause derangements in proper protein expression. Therefore, receptors already present in nerves are safer targets. P2X receptors are choice candidates for light stimulation because they are functionally expressed in the periphery and CNS to govern inflammatory and nociceptive pathways. For a long time, uncaging of ATP by UV light has been the primary method used to activate P2X receptors. Improved caging compounds are now used to reduce by-products. The isomerizable property of azobenzene introduced a new way to construct photoswitches that can reversibly change conformations. Photoswitches can be incorporated into receptor subunits or used as light sensitive ligands. Controlling both receptors and ligands by light stimulation enables many options for combinatorial manipulation of neuronal activation. Even intact receptors can receive light stimulation. The novel concept of applying gold particles to convert light energy into heat proved the possibility of activating neurons by increasing membrane conductance. Using specific antibodies for pain receptors such as P2X, gold nanoparticles target intact receptors to activate nerves.

Although there are diverse benefits with light stimulation techniques, every method inevitably introduces limitations also. Our goal is to improve current methods to increase specificity while developing safer applications in humans.

The fields of optics and material science suggest innovative technical advances for new ideas.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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References

[1] G. Burnstock and H. Wong, "Comparison of the effects of ultraviolet light and purinergic nerve stimulation on the guinea-pig taenia coli," *British Journal of Pharmacology*, vol. 62, no. 2, pp. 293–302, 1978.

[2] B. S. Khakh and R. A. North, "Neuromodulation by extracellular ATP and P2X Receptors in the CNS," *Neuron*, vol. 76, no. 1, pp. 51–69, 2012.

[3] C. Coddou, Z. Yan, T. Obsil, J. Pablo Huidobro-Toro, and S. S. Stojilkovic, "Activation and regulation of purinergic P2X receptor channels," *Pharmacological Reviews*, vol. 63, no. 3, pp. 641–683, 2011.

[4] K. Kaczmarek-Hájek, É. Lörinczi, R. Hausmann, and A. Nicke, "Molecular and functional properties of P2X receptors—recent progress and persisting challenges," *Purinergic Signalling*, vol. 8, no. 3, pp. 375–417, 2012.

[5] S. Beggs, T. Trang, and M. W. Salter, "P2X4R$^+$ microglia drive neuropathic pain," *Nature Neuroscience*, vol. 15, no. 8, pp. 1068–1073, 2012.

[6] B. A. Chizh and P. Illes, "P2X receptors and nociception," *Pharmacological Reviews*, vol. 53, no. 4, pp. 553–568, 2001.

[7] N. White and G. Burnstock, "P2 receptors and cancer," *Trends in Pharmacological Sciences*, vol. 27, no. 4, pp. 211–217, 2006.

[8] G. Burnstock, "Purinergic signalling: its unpopular beginning, its acceptance and its exciting future," *BioEssays*, vol. 34, no. 3, pp. 218–225, 2012.

[9] F. Di Virgilio, "Liaisons dangereuses: $P2X_7$ and the inflammasome," *Trends in Pharmacological Sciences*, vol. 28, no. 9, pp. 465–472, 2007.

[10] F. Di Virgilio, "P2X receptors and inflammation," *Current Medicinal Chemistry*, vol. 22, no. 7, pp. 866–877, 2015.

[11] E. Beamer, F. Gölöncsér, G. Horváth et al., "Purinergic mechanisms in neuroinflammation: an update from molecules to behavior," *Neuropharmacology*, 2015.

[12] M. Idzko, D. Ferrari, and H. K. Eltzschig, "Nucleotide signalling during inflammation," *Nature*, vol. 509, no. 7500, pp. 310–317, 2014.

[13] W. G. Junger, "Immune cell regulation by autocrine purinergic signalling," *Nature Reviews Immunology*, vol. 11, no. 3, pp. 201–212, 2011.

[14] H. K. Eltzschig, M. V. Sitkovsky, and S. C. Robson, "Purinergic signaling during inflammation," *The New England Journal of Medicine*, vol. 367, no. 24, pp. 2322–2333, 2012.

[15] H. K. Eltzschig, T. Eckle, A. Mager et al., "ATP release from activated neutrophils occurs via connexin 43 and modulates adenosine-dependent endothelial cell function," *Circulation Research*, vol. 99, no. 10, pp. 1100–1108, 2006.

[16] W. Poelchen, D. Sieler, K. Wirkner, and P. Illes, "Co-transmitter function of ATP in central catecholaminergic neurons of the rat," *Neuroscience*, vol. 102, no. 3, pp. 593–602, 2001.

[17] W. Norenberg and P. Illes, "Neuronal P2X receptors: localisation and functional properties," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 362, no. 4-5, pp. 324–339, 2000.

[18] Z. Xiang, X. Bo, and G. Burnstock, "Localization of ATP-gated P2X receptor immunoreactivity in rat sensory and sympathetic ganglia," *Neuroscience Letters*, vol. 256, no. 2, pp. 105–108, 1998.

[19] A. Guo, L. Vulchanova, J. Wang, X. Li, and R. Elde, "Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the $P2X_3$ purinoceptor and IB4 binding sites," *European Journal of Neuroscience*, vol. 11, no. 3, pp. 946–958, 1999.

[20] E. Fabbretti, M. D'Arco, A. Fabbro, M. Simonetti, A. Nistri, and R. Giniatullin, "Delayed upregulation of ATP P2X3 receptors of trigeminal sensory neurons by calcitonin gene-related peptide," *Journal of Neuroscience*, vol. 26, no. 23, pp. 6163–6171, 2006.

[21] S. J. Fountain and G. Burnstock, "An evolutionary history of P2X receptors," *Purinergic Signalling*, vol. 5, no. 3, pp. 269–272, 2009.

[22] T. Chataigneau, D. Lemoine, and T. Grutter, "Exploring the ATP-binding site of P2X receptors," *Frontiers in Cellular Neuroscience*, vol. 7, article 273, 2013.

[23] R. J. Evans, "Structural interpretation of P2X receptor mutagenesis studies on drug action," *British Journal of Pharmacology*, vol. 161, no. 5, pp. 961–971, 2010.

[24] J. Rettinger and G. Schmalzing, "Activation and desensitization of the recombinant P2X1 receptor at nanomolar ATP concentrations," *Journal of General Physiology*, vol. 121, no. 5, pp. 451–461, 2003.

[25] J. A. McCray, L. Herbette, T. Kihara, and D. R. Trentham, "A new approach to time-resolved studies of ATP-requiring biological systems; laser flash photolysis of caged ATP," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 77, no. 12, pp. 7237–7241, 1980.

[26] J. H. Kaplan, B. Forbush III, and J. F. Hoffman, "Rapid photolytic release of adenosine 5′-triphosphate from a protected analogue: Utilization by the Na:K pump of human red blood cell ghosts," *Biochemistry*, vol. 17, no. 10, pp. 1929–1935, 1978.

[27] J. M. Amatrudo, J. P. Olson, H. K. Agarwal, and G. C. R. Ellis-Davies, "Caged compounds for multichromic optical interrogation of neural systems," *European Journal of Neuroscience*, vol. 41, no. 1, pp. 5–16, 2015.

[28] B. V. Zemelman, N. Nesnas, G. A. Lee, and G. Miesenböck, "Photochemical gating of heterologous ion channels: remote control over genetically designated populations of neurons," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 3, pp. 1352–1357, 2003.

[29] S. Q. Lima and G. Miesenböck, "Remote control of behavior through genetically targeted photostimulation of neurons," *Cell*, vol. 121, no. 1, pp. 141–152, 2005.

[30] A. M. Binshtok, B. P. Bean, and C. J. Woolf, "Inhibition of nociceptors by TRPV1-mediated entry of impermeant sodium channel blockers," *Nature*, vol. 449, no. 7162, pp. 607–610, 2007.

[31] A. M. Binshtok, P. Gerner, S. B. Oh et al., "Coapplication of lidocaine and the permanently charged sodium channel blocker QX-314 produces a long-lasting nociceptive blockade in rodents," *Anesthesiology*, vol. 111, no. 1, pp. 127–137, 2009.

[32] A. Mourot, T. Fehrentz, Y. Le Feuvre et al., "Rapid optical control of nociception with an ion-channel photoswitch," *Nature Methods*, vol. 9, no. 4, pp. 396–402, 2012.

[33] L. E. Browne, V. Compan, L. Bragg, and R. A. North, "P2X7 receptor channels allow direct permeation of nanometer-sized dyes," *The Journal of Neuroscience*, vol. 33, no. 8, pp. 3557–3566, 2013.

[34] D. Lemoine, C. Habermacher, A. Martz et al., "Optical control of an ion channel gate," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 51, pp. 20813–20818, 2013.

[35] M. Li, T.-H. Chang, S. D. Silberberg, and K. J. Swartz, "Gating the pore of P2X receptor channels," *Nature Neuroscience*, vol. 11, no. 8, pp. 883–887, 2008.

[36] B. S. Khakh, X. R. Bao, C. Labarca, and H. A. Lester, "Neuronal P2X transmitter-gated cation channels change their ion selectivity in seconds," *Nature Neuroscience*, vol. 2, no. 4, pp. 322–330, 1999.

[37] C. Virginio, A. MacKenzie, F. A. Rassendren, R. A. North, and A. Surprenant, "Pore dilation of neuronal P2X receptor channels," *Nature Neuroscience*, vol. 2, no. 4, pp. 315–321, 1999.

[38] L. E. Browne, J. P. Nunes, J. A. Sim et al., "Optical control of trimeric P2X receptors and acid-sensing ion channels," *Proceedings of the National Academy of Sciences*, vol. 111, no. 1, pp. 521–526, 2014.

[39] J. Wells, C. Kao, K. Mariappan et al., "Optical stimulation of neural tissue *in vivo*," *Optics Letters*, vol. 30, no. 5, pp. 504–506, 2005.

[40] M. G. Shapiro, K. Homma, S. Villarreal, C.-P. Richter, and F. Bezanilla, "Infrared light excites cells by changing their electrical capacitance," *Nature Communications*, vol. 3, article 736, 2012.

[41] K. Eom, J. Kim, J. M. Choi et al., "Enhanced infrared neural stimulation using localized surface plasmon resonance of gold nanorods," *Small*, vol. 10, no. 19, pp. 3853–3857, 2014.

[42] J. Yong, K. Needham, W. G. Brown et al., "Gold-nanorod-assisted near-infrared stimulation of primary auditory neurons," *Advanced Healthcare Materials*, vol. 3, no. 11, pp. 1862–1868, 2014.

[43] D. K. Roper, W. Ahn, and M. Hoepfner, "Microscale heat transfer transduced by surface plasmon resonant gold nanoparticles," *The Journal of Physical Chemistry C*, vol. 111, no. 9, pp. 3636–3641, 2007.

[44] J. L. Carvalho-de-Souza, J. S. Treger, B. Dang, S. B. H. Kent, D. R. Pepperberg, and F. Bezanilla, "Photosensitivity of neurons enabled by cell-targeted gold nanoparticles," *Neuron*, vol. 86, no. 1, pp. 207–217, 2015.

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Review Article

Novel Roles for Chloride Channels, Exchangers, and Regulators in Chronic Inflammatory Airway Diseases

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Chloride transport proteins play critical roles in inflammatory airway diseases, contributing to the detrimental aspects of mucus overproduction, mucus secretion, and airway constriction. However, they also play crucial roles in contributing to the innate immune properties of mucus and mucociliary clearance. In this review, we focus on the emerging novel roles for a chloride channel regulator (CLCA1), a calcium-activated chloride channel (TMEM16A), and two chloride exchangers (SLC26A4/pendrin and SLC26A9) in chronic inflammatory airway diseases.

1. Introduction

The chronic inflammatory airway disease, asthma, and chronic obstructive pulmonary disease (COPD) are significant causes of morbidity and mortality in children and adults. Asthma affects over 300 million people worldwide, and the prevalence is increasing among all demographics. COPD is currently the third leading cause of death in USA. These diseases are hallmarked by a Th2-mediated inflammatory response which drives the three pathologies that contribute to airway obstruction in these diseases: chronic inflammation; airway muscle constriction due to airway hyperreactivity (AHR); and mucus overproduction due to mucous cell metaplasia (MCM). A central feature of these diseases is production of the inflammatory cytokines IL-4 and IL-13, which drive MCM and contribute to AHR.

The inflammatory signaling upregulates the expression of hundreds of proteins in the airway epithelia. A number of these proteins have roles in anion transport across membranes, including chloride channels, channel regulators, and transporters. The identity, function, and elucidated mechanism of action of these proteins have lagged behind their cation channel counterparts. However, recent advances in several technologies, including high throughput screening, have made it possible to consider the development of specific inhibitors and activators for these classes of proteins [1]. The development of such therapeutics, however, requires an intimate knowledge of the roles these proteins play in airway homeostasis and mucociliary clearance. Anion channels play very crucial roles in mucus function. Mucus is composed of 97% water and 3% solids, with the main solid component being the mucin proteins [2]. Mucin proteins are secreted in a dehydrated form and require anion channel activity to instill chloride and bicarbonate ions that ensure proper salination, hydration, and pH of the mucus gel layer. Proper control of this is crucial as is exemplified by the disease cystic fibrosis

(CF), which is caused by loss of function mutations to the chloride channel cystic fibrosis transmembrane conductance regulator (CFTR) that produces thick, sticky mucus deficient in mucociliary clearance or innate antimicrobial properties [3].

Here we discuss what is currently known about the function of four exciting, new, and emerging proteins affecting anion channel activity in inflammatory airway epithelia: a chloride channel regulator (CLCA1), a calcium-activated chloride channel (TMEM16A), and two chloride exchangers (SLC26A4/pendrin and SLC26A9). In particular, we focus on recently uncovered contributions to airway diseases and mucus function, in order to answer whether they can be targeted by inhibitors or activators and whether they should be.

2. The CLCA Family of Chloride Channel Regulators

The CLCA family of proteins was originally misidentified as calcium-activated chloride channels and has long been associated with chronic inflammatory airway diseases. Their evolving functional identity and the possible role they play in these diseases have only recently been elucidated.

2.1. CLCAs: Association with Chronic Inflammatory Airway Diseases. Asthmatic inflammation results from a Th2-mediated mechanism, where the cytokines IL-4 and IL-13 bind their receptors and activate the transcription factor STAT6 to drive inflammation and mucus overproduction in the airways [4, 5]. In mouse models of both allergic and respiratory virus induced-asthma, CLCA1 (previously known as mCLCA3 or gob-5) expression has been solidly linked to IL-13 driven MCM [6, 7] and controversially linked to AHR [6], both hallmarks of asthma and COPD. Similar results were observed *in vitro* with the human pulmonary mucoepidermoid cell line NCI-H292, in which expression of the protein significantly increased mucin gene MUC5AC expression and subsequent mucus production [8, 9], implying that CLCA1 can drive MCM. Studies using *Clca1*$^{-/-}$ mice, however, have failed to show reduced response to IL-13 stimulation, as these mice showed the same phenotype as wild-type (WT) mice [7, 10]. Other members of the family, particularly CLCA2 and potentially CLCA4A and CLCA4B, have also been observed to be upregulated and to induce and colocalize with the inflammatory mucin protein, MUC5AC [7]. These results indicate a possible functional redundancy between members of the mouse CLCA family, which is unlikely to translate to human biology, as there are only three human CLCA proteins, compared to the seven mouse CLCA proteins [11] (Figure 1).

Of the three human CLCA proteins, CLCA1 has been identified as a potential biomarker of inflammation and MCM in the airways [12] and suggested as a potential drug target for treatment of asthma and COPD. Similar to mouse CLCA1, overexpression of human CLCA1 in NCI-H292 cells increases MUC5AC expression and mucus levels [6, 8, 9]. Its expression is upregulated in primary cell models of IL-13 driven MCM and siRNA-mediated knockdown of CLCA1 prevents IL-13-driven mucus overproduction [9]. These experimental observations suggest a central role for CLCA1 function in IL-13-mediated MCM (Figure 2). Additionally, it is highly overexpressed in the airway epithelia of asthmatic patients [8] and can be found in the bronchoalveolar lavage fluid (BALF) at high levels [13]. In contrast, other members of the human CLCA family are not upregulated in response to IL-13 [8, 9], suggesting that CLCA1 is the sole family member with an essential role in MCM in human airways.

2.2. The Conceptual Evolution of CLCA Proteins from "Channels" into "Channel Regulators". The family of CLCA proteins were first cloned from bovine and murine samples, where overexpression of these proteins increased Ca^{2+}-sensitive Cl^- conductance, which led to their initial misannotation as Ca^{2+}-activated Cl^- channels (CaCCs) [14–16]. In addition, observations that nonspecific chloride channel blockers, such as niflumic acid and DIDS, seemed to reduce both currents and the mucus production [17] erroneously supported this hypothesis. However, modern bioinformatics algorithms and experimental approaches have definitively demonstrated that CLCA proteins are soluble, secreted proteins that do not constitute ion channels themselves [13, 18], and subsequent studies demonstrated that CLCA proteins activate currents through an endogenous CaCC [18–20]. As a consequence, the CLCA nomenclature has been updated and the family is now recognized as Ca^{2+}-activated Cl^- channel regulator proteins. All members of this family (with the exception of the likely pseudogenes human CLCA3 and mouse CLCA4C) are synthesized as full-length proteins and proteolytically cleaved into two fragments. It has been demonstrated that, for some of the family members, the cleavage is carried out by a zinc-dependent matrix metalloprotease-like (MMP-like) domain located in the N-terminus of the protein [18, 21] (Figure 1). However, sequence analysis has revealed that all CLCA family members contain the required MMP active site motif [18]; thus self-cleavage is likely a conserved feature of all CLCAs. Self-cleavage is required to unmask the N-terminal fragment of CLCA1, which then interacts with the CaCC [18] (Figure 2). The molecular identity of this previously unknown CaCC has been shown to be TMEM16A and the direct interaction between the channel and the N-terminal fragment stabilizes and increases the cell surface expression of the channel, thereby increasing currents [20].

2.3. CLCA1 as a Potential Regulator of Cytokine Expression. Upstream of CLCA1 expression, the involvement of Th2-cytokines IL-4 and IL-13 has been shown in cellular and animal models as discussed above. The relationship between CLCA1 and downstream cytokine signaling, however, is still under investigation. The few articles that do exist report contradictory observations regarding the role of CLCA1 as a signaling molecule for cytokine expression. Challenging *Clca1*$^{-/-}$ mice with lipopolysaccharide (LPS), Long et al. observed increased KC (keratinocyte-chemoattractant) levels in BALF, but no change in MIP-2 (macrophage inflammatory protein 2) or IL-17 levels in the knockout mice compared

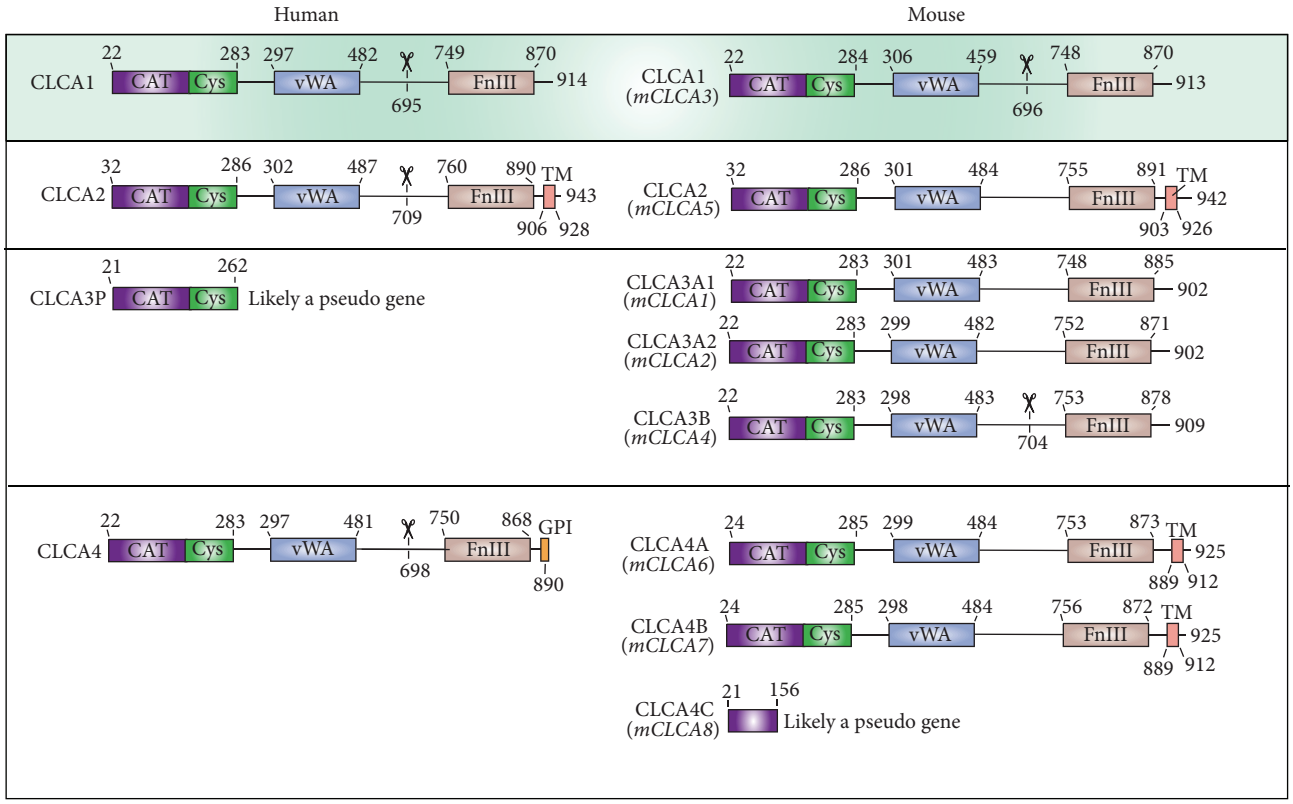

Figure 1: Domain architecture schematic for human and mouse CLCA proteins. Each row contains the corresponding human and mouse homologs. Mouse CLCA proteins are labeled according to the recently updated naming commissioned by the Mouse Gene Nomenclature Committee (MGNC) in order to align the numbering established by the Human Gene Nomenclature Committee and the Rat Genome Database. The previously used names for the mouse proteins are shown below the current names and are in italics. Scissors denote the experimentally determined location of proteolytic cleavage sites [18]. Human CLCA3 and mouse CLCA4C are likely pseudogenes because they contain premature stop codons. Labels denote the following domains: CAT: matrix-metalloprotease-like catalytic domain; CYS: matrix-metalloprotease-like cysteine rich domain; vWA: von Willebrand factor type A domain; FnIII: fibronectin type III domain; TM: transmembrane domain; GPI: glycosylphosphatidylinositol anchor.

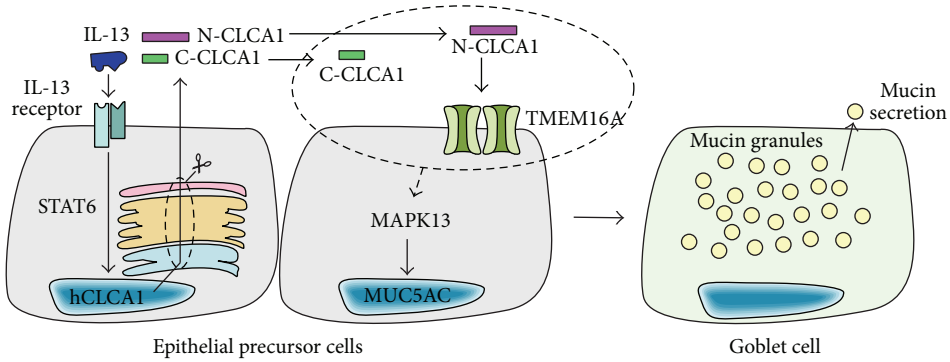

Figure 2: Schematic of CLCA1-driven MCM in human airways based on the current literature. IL-13 induces *CLCA1* gene expression through activated STAT6. CLCA1 protein is expressed, is secreted, and undergoes proteolytic self-cleavage to yield two fragments (N-CLCA1: N-terminal fragment; C-CLCA1: C-terminal fragment). N-CLCA1 engages and activates the CaCC TMEM16A. Downstream, a signaling pathway is activated through MAPK13 which leads to induction of the inflammatory mucin *MUC5AC*, followed by goblet cell differentiation and subsequent MCM. It is currently unknown whether or how the steps highlighted in the dashed ellipse (CLCA1 cleavage and activation of TMEM16A) contribute to the activation of MAPK13.

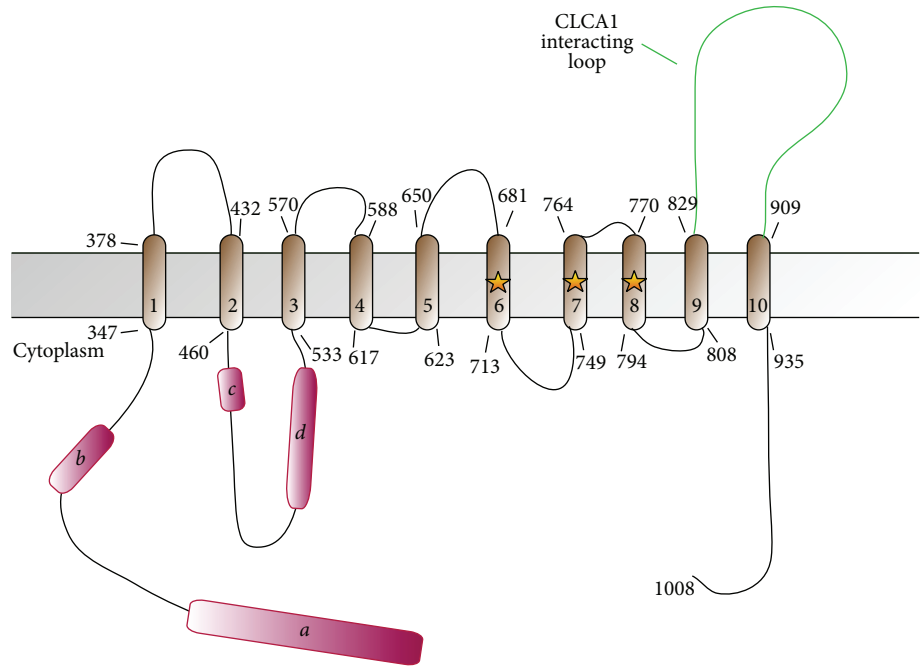

Figure 3: Domain architecture schematic for human TMEM16A. Topology shown is predicted from structure-based alignment to the crystal structure of the fungal *Nectria haematococca* TMEM16 [31]. Alternative splicing segments *a*, *b*, *c*, and *d* are shown in magenta. The location of residues of a crystallographically determined Ca^{2+} binding site is highlighted with stars. The extracellular loop mediating interaction with CLCA1 is highlighted in green.

to WT mice [22]. In contrast, Dietert et al. observed significantly decreased levels of IL-17 and CXCL-1 in BALF from the *Clca1*$^{-/-}$ mice infected with *Staphylococcus aureus* [23]. Using a cellular model of inflammation, Ching et al. showed that CLCA1-conditioned media increased proinflammatory cytokine (IL-6, IL-8, IL-1β, and TNF α) mRNA levels in monocyte cell line U-937 and primary porcine alveolar macrophages. Immunopurified CLCA1 protein only increased IL-8 and IL-1β levels significantly [24]. If such a regulatory mechanism exists for cytokine expression, modulation of CLCA1 function with small molecules to treat mucus cell metaplasia might also alter the inflammatory response in the airways.

3. TMEM16: The First Family of CaCCs

While CaCC conductance was a long-observed phenomenon in the airways and could be separated from CFTR currents, the molecular identity of the channels responsible for these currents remained elusive until the late 2000s. The TMEM16/Anoctamin family was identified in 2008 as the first bona fide CaCCs [25–27]. However, based on their electrical and pharmacological characterization, only two of the ten family members, TMEM16A and TMEM16B, displayed properties previously observed for CaCCs in the airways [28, 29], whereas most of the other members function as lipid scramblases. Of these two, TMEM16A expression has been verified in airway epithelium and airway smooth muscle cells [30].

3.1. TMEM16A Is Linked to Chronic Inflammatory Airway Diseases. The predicted topology for TMEM16 family members is based on the recent landmark crystal structure of the fungal *Nectria haematococca* TMEM16 (nhTMEM16) which has 10 transmembrane domains instead of the previously predicted 8 (Figure 3) [31]. The purified and reconstituted protein, which was shown to be a homodimer [32], constitutes a channel on its own and does not require other proteins to be active [33]. While the Ca^{2+} sensitivity of the channel is well documented [31, 34, 35] and the protein directly binds Ca^{2+}, the possible involvement and mechanism of interaction with calmodulin as a calcium sensor and binding partner are still controversial [35–37].

Much like CLCA1, expression of TMEM16A is upregulated by IL-4/IL-13 stimulation [38–41]. Upon upregulation, TMEM16A colocalizes to the apical plasma membrane of goblet cells along with the mucin MUC5AC [41–43]. It is expressed in airway smooth muscle cells and has been shown to play a role in AHR [42]. Additionally, inhibitors of TMEM16A have been shown to block mucus secretion [42, 44] whereas small molecule activators increase secretion [45].

3.2. The Potential for Targeting CLCA1 and TMEM16A in Chronic Inflammatory Airway Diseases. CLCA1 is a promising therapeutic target for asthma and COPD, as it is the only member of its family to be upregulated in models of IL-13 mediated mucus overproduction [9], is a secreted protein [18], is expressed in goblet cells [7, 9, 16], and associates with mucin granules [46, 47]. Overexpression of CLCA1 in airway

epithelium induces MUC5AC expression via a signaling cascade involving the kinase MAPK13, and siRNA-knockdown of CLCA1 blocks mucus production in these models [9], implying a critical role for CLCA1 function in inflammatory mucus production. Consistent with these observations, DNA vaccines [48] and antibodies targeting mouse CLCA1 [49] have displayed some effectiveness in reducing airway inflammation and MUC5AC levels in mouse asthma models. Similarly, as mentioned above, TMEM16A inhibitors block ATP-dependent mucus secretion, suggesting a central role for this pair in inflammatory mucus overproduction. However, it should be noted that these small molecules have relatively low potency and questionable selectivity and these findings need to be supported by additional experiments to determine the role TMEM16A plays in mucus secretion [50]. An important question that currently remains unanswered is what role this pair of molecules plays in mucociliary clearance. It is well known that anion channel activity is required for secreted mucins (MUC5AC and MUC5B) to function properly in a mucosal immunity and mucociliary clearance capacity [51]. Mucin proteins are secreted in dense, dehydrated granules and require anion channel passage of chloride and bicarbonate ions to ensure proper hydration, salination, and pH control [2]. Thus, any therapy targeting anion channel activity in airway diseases should proceed with caution to avoid any potential detrimental impacts to mucociliary clearance and innate mucosal immunological properties. Along these lines, exploiting the mechanism CLCA1-mediated regulation of TMEM16A action might be a possible therapeutic route for CF, utilizing a potential compensatory channel to make up for loss of CFTR activity [52]. Further along these lines, TMEM16A has been shown to carry not only chloride ions but also bicarbonate ions [36]; thus activation of TMEM16A in the setting of CF could be beneficial to adjust mucus hydration and pH.

4. SLC26: An Ancient Family with Unexpected New Roles

Over the last decade the anion exchanger pendrin (PDS, *SLC26A4*), once thought to be limited mainly to the inner ear, kidney, and thyroid, has been found to be upregulated by inflammatory cytokines in the bronchial epithelium, where it contributes to the pathogenesis of inflammatory airway diseases [53–55] and also to the host response to bacterial infections [56, 57]. Another member of the family, SLC26A9, is prominently expressed in the airway epithelia, where it interacts with CFTR to modulate mucus production [58]. The discovery of these crucial roles in lung physiology and pathophysiology makes these anion transporters intriguing new biomarkers for airway disease and promising novel pharmacological targets.

4.1. Pendrin: An Anion Exchanger with Critical Roles in Ear, Kidney, and Lung Physiology. Pendrin (PDS, *SLC26A4*) is a member of the SLC26 family of multifunctional anion transporters and channels [59, 60]. The eleven mammalian *SLC26* genes encode proteins with cytoplasmic N-termini and C-termini flanking a transmembrane core of unknown structure, predicted to span the lipid bilayer 10 to 14 times (Figure 4). Mutagenesis, homology modeling, and molecular dynamics simulation data are consistent with the hypothesis that the SLC26 transmembrane fold consists of two nesting, inverted repeats of 5–7 helices, resembling that of the CLC Cl^-/H^+ antiporter channel proteins [61, 62] and the recently solved three-dimensional structure of SLC26Dg, a bacterial H^+-coupled fumarate symporter, has clarified this [63]. It has been suggested that SLC26 proteins organize in functional homodimers or homotetramers [64], though each subunit is thought to constitute an independent translocation pathway. The C-terminal cytoplasmic region of all SLC26 proteins includes a sulfate transporter and antisigma factor antagonist (STAS) domain (Figure 4), which has been implicated in nucleotide binding and hydrolysis [65]. SLC26A4, or pendrin, functions as an electroneutral exchanger of Cl^-, HCO_3^-, I^-, NO_3^-, formate, SCN^-, and other monovalent anions. It is expressed in cochlear epithelial cells of the spiral prominence, in root cells, in spindle cells of the stria vascularis, in epithelial cells of the endolymphatic sac, and in epithelial cells surrounding the hair cells of the saccule, utricle, and ampulla [66]. Additionally, pendrin is expressed in the apical membrane of thyrocytes [67, 68], renal collecting duct Type B intercalated cells [69], salivary gland cells [70], and airway epithelia [53].

Pendrin function is important in several settings. In the inner ear, pendrin helps maintain Cl^- and HCO_3^- homeostasis, which is crucial for normal hearing and for the development of bony structures such as the cochlea and the vestibular aqueduct [71]. In the thyroid gland, pendrin contributes I^- to the follicle for thyroxine biosynthesis [72], and in the cortical collecting duct the transporter is implicated in Cl^- reabsorption through functional coupling with the epithelial Na^+ channel ENaC and the Na^+-dependent Cl^-/HCO_3^- exchanger NDCBE/SLC4A8 [73]. Most interestingly, it has been shown that in the bronchial epithelium pendrin mediates an increase in Cl^-/SCN^- exchange in response to IL-4 stimulation [74] to provide SCN^- substrate to lactoperoxidase for the synthesis of hypothiocyanite ($OSCN^-$), a molecule with antimicrobial properties [75], and this underscores the emerging role of pendrin in innate airway defense mechanisms (Figure 5).

4.2. Pendrin and the Pathogenesis of Inflammatory Lung Disease: Too Much of a Good Thing? Pendrin was first identified by positional cloning as the disease gene for Pendred syndrome (OMIM number 247600), an autosomal recessive condition characterized by deafness with enlargement of the vestibular aqueduct, complex abnormalities in cochlear structure, and variably penetrant euthyroid goiter [76–78]. Pendrin is also implicated in DFNB4, an autosomal recessive form of nonsyndromic deafness [79]. *SLC26A4* mutations that are clinically associated with Pendred syndrome cause complete loss of transport function when studied in heterologous expression systems, mostly due to retention in various intracellular compartments, whereas those exclusively associated with DFNB4 have residual transport activity [80]. In recent years, pendrin *gain* of function, mainly due to increased surface expression, has been linked to respiratory

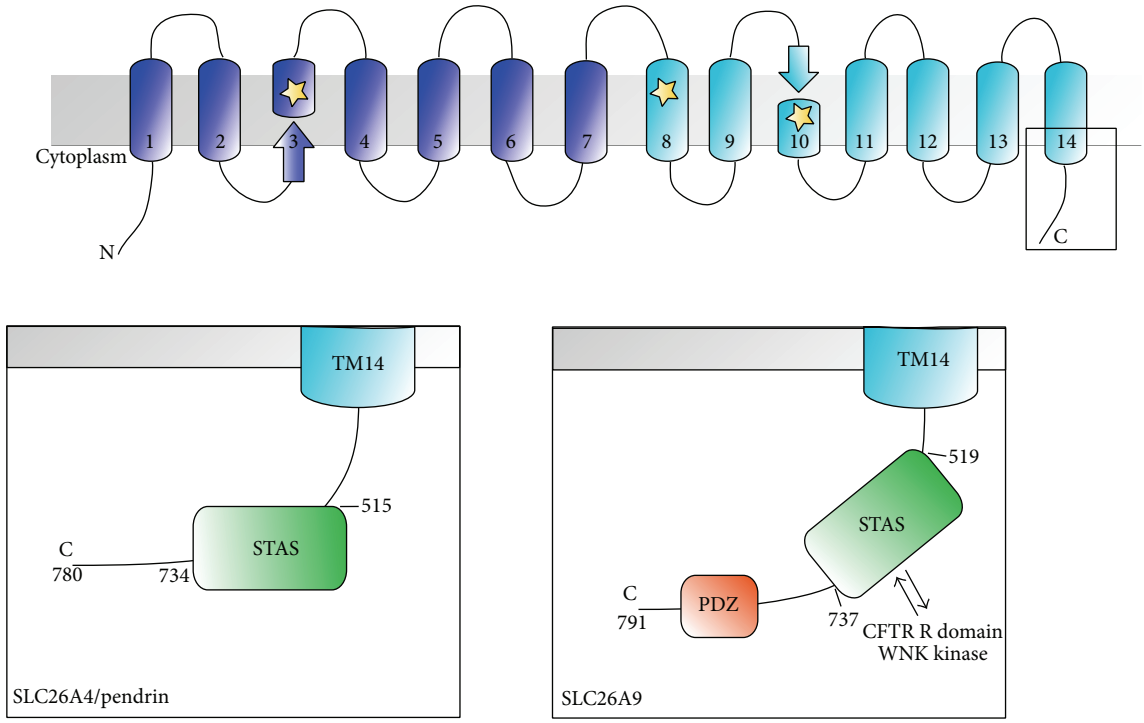

Figure 4: Domain architecture schematic for human SLC26 family proteins discussed here, SLC26A4 (pendrin) and SLC26A9, based on the crystal structure of SLC26Dg (PDB ID 5DA0). Upper inset shows general schematic while the lower insets show details of the C-terminal cytoplasmic region for each protein. Labels denote the following domains: STAS: sulfate transporter and antisigma factor antagonist domain; PDZ: PSD95-Dlg1-Zo-1 domain. Locations of SLC26A9 interaction with CFTR R domains and WNK kinases are denoted. The location of residues of a crystallographically determined ligand binding site is highlighted with stars.

diseases including bronchial asthma, COPD, and rhinovirus infection, rhinitis, and chronic rhinosinusitis [54, 55, 81–86].

The association between pendrin and inflammatory airway disease was first proposed in 2005, when it was observed that pendrin expression was upregulated in three different murine asthma models, including transgenic overexpression of IL-13 in lung [83]. Later, it was reported that induction of asthma or COPD in mice by inhalation of ovalbumin or elastase, respectively, resulted in increased pendrin expression; direct overexpression of pendrin in the lung led to increased mucus production and secretion and neutrophilic infiltration [86]. In subsequent works, the link between inflammatory cytokines, in particular IL-4 and IL-13, and pendrin overexpression has been cemented [81, 85, 87], and in a recent study *SLC26A4* was identified as the most upregulated gene in human asthmatic bronchi [88]. A major downstream effect of IL-4 and IL-13 signaling is the activation of the signal transducer and activator of transcription 6 (STAT6). Following ligand-receptor binding, associated Janus kinases (JAKs) activate the receptor, allowing STAT6 to then be recruited and activated by phosphorylation. Once phosphorylated, STAT6 homodimerizes and translocates to the nucleus where it regulates the transcription of target genes via binding to N4 interferon-γ activated sequences (N_4 GAS) in the promoter region [89]. The pendrin promoter contains at least one N_4 GAS motif, and STAT6 has been shown to bind this sequence *in vitro*, thus suggesting that increases in pendrin promoter activity via STAT6 represent at least one mechanism by which IL-4 and IL-13 increase pendrin activity [87]. Cytokines other than IL-4 and IL-13 may be responsible for increases in pendrin expression; IL-1β, a macrophage-secreted cytokine involved in the immunopathogenesis of asthma and COPD, has also been shown to increase pendrin levels in rodent and human bronchial epithelial cells [74, 90].

Signaling through IL-4/IL-13 mediates airway hyperresponsiveness, eosinophilic inflammation, mucus cell metaplasia and mucus overproduction, subepithelial fibrosis, and increased viscosity of the airway surface liquid (ASL), all of which are common to bronchial asthma and COPD [91]. Pendrin may play a major role in the pathogenesis of asthma or COPD by regulating some of these responses, in particular ASL thickness and mucus production (Figure 5). In lung epithelial cells, reabsorptive Na^+ transport through ENaC is suppressed whereas secretory Cl^- transport through CFTR and CaCCs is stimulated, which collectively results in a net secretory phenotype whereby water osmotically flows into the lumen and ASL viscosity decreases. On the other hand, pendrin imports Cl^- in exchange for other anions, and thus an IL-4/IL-13-mediated increase in pendrin activity may shift the equilibrium towards a reabsorptive phenotype, resulting in the osmotic flow of water into the interstitium and the thinning of the ASL [85]. In asthmatic mice, mucus overproduction is accompanied by an increased pendrin expression at the apical surface of bronchial epithelial cells,

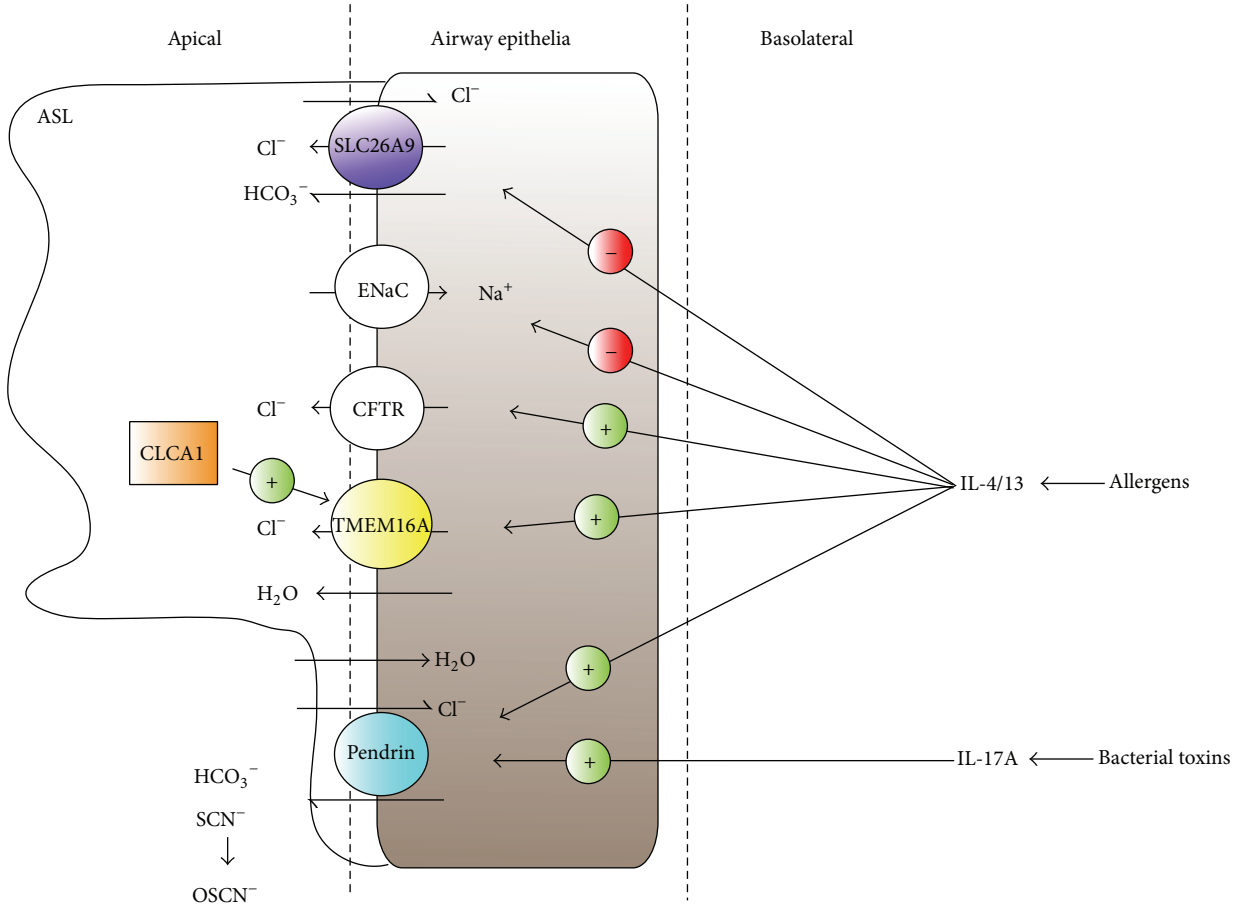

Figure 5: Roles of ion channels in the airway epithelium. In response to allergens and other asthma and COPD exacerbating factors, Th2-cytokines IL-4 and IL-13 induce a water secretory phenotype by stimulating Cl^- secretion via CFTR and Ca^{2+}-activated Cl^- channels such as TMEM16A and by decreasing Na^+ and Cl^- reabsorption via ENaC and SLC26A9, respectively, which leads to the thickening of the airway surface liquid (ASL). TMEM16A activity can be increased by secreted CLCA1 protein. In concert, signaling through IL-4/IL-13 increases the functional expression of pendrin, which results in reabsorption of water and thinning of the ASL. Pendrin can also increase the secretion of thiocyanate (SCN^-), a substrate for the production of the antimicrobial agent hypothiocyanite ($OSCN^-$) by the lactoperoxidase system. Toxins from *B. pertussis* and other bacteria trigger an IL-17A-mediated inflammatory host response in the lung epithelium, which is characterized by a significant upregulation of pendrin activity.

and in pendrin overexpression cell models, production of MUC5AC, a major mucus protein in asthma and COPD patients, is increased [86]. In mice, pendrin overexpression is also accompanied by neutrophil-dominant inflammation, suggesting that, in this system, mucus production may be induced not only by a direct effect of pendrin on airway epithelial cells, but also by an indirect effect of pendrin by recruiting inflammatory neutrophils [86].

In bronchial epithelial cells, IL-4/IL-13 signaling upregulates the expression of CLCA1 [9], CFTR [92], and pendrin [87], whereas it downregulates the expression of the β and γ subunits of ENaC. Because upregulation of certain proteins, such as pendrin, might aggravate asthma or COPD symptoms, whereas the downregulation of ENaC and the downregulation of CLCA1 might be protective (Figure 5), it is unsurprising that pharmacological strategies aimed at the blocking of the IL-4/IL-13 pathway are not as successful as they were anticipated to be [93]. Selective inhibition of pendrin could be an intriguing new strategy for asthma/COPD therapy, but as noted above, pendrin contributes to the secretion of SCN^-, a substrate of lactoperoxidase for the production of the protective, antimicrobial $OSCN^-$ [74], and this should be taken into account when exploring novel treatment avenues.

4.3. The Next Frontier: Pendrin and Infectious Lung Disease. Most recently, pendrin has been implicated in the IL-17A-dependent host inflammatory response to bacterial airway infections (Figure 5) [56, 57]. IL-17A is critical for the immune response of the lung to infectious bacteria, for example, *Haemophilus influenzae*, *Staphylococcus aureus*, *Klebsiella pneumoniae,* and *Bordetella pertussis* [94]. The latter is the etiologic agent of whooping cough, or pertussis disease, which is a resurgent condition of great clinical concern as it can progress to pulmonary inflammation and death in infants and for which there is no effective treatment [95]. Pertussis toxin (PT), the virulence factor of *B. pertussis*, undermines the host immune system by inhibiting macrophage and neutrophil

responses, suppressing the production of antibodies against the bacteria, and inducing proinflammatory cytokines, in particular IL-17A [96]. One of the most highly upregulated genes in association with PT activity is *SLC26A4* [97, 98], and pendrin is upregulated in human bronchial epithelial cells exposed to IL-17A [56]. In the lungs of *B. pertussis*-infected mice there is an increase in pendrin levels that is concomitant with an increase in IL-17A but not in IL-4/IL-13 levels, and pendrin upregulation is significantly hampered in *Il-17a*-null mice [57]. Other host factors may be involved in PT-dependent upregulation of pendrin, such as IL-1β and IFN-γ, as both are upregulated during *B. pertussis* infection and have been linked with pendrin upregulation [85, 98, 99]. Taken together, these recent advances suggest that the upregulation of pendrin, with its associated inflammatory pathology, is a major mechanism of virulence for the pertussis toxin and position pendrin as a potential novel therapeutic target for the treatment of whooping cough.

4.4. SLC26A9: A Novel CFTR Regulator. SLC26A9 is another member of the SLC26 family of anion exchangers and channels. It is robustly expressed in apical airway epithelia [100] and gastric parietal cells [101] and to a lesser extent in the kidney, brain, and reproductive tracts [59, 102–104]. The function of SLC26A9 is still unclear: it has been described as a Cl^- channel with a small degree of bicarbonate transport [104], a Cl^- channel or a Cl^-/HCO_3^- exchanger [105, 106]. One group reported increased Cl^- conductance particularly in high bicarbonate conditions [107], and others have found that SLC26A9 activity is coupled to Na^+ transport [58, 103, 108]. Like other members of the family, SLC26A9 is predicted to span the membrane 14 times, and it contains a STAS domain followed by a PSD95-Dlg1-Zo-1 (PDZ) domain in the C-terminal cytoplasmic region (Figure 4) [59, 109]. Two groups propose that SLC26 proteins, including SLC26A9, interact with CFTR initially via their PDZ domains [110, 111]. This is followed by a stronger interaction between the CFTR R domain and the SLC26 STAS domain, which is enhanced by PKA-dependent phosphorylation of the R domain [111–113]. The interaction between SLC26A9 and CFTR has been described in multiple studies. However, whether the interaction is stimulatory or inhibitory is still controversial and may be cell type-dependent [108, 114]. Not much is known about the regulation of SLC26A9, but WNK kinases, also known to regulate other transporters and channels involved in osmoregulation, have been shown to inhibit SLC26A9 activity via interaction with the STAS domain [109]. Though the influence of SLC26A9 on CFTR has been reported, the reciprocal interaction is less clear. Multiple groups have described CFTR regulation of SLC26A9 activity and expression, but the results are not consistent [58, 114–118]. Evidence of CFTR and SLC26A9 coexpression has been found in the lung, trachea, stomach, and sweat gland [119].

Due to its high expression in the lung, numerous studies have investigated the role of SLC26A9 in lung disease. Anagnostopoulou and colleagues [120] first reported that SLC26A9 activity is responsible for increased constitutive Cl^- current under Th2 inflammatory conditions, but not in normal physiology. The authors also found that SLC26A9 prevents airway mucous obstruction after stimulation with IL-13. These changes were due to increased SLC26A9 activity, which may be due to changes in regulation by WNK kinases [104]. Unlike CFTR and TMEM16A, SLC26A9 is downregulated in patients with allergic asthma (Figure 4). Further investigation revealed that a SNP in the $3'$ UTR of the *SLC26A9* gene likely reduced expression levels in these patients, possibly through enhanced binding of hsa-miR-632 [120].

SLC26A9 has also been implicated in the pathogenesis of bronchiectasis, the widening of airways frequently due to mucous obstruction, a condition often seen in patients with cystic fibrosis. A recent report [119] identified two patients with diffuse idiopathic bronchiectasis who also had mutations in the *SLC26A9* gene. One patient presented with a mutation in a transmembrane domain of SLC26A9 (V486I); the patient's brother was asymptomatic, though he had the same mutation. The second patient presented with a mutation in the STAS domain of SLC26A9 (R575W) in addition to the Fdel508 mutation in CFTR. Coexpression of both mutants in *Xenopus* oocytes provided evidence of a decreased interaction between the SLC26A9 STAS domain and the R domain of CFTR. It is thought that wild-type SLC26A9, in conjunction with CFTR loss, may enhance ion conductance and fluid secretion [121]. Thus, loss of SLC26A9, in the setting of CFTR loss, may result in reduced airway surface liquid hydration, mucous blockage, and consequent bronchiectasis. The authors of the study further narrow the CFTR-SLC26A9 interaction region to a peptide within the STAS domain but do not confirm that the R575W mutation in this peptide disrupts the interaction and activation [119]. In contrast, a second group did not report changes in Cl^- transport with the R575W mutation [122]. As the second patient's daughter only carried the Fdel508 mutation and was asymptomatic, the authors speculate that one mutation in CFTR is not sufficient to produce the CF phenotype [119]. However, mutations in modifier genes, such as SLC26A9, may contribute to CF in those heterozygous for CFTR mutations. Thus, SLC26A9 may influence phenotypic expression of heterozygous mutations in ion channels (CFTR, ENaC, and others) involved in airway surface liquid hydration [119].

Further supporting the modifier gene hypothesis, some SLC26A9 mutations have been shown to increase the risk of meconium ileus in patients with CF [123], as well as CF-related diabetes onset [124] and pancreatic disease severity [108, 125]. In the case of CFTR and SLC26A9 double mutations, the exact mechanism causing the phenotype must be further investigated as Cl^- transport could be due to altered CFTR and/or SLC26A9 function or to impaired regulation of CFTR by SLC26A9 or vice versa [119, 124]. Understanding the various mechanisms of SLC26A9 mutations will be important towards developing therapies that can improve lung diseases such as asthma, CF, and bronchiectasis.

5. Conclusions

Chloride transport proteins play crucial roles in airway health and disease. On one hand, they contribute to proper

mucus function by controlling mucus hydration and pH via controlling chloride and bicarbonate ion transport. On the other hand, they may play a direct role in mucus synthesis, secretion, and AHR. Recent animal model studies have emphasized the crucial role that mucus and mucin proteins play in innate mucosal immunology. Deletion of MUC5B (the main secreted mucin protein produced in the airway under homeostatic conditions) results in impaired mucociliary clearance and increased microbial infection [126]. Knockout of CFTR in pig results in airway mucus that is more acidic and deficient in antimicrobial activity due to loss of defensin function at low pH [3, 127] and is also deficient in mucociliary clearance as it remains tethered to secreting cells [128]. This could be due to improper proteolytic processing of mucin proteins, since loss of CFTR function in the intestine impairs β-meprin processing and release of secreted mucins in that setting [129]. Thus a complete understanding of how these channels contribute to mucus synthesis, secretion, function, and mucociliary clearance is required to understand the impact of modulating their activity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding publication of this paper.

Authors' Contribution

Monica Sala-Rabanal and Zeynep Yurtsever contributed equally to this work.

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References

[1] A. S. Verkman and L. J. Galietta, "Chloride channels as drug targets," *Nature Reviews Drug Discovery*, vol. 8, pp. 153–171, 2009.

[2] J. V. Fahy and B. F. Dickey, "Airway mucus function and dysfunction," *The New England Journal of Medicine*, vol. 363, no. 23, pp. 2233–2247, 2010.

[3] A. A. Pezzulo, X. X. Tang, M. J. Hoegger et al., "Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung," *Nature*, vol. 487, no. 7405, pp. 109–113, 2012.

[4] W. W. Busse and R. F. Lemanske Jr., "Asthma," *The New England Journal of Medicine*, vol. 344, pp. 350–362, 2001.

[5] M. E. Loewen and G. W. Forsyth, "Structure and function of CLCA proteins," *Physiological Reviews*, vol. 85, no. 3, pp. 1061–1092, 2005.

[6] A. Nakanishi, S. Morita, H. Iwashita et al., "Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 9, pp. 5175–5180, 2001.

[7] A. C. Patel, J. D. Morton, E. Y. Kim et al., "Genetic segregation of airway disease traits despite redundancy of calcium-activated chloride channel family members," *Physiological Genomics*, vol. 25, no. 3, pp. 502–513, 2006.

[8] M. Hoshino, S. Morita, H. Iwashita et al., "Increased expression of the human Ca^{2+}-activated Cl^- channel 1 (CaCC1) gene in the asthmatic airway," *American Journal of Respiratory and Critical Care Medicine*, vol. 165, no. 8, pp. 1132–1136, 2002.

[9] Y. G. Alevy, A. C. Patel, A. G. Romero et al., "IL-13-induced airway mucus production is attenuated by MAPK13 inhibition," *The Journal of Clinical Investigation*, vol. 122, pp. 4555–4568, 2012.

[10] L. Mundhenk, B. Johannesson, P. Anagnostopoulou et al., "mCLCA3 does not contribute to calcium-activated chloride conductance in murine airways," *American Journal of Respiratory Cell and Molecular Biology*, vol. 47, no. 1, pp. 87–93, 2012.

[11] A. C. Patel, T. J. Brett, and M. J. Holtzman, "The role of CLCA proteins in inflammatory airway disease," *Annual Review of Physiology*, vol. 71, pp. 425–449, 2009.

[12] M. C. Peters, Z. K. Mekonnen, S. Yuan, N. R. Bhakta, P. G. Woodruff, and J. V. Fahy, "Measures of gene expression in sputum cells can identify T_H2-high and T_H2-low subtypes of asthma," *Journal of Allergy and Clinical Immunology*, vol. 133, no. 2, pp. 388.e5–394.e5, 2014.

[13] A. Gibson, A. P. Lewis, K. Affleck, A. J. Aitken, E. Meldrum, and N. Thompson, "HCLCA1 and mCLCA3 are secreted non-integral membrane proteins and therefore are not ion channels," *Journal of Biological Chemistry*, vol. 280, no. 29, pp. 27205–27212, 2005.

[14] S. A. Cunningham, M. S. Awayda, J. K. Bubien et al., "Cloning of an epithelial chloride channel from bovine trachea," *The Journal of Biological Chemistry*, vol. 270, no. 52, pp. 31016–31026, 1995.

[15] R. Gandhi, R. C. Elble, A. D. Gruber et al., "Molecular and functional characterization of a calcium-sensitive chloride channel from mouse lung," *Journal of Biological Chemistry*, vol. 273, no. 48, pp. 32096–32101, 1999.

[16] A. D. Gruber, R. C. Elble, H.-L. Ji, K. D. Schreur, C. M. Fuller, and B. U. Pauli, "Genomic cloning, molecular characterization, and functional analysis of human CLCA1, the first human member of the family of Ca^{2+}-activated Cl- channel proteins," *Genomics*, vol. 54, no. 2, pp. 200–214, 1998.

[17] T. Nakano, H. Inoue, S. Fukuyama et al., "Niflumic acid suppresses interleukin-13–induced asthma phenotypes," *American Journal of Respiratory and Critical Care Medicine*, vol. 173, no. 11, pp. 1216–1221, 2006.

[18] Z. Yurtsever, M. Sala-Rabanal, D. T. Randolph et al., "Self-cleavage of human CLCA1 protein by a novel internal metalloprotease domain controls calcium-activated chloride channel activation," *The Journal of Biological Chemistry*, vol. 287, no. 50, pp. 42138–42149, 2012.

[19] M. Hamann, A. Gibson, N. Davies et al., "Human ClCa1 modulates anionic conduction of calcium-dependent chloride currents," *Journal of Physiology*, vol. 587, no. 10, pp. 2255–2274, 2009.

[20] M. Sala-Rabanal, Z. Yurtsever, C. G. Nichols, and T. J. Brett, "Secreted CLCA1 modulates TMEM16A to activate Ca^{2+}-dependent chloride currents in human cells," *eLife*, vol. 4, Article ID e05875, 2015.

[21] M. K. Bothe, L. Mundhenk, M. Kaup, C. Weise, and A. D. Gruber, "The murine goblet cell protein mCLCA3 is a zinc-dependent metalloprotease with autoproteolytic activity," *Molecules and Cells*, vol. 32, no. 6, pp. 535–541, 2011.

[22] A. J. Long, J. P. Sypek, R. Askew et al., "Gob-5 contributes to goblet cell hyperplasia and modulates pulmonary tissue inflammation," *American Journal of Respiratory Cell and Molecular Biology*, vol. 35, no. 3, pp. 357–365, 2006.

[23] K. Dietert, K. Reppe, L. Mundhenk, M. Witzenrath, and A. D. Gruber, "mCLCA3 modulates IL-17 and CXCL-1 induction and leukocyte recruitment in murine *Staphylococcus aureus* pneumonia," *PLoS ONE*, vol. 9, no. 7, Article ID e102606, 2014.

[24] J. C. H. Ching, L. Lobanova, and M. E. Loewen, "Secreted hCLCA1 is a signaling molecule that activates airway macrophages," *PLoS ONE*, vol. 8, no. 12, Article ID e83130, 2013.

[25] A. Caputo, E. Caci, L. Ferrera et al., "TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity," *Science*, vol. 322, no. 5901, pp. 590–594, 2008.

[26] B. C. Schroeder, T. Cheng, Y. N. Jan, and L. Y. Jan, "Expression cloning of TMEM16A as a calcium-activated chloride channel subunit," *Cell*, vol. 134, no. 6, pp. 1019–1029, 2008.

[27] Y. D. Yang, H. Cho, J. Y. Koo et al., "TMEM16A confers receptor-activated calcium-dependent chloride conductance," *Nature*, vol. 455, no. 7217, pp. 1210–1215, 2008.

[28] R. Schreiber, I. Uliyakina, P. Kongsuphol et al., "Expression and function of epithelial anoctamins," *The Journal of Biological Chemistry*, vol. 285, no. 10, pp. 7838–7845, 2010.

[29] Y. Tian, R. Schreiber, and K. Kunzelmann, "Anoctamins are a family of Ca^{2+}-activated Cl^- channels," *Journal of Cell Science*, vol. 125, no. 21, pp. 4991–4998, 2012.

[30] F. Huang, J. R. Rock, B. D. Harfe et al., "Studies on expression and function of the TMEM16A calcium-activated chloride channel," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 50, pp. 21413–21418, 2009.

[31] J. D. Brunner, N. K. Lim, S. Schenck, A. Duerst, and R. Dutzler, "X-ray structure of a calcium-activated TMEM16 lipid scramblase," *Nature*, vol. 516, no. 7530, pp. 207–212, 2014.

[32] J. Tien, H. Y. Lee, D. L. Minor Jr., Y. N. Jan, and L. Y. Jan, "Identification of a dimerization domain in the TMEM16A calcium-activated chloride channel (CaCC)," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 16, pp. 6352–6357, 2013.

[33] H. Terashima, A. Picollo, and A. Accardi, "Purified TMEM16A is sufficient to form Ca^{2+}-activated Cl^- channels," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 48, pp. 19354–19359, 2013.

[34] C. J. Peters, H. Yu, J. Tien, Y. N. Jan, M. Li, and L. Y. Jan, "Four basic residues critical for the ion selectivity and pore blocker sensitivity of TMEM16A calcium-activated chloride channels," *Proceedings of the National Academy of Sciences*, vol. 112, no. 11, pp. 3547–3552, 2015.

[35] J. Tien, C. J. Peters, X. M. Wong et al., "A comprehensive search for calcium binding sites critical for TMEM16A calcium-activated chloride channel activity," *eLife*, 2014.

[36] J. Jung, J. H. Nam, H. W. Park, U. Oh, J.-H. Yoon, and M. G. Lee, "Dynamic modulation of ANO1/TMEM16A HCO_3^- permeability by Ca^{2+}/calmodulin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 1, pp. 360–365, 2013.

[37] Y. Yu, A.-S. Kuan, and T.-Y. Chen, "Calcium-calmodulin does not alter the anion permeability of the mouse TMEM16A calcium-activated chloride channel," *Journal of General Physiology*, vol. 144, no. 1, pp. 115–124, 2014.

[38] J. Lin, Y. Jiang, L. Li, Y. Liu, H. Tang, and D. Jiang, "TMEM16A mediates the hypersecretion of mucus induced by Interleukin-13," *Experimental Cell Research*, vol. 334, no. 2, pp. 260–269, 2015.

[39] A. Mazzone, S. J. Gibbons, C. E. Bernard et al., "Identification and characterization of a novel promoter for the human *ANO1* gene regulated by the transcription factor signal transducer and activator of transcription 6 (STAT6)," *The FASEB Journal*, vol. 29, no. 1, pp. 152–163, 2015.

[40] W. Namkung, P.-W. Phuan, and A. S. Verkman, "TMEM16A inhibitors reveal TMEM16A as a minor component of calcium-activated chloride channel conductance in airway and intestinal epithelial cells," *Journal of Biological Chemistry*, vol. 286, no. 3, pp. 2365–2374, 2011.

[41] P. Scudieri, E. Caci, S. Bruno et al., "Association of TMEM16A chloride channel overexpression with airway goblet cell metaplasia," *Journal of Physiology*, vol. 590, no. 23, pp. 6141–6155, 2012.

[42] F. Huang, H. Zhang, M. Wu et al., "Calcium-activated chloride channel TMEM16A modulates mucin secretion and airway smooth muscle contraction," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 40, pp. 16354–16359, 2012.

[43] R. J. Lee and J. K. Foskett, "Mechanisms of Ca^{2+}-stimulated fluid secretion by porcine bronchial submucosal gland serous acinar cells," *The American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 298, no. 2, pp. L210–L231, 2010.

[44] Y. Zhang, X. Wang, H. Wang et al., "TMEM16A-mediated mucin secretion in IL-13-induced nasal epithelial cells from chronic rhinosinusitis patients," *Allergy, Asthma & Immunology Research*, vol. 7, no. 4, pp. 367–375, 2015.

[45] W. Namkung, Z. Yao, W. E. Finkbeiner, and A. S. Verkman, "Small-molecule activators of TMEM16A, a calcium-activated chloride channel, stimulate epithelial chloride secretion and intestinal contraction," *The FASEB Journal*, vol. 25, no. 11, pp. 4048–4062, 2011.

[46] I. Leverkoehne and A. D. Gruber, "The murine mCLCA3 (alias gob-5) protein is located in the mucin granule membranes of intestinal, respiratory, and uterine goblet cells," *Journal of Histochemistry and Cytochemistry*, vol. 50, no. 6, pp. 829–838, 2002.

[47] K. L. Raiford, J. Park, K.-W. Lin, S. Fang, A. L. Crews, and K. B. Adler, "Mucin granule-associated proteins in human bronchial epithelial cells: the airway goblet cell 'granulome'," *Respiratory Research*, vol. 12, article 118, 2011.

[48] L. Q. Song, Y. Li, W. N. Li, W. Zhang, H. W. Qi, and C. G. Wu, "Safety and immunogenicity of a DNA vaccine encoding human calcium-activated chloride channel 1 (hCLCA1) in asthmatic mice," *International Archives of Allergy and Immunology*, vol. 161, no. 3, pp. 243–251, 2013.

[49] L. Song, D. Liu, C. Wu et al., "Antibody to mCLCA3 suppresses symptoms in a mouse model of asthma," *PLoS ONE*, vol. 8, no. 12, Article ID e82367, 2013.

[50] D. M. Boedtkjer, S. Kim, A. B. Jensen, V. M. Matchkov, and K. E. Andersson, "New selective inhibitors of calcium-activated chloride channels—T16Ainh-A01, CaCCinh-A01 and MONNA—what do they inhibit?" *British Journal of Pharmacology*, vol. 172, no. 16, pp. 4158–4172, 2015.

[51] A. Ghosh, R. C. Boucher, and R. Tarran, "Airway hydration and COPD," *Cellular and Molecular Life Sciences*, vol. 72, no. 19, pp. 3637–3652, 2015.

[52] E. Sondo, E. Caci, and L. J. V. Galietta, "The TMEM16A chloride channel as an alternative therapeutic target in cystic fibrosis," *International Journal of Biochemistry and Cell Biology*, vol. 52, pp. 73–76, 2014.

[53] C. Nofziger, S. Dossena, S. Suzuki, K. Izuhara, and M. Paulmichl, "Pendrin function in airway epithelia," *Cellular Physiology and Biochemistry*, vol. 28, no. 3, pp. 571–578, 2011.

[54] S. Seshadri, X. Lu, M. R. Purkey et al., "Increased expression of the epithelial anion transporter pendrin/SLC26A4 in nasal polyps of patients with chronic rhinosinusitis," *The Journal of Allergy and Clinical Immunology*, 2015.

[55] A. Ishida, N. Ohta, Y. Suzuki et al., "Expression of pendrin periostin in allergic rhinitis chronic rhinosinusitis," *Allergology International*, vol. 61, no. 4, pp. 589–595, 2012.

[56] K. M. Adams, V. Abraham, D. Spielman et al., "IL-17A induces Pendrin expression and chloride-bicarbonate exchange in human bronchial epithelial cells," *PLoS ONE*, vol. 9, no. 8, Article ID e103263, 2014.

[57] K. M. Scanlon, Y. Gau, J. Zhu et al., "Epithelial anion transporter pendrin contributes to inflammatory lung pathology in mouse models of *Bordetella pertussis* infection," *Infection and Immunity*, vol. 82, no. 10, pp. 4212–4221, 2014.

[58] E. El Khouri and A. Touré, "Functional interaction of the cystic fibrosis transmembrane conductance regulator with members of the SLC26 family of anion transporters (SLC26A8 and SLC26A9): physiological and pathophysiological relevance," *International Journal of Biochemistry and Cell Biology*, vol. 52, pp. 58–67, 2014.

[59] S. L. Alper and A. K. Sharma, "The SLC26 gene family of anion transporters and channels," *Molecular Aspects of Medicine*, vol. 34, no. 2-3, pp. 494–515, 2013.

[60] D. B. Mount and M. F. Romero, "The SLC26 gene family of multifunctional anion exchangers," *Pflügers Archiv*, vol. 447, no. 5, pp. 710–721, 2004.

[61] D. Gorbunov, M. Sturlese, F. Nies et al., "Molecular architecture and the structural basis for anion interaction in prestin and SLC26 transporters," *Nature Communications*, vol. 5, article 3622, 2014.

[62] E. Ohana, D. Yang, N. Shcheynikov, and S. Muallem, "Diverse transport modes by the solute carrier 26 family of anion transporters," *The Journal of Physiology*, vol. 587, no. 10, pp. 2179–2185, 2009.

[63] E. R. Geertsma, Y. Chang, F. R. Shaik et al., "Structure of a prokaryotic fumarate transporter reveals the architecture of the SLC26 family," *Nature Structural & Molecular Biology*, vol. 22, no. 10, pp. 803–808, 2015.

[64] R. Hallworth and M. G. Nichols, "Prestin in HEK cells is an obligate tetramer," *Journal of Neurophysiology*, vol. 107, no. 1, pp. 5–11, 2012.

[65] L. Aravind and E. V. Koonin, "The STAS domain—a link between anion transporters and antisigma-factor antagonists," *Current Biology*, vol. 10, no. 2, pp. R53–R54, 2000.

[66] B. Y. Choi, H.-M. Kim, T. Ito et al., "Mouse model of enlarged vestibular aqueducts defines temporal requirement of Slc26a4 expression for hearing acquisition," *The Journal of Clinical Investigation*, vol. 121, no. 11, pp. 4516–4525, 2011.

[67] J.-M. Bidart, C. Mian, V. Lazar et al., "Expression of pendrin and the Pendred syndrome (PDS) gene in human thyroid tissues," *Journal of Clinical Endocrinology and Metabolism*, vol. 85, no. 5, pp. 2028–2033, 2000.

[68] I. E. Royaux, K. Suzuki, A. Mori et al., "Pendrin, the protein encoded by the pendred syndrome gene (PDS), is an apical porter of iodide in the thyroid and is regulated by thyroglobulin in FRTL-5 cells," *Endocrinology*, vol. 141, no. 2, pp. 839–845, 2000.

[69] I. E. Royaux, S. M. Wall, L. P. Karniski et al., "Pendrin, encoded by the pendred syndrome gene, resides in the apical region of renal intercalated cells and mediates bicarbonate secretion," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 7, pp. 4221–4226, 2001.

[70] N. Shcheynikov, D. Yang, Y. Wang et al., "The Slc26a4 transporter functions as an electroneutral $Cl^-/I^-/HCO_3^-$ exchanger: role of Slc26a4 and Slc26a6 in I^- and HCO_3^- secretion and in regulation of CFTR in the parotid duct," *Journal of Physiology*, vol. 586, no. 16, pp. 3813–3824, 2008.

[71] P. Wangemann, "Mouse models for pendrin-associated loss of cochlear and vestibular function," *Cellular Physiology and Biochemistry*, vol. 32, no. 7, pp. 157–165, 2013.

[72] P. Fong, "Thyroid iodide efflux: a team effort?" *Journal of Physiology*, vol. 589, no. 24, pp. 5929–5939, 2011.

[73] J. Rozenfeld, O. Tal, O. Kladnitsky et al., "Pendrin, a novel transcriptional target of the uroguanylin system," *Cellular Physiology and Biochemistry*, vol. 32, no. 7, pp. 221–237, 2013.

[74] N. Pedemonte, E. Caci, E. Sondo et al., "Thiocyanate transport in resting and IL-4-stimulated human bronchial epithelial cells: role of pendrin and anion channels," *The Journal of Immunology*, vol. 178, no. 8, pp. 5144–5153, 2007.

[75] A. J. Ratner and A. Prince, "Lactoperoxidase: new recognition of an "old" enzyme in airway defenses," *American Journal of Respiratory Cell and Molecular Biology*, vol. 22, no. 6, pp. 642–644, 2000.

[76] B. Coyle, R. Coffey, J. A. L. Armour et al., "Pendred syndrome (goitre and sensorineural hearing loss) maps to chromosome 7 in the region containing the nonsyndromic deafness gene DFNB4," *Nature Genetics*, vol. 12, no. 4, pp. 421–423, 1996.

[77] P. Kopp and A. Bizhanova, "Clinical and molecular characteristics of Pendred syndrome," *Annales d'Endocrinologie*, vol. 72, no. 2, pp. 88–94, 2011.

[78] V. C. Sheffield, Z. Kraiem, J. C. Beck et al., "Pendred syndrome maps to chromosome 7q21-34 and is caused by an intrinsic defect in thyroid iodine organification," *Nature Genetics*, vol. 12, no. 4, pp. 424–426, 1996.

[79] X. C. Li, L. A. Everett, A. K. Lalwani et al., "A mutation in PDS causes non-syndromic recessive deafness," *Nature Genetics*, vol. 18, no. 3, pp. 215–217, 1998.

[80] A. Bizhanova and P. Kopp, "Genetics and phenomics of Pendred syndrome," *Molecular and Cellular Endocrinology*, vol. 322, no. 1-2, pp. 83–90, 2010.

[81] E. Di Valentin, C. Crahay, N. Garbacki et al., "New asthma biomarkers: lessons from murine models of acute and chronic asthma," *The American Journal of Physiology: Lung Cellular and Molecular Physiology*, vol. 296, no. 2, pp. L185–L197, 2009.

[82] K. Izuhara, S. Ohta, H. Shiraishi et al., "The mechanism of mucus production in bronchial asthma," *Current Medicinal Chemistry*, vol. 16, no. 22, pp. 2867–2875, 2009.

[83] D. A. Kuperman, C. C. Lewis, and P. G. Woodruff, "Dissecting asthma using focused transgenic modeling and functional genomics," *The Journal of Allergy and Clinical Immunology*, vol. 116, no. 2, pp. 305–311, 2005.

[84] C. C. Lewis, J. Y. Yang, X. Huang et al., "Disease-specific gene expression profiling in multiple models of lung disease," *American Journal of Respiratory and Critical Care Medicine*, vol. 177, no. 4, pp. 376–387, 2008.

[85] Y. Nakagami, S. Favoreto Jr., G. Zhen et al., "The epithelial anion transporter pendrin is induced by allergy and rhinovirus infection, regulates airway surface liquid, and increases airway reactivity and inflammation in an asthma model," *Journal of Immunology*, vol. 181, no. 3, pp. 2203–2210, 2008.

[86] I. Nakao, S. Kanaji, S. Ohta et al., "Identification of pendrin as a common mediator for mucus production in bronchial asthma and chronic obstructive pulmonary disease," *Journal of Immunology*, vol. 180, no. 9, pp. 6262–6269, 2008.

[87] C. Nofziger, V. Vezzoli, S. Dossena et al., "STAT6 links IL-4/IL-13 stimulation with pendrin expression in asthma and chronic obstructive pulmonary disease," *Clinical Pharmacology and Therapeutics*, vol. 90, no. 3, pp. 399–405, 2011.

[88] C. Y. Yick, A. H. Zwinderman, P. W. Kunst et al., "Transcriptome sequencing (RNA-Seq) of human endobronchial biopsies: asthma versus controls," *European Respiratory Journal*, vol. 42, no. 3, pp. 662–670, 2013.

[89] N. C. Reich, "STATs get their move on," *JAK-STAT*, vol. 2, no. 4, Article ID e27080, 2013.

[90] U. Lappalainen, J. A. Whitsett, S. E. Wert, J. W. Tichelaar, and K. Bry, "Interleukin-1β causes pulmonary inflammation, emphysema, and airway remodeling in the adult murine lung," *American Journal of Respiratory Cell and Molecular Biology*, vol. 32, no. 4, pp. 311–318, 2005.

[91] D. F. Roger, "Physiology of airway mucus secretion and pathophysiology of hypersecretion," *Respiratory Care*, vol. 52, no. 9, pp. 1134–1146, 2007.

[92] L. J. V. Galietta, P. Pagesy, C. Folli et al., "IL-4 is a potent modulator of ion transport in the human bronchial epithelium in vitro," *Journal of Immunology*, vol. 168, no. 2, pp. 839–845, 2002.

[93] S. T. Holgate, "A look at the pathogenesis of asthma: the need for a change in direction," *Discovery Medicine*, vol. 9, no. 48, pp. 439–447, 2010.

[94] J. P. McAleer and J. K. Kolls, "Directing traffic: IL-17 and IL-22 coordinate pulmonary immune defense," *Immunological Reviews*, vol. 260, no. 1, pp. 129–144, 2014.

[95] J. T. Berger, J. A. Carcillo, T. P. Shanley et al., "Critical pertussis illness in children: a multicenter prospective cohort study," *Pediatric Critical Care Medicine*, vol. 14, no. 4, pp. 356–365, 2013.

[96] C. Andreasen, D. A. Powell, and N. H. Carbonetti, "Pertussis toxin stimulates IL-17 production in response to *Bordetella pertussis* infection in mice," *PLoS ONE*, vol. 4, no. 9, Article ID e7079, 2009.

[97] S. Banus, J. Pennings, R. Vandebriel et al., "Lung response to *Bordetella pertussis* infection in mice identified by gene-expression profiling," *Immunogenetics*, vol. 59, no. 7, pp. 555–564, 2007.

[98] C. E. Connelly, Y. Sun, and N. H. Carbonetti, "Pertussis toxin exacerbates and prolongs airway inflammatory responses during *Bordetella pertussis* infection," *Infection and Immunity*, vol. 80, no. 12, pp. 4317–4332, 2012.

[99] A. Hogmalm, M. Bry, B. Strandvik, and K. Bry, "IL-1β expression in the distal lung epithelium disrupts lung morphogenesis and epithelial cell differentiation in fetal mice," *American Journal of Physiology: Lung Cellular and Molecular Physiology*, vol. 306, no. 1, pp. L23–L34, 2014.

[100] H. Lohi, M. Kujala, S. Mäkelä et al., "Functional characterization of three novel tissue-specific anion exchangers SLC26A7, -A8, and -A9," *The Journal of Biological Chemistry*, vol. 277, no. 16, pp. 14246–14254, 2002.

[101] J. Xu, P. Song, M. L. Miller et al., "Deletion of the chloride transporter Slc26a9 causes loss of tubulovesicles in parietal cells and impairs acid secretion in the stomach," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 46, pp. 17955–17960, 2008.

[102] H. Amlal, J. Xu, S. Barone, K. Zahedi, and M. Soleimani, "The chloride channel/transporter Slc26a9 regulates the systemic arterial pressure and renal chloride excretion," *Journal of Molecular Medicine*, vol. 91, no. 5, pp. 561–572, 2013.

[103] M. H. Chang, C. Plata, K. Zandi-Nejad et al., "Slc26a9—anion exchanger, channel and Na^+ transporter," *Journal of Membrane Biology*, vol. 228, no. 3, pp. 125–140, 2009.

[104] M. R. Dorwart, N. Shcheynikov, Y. Wang, S. Stippec, and S. Muallem, "SLC26A9 is a Cl^- channel regulated by the WNK kinases," *The Journal of Physiology*, vol. 584, no. 1, pp. 333–345, 2007.

[105] E. S. Demitrack, M. Soleimani, and M. H. Montrose, "Damage to the gastric epithelium activates cellular bicarbonate secretion via SLC26A9 Cl^-/HCO_3^- exchange," *American Journal of Physiology: Gastrointestinal and Liver Physiology*, vol. 299, no. 1, pp. G255–G264, 2010.

[106] J. Xu, J. Henriksnäs, S. Barone et al., "SLC26A9 is expressed in gastric surface epithelial cells, mediates Cl^-/HCO_3^- exchange, and is inhibited by NH_4^+," *American Journal of Physiology—Cell Physiology*, vol. 289, no. 2, pp. C493–C505, 2005.

[107] C. Loriol, S. Dulong, M. Avella et al., "Characterization of SLC26A9, facilitation of Cl^- transport by bicarbonate," *Cellular Physiology and Biochemistry*, vol. 22, no. 1–4, pp. 15–30, 2008.

[108] X. Liu, T. Li, B. Riederer et al., "Loss of Slc26a9 anion transporter alters intestinal electrolyte and HCO_3^- transport and reduces survival in CFTR-deficient mice," *Pflügers Archiv—European Journal of Physiology*, vol. 467, no. 6, pp. 1261–1275, 2015.

[109] M. A. Mall and L. J. Galietta, "Targeting ion channels in cystic fibrosis," *Journal of Cystic Fibrosis*, vol. 14, no. 5, pp. 561–570, 2015.

[110] M. N. Chernova, L. Jiang, B. E. Shmukler et al., "Acute regulation of the SLC26A3 congenital chloride diarrhoea anion exchanger (DRA) expressed in *Xenopus oocytes*," *The Journal of Physiology*, vol. 549, no. 1, pp. 3–19, 2003.

[111] S. B. Ko, W. Zeng, M. R. Dorwart et al., "Gating of CFTR by the STAS domain of SLC26 transporters," *Nature Cell Biology*, vol. 6, no. 4, pp. 343–350, 2004.

[112] S. B. H. Ko, N. Shcheynikov, J. Y. Choi et al., "A molecular mechanism for aberrant CFTR-dependent HCO_3^- transport in cystic fibrosis," *The EMBO Journal*, vol. 21, no. 21, pp. 5662–5672, 2002.

[113] N. Shcheynikov, S. B. H. Ko, W. Zeng et al., "Regulatory interaction between CFTR and the SLC26 transporters," *Novartis Foundation Symposium*, vol. 273, pp. 177–186, 2006.

[114] J. Ousingsawat, R. Schreiber, and K. Kunzelmann, "Differential contribution of SLC26A9 to Cl^- conductance in polarized and non-polarized epithelial cells," *Journal of Cellular Physiology*, vol. 227, no. 6, pp. 2323–2329, 2012.

[115] M. Avella, C. Loriol, K. Boulukos, F. Borgese, and J. Ehrenfeld, "SLC26A9 stimulates CFTR expression and function in human bronchial cell lines," *Journal of Cellular Physiology*, vol. 226, no. 1, pp. 212–223, 2011.

[116] C. A. Bertrand, R. Zhang, J. M. Pilewski, and R. A. Frizzell, "SLC26A9 is a constitutively active, CFTR-regulated anion conductance in human bronchial epithelia," *Journal of General Physiology*, vol. 133, no. 4, pp. 421–438, 2009.

[117] J. Braun, L. Mundhenk, F. Range, and A. D. Gruber, "Quantitative expression analyses of candidates for alternative anion conductance in cystic fibrosis mouse models," *Journal of Cystic Fibrosis*, vol. 9, no. 5, pp. 351–364, 2010.

[118] M.-H. Chang, C. Plata, A. Sindic et al., "Slc26a9 is inhibited by the R-region of the cystic fibrosis transmembrane conductance regulator via the STAS domain," *The Journal of Biological Chemistry*, vol. 284, no. 41, pp. 28306–28318, 2009.

[119] N. Bakouh, T. Bienvenu, A. Thomas et al., "Characterization of SLC26A9 in patients with CF-like lung disease," *Human Mutation*, vol. 34, no. 10, pp. 1404–1414, 2013.

[120] P. Anagnostopoulou, B. Riederer, J. Duerr et al., "SLC26A9-mediated chloride secretion prevents mucus obstruction in airway inflammation," *Journal of Clinical Investigation*, vol. 122, no. 10, pp. 3629–3634, 2012.

[121] M. R. Miller, D. Soave, W. Li et al., "Variants in solute carrier *SLC26A9* modify prenatal exocrine pancreatic damage in cystic fibrosis," *Journal of Pediatrics*, vol. 166, no. 5, pp. 1152–1157.e6, 2015.

[122] A.-P. Chen, M.-H. Chang, and M. F. Romero, "Functional analysis of nonsynonymous single nucleotide polymorphisms in human SLC26A9," *Human Mutation*, vol. 33, no. 8, pp. 1275–1284, 2012.

[123] L. Sun, J. M. Rommens, H. Corvol et al., "Multiple apical plasma membrane constituents are associated with susceptibility to meconium ileus in individuals with cystic fibrosis," *Nature Genetics*, vol. 44, no. 5, pp. 562–569, 2012.

[124] S. M. Blackman, C. W. Commander, C. Watson et al., "Genetic modifiers of cystic fibrosis-related diabetes," *Diabetes*, vol. 62, no. 10, pp. 3627–3635, 2013.

[125] W. Li, D. Soave, M. R. Miller et al., "Unraveling the complex genetic model for cystic fibrosis: pleiotropic effects of modifier genes on early cystic fibrosis-related morbidities," *Human Genetics*, vol. 133, no. 2, pp. 151–161, 2014.

[126] M. G. Roy, A. Livraghi-Butrico, A. A. Fletcher et al., "Muc5b is required for airway defence," *Nature*, vol. 505, no. 7483, pp. 412–416, 2014.

[127] M. H. Abou Alaiwa, L. R. Reznikov, N. D. Gansemer et al., "pH modulates the activity and synergism of the airway surface liquid antimicrobials beta-defensin-3 and LL-37," *Proceedings of the National Academy of Sciences*, vol. 111, no. 52, pp. 18703–18708, 2014.

[128] M. J. Hoegger, A. J. Fischer, J. D. McMenimen et al., "Impaired mucus detachment disrupts mucociliary transport in a piglet model of cystic fibrosis," *Science*, vol. 345, no. 6198, pp. 818–822, 2014.

[129] A. Schütte, A. Ermund, C. Becker-Pauly et al., "Microbial-induced meprin β cleavage in MUC2 mucin and a functional CFTR channel are required to release anchored small intestinal mucus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 34, pp. 12396–12401, 2014.

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Review Article

Proinflammatory Cytokines and Potassium Channels in the Kidney

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Proinflammatory cytokines affect several cell functions via receptor-mediated processes. In the kidney, functions of transporters and ion channels along the nephron are also affected by some cytokines. Among these, alteration of activity of potassium ion (K^+) channels induces changes in transepithelial transport of solutes and water in the kidney, since K^+ channels in tubule cells are indispensable for formation of membrane potential which serves as a driving force for the transepithelial transport. Altered K^+ channel activity may be involved in renal cell dysfunction during inflammation. Although little information was available regarding the effects of proinflammatory cytokines on renal K^+ channels, reports have emerged during the last decade. In human proximal tubule cells, interferon-γ showed a time-dependent biphasic effect on a 40 pS K^+ channel, that is, delayed suppression and acute stimulation, and interleukin-1β acutely suppressed the channel activity. Transforming growth factor-β1 activated KCa3.1 K^+ channel in immortalized human proximal tubule cells, which would be involved in the pathogenesis of renal fibrosis. This review discusses the effects of proinflammatory cytokines on renal K^+ channels and the causal relationship between the cytokine-induced changes in K^+ channel activity and renal dysfunction.

1. Introduction

Renal tubular potassium (K^+) channels are involved in a wide spectrum of the transepithelial transport in the kidney [1, 2]. They contribute to the formation of the cell-negative potential, which serves as a driving force for the electrogenic passive transport of solutes, such as apical Na^+ entries through the Na^+-glucose cotransporter (SGLT) in proximal tubule cells and the epithelial Na^+ channel (ENaC) in the principal cells of cortical collecting duct (CCD) [1, 2]. The apical K^+ channels in the principal cells of CCD are the major pathway of K^+ secretion [1, 2]. The renal tubular K^+ channels also play important roles in K^+ recycling for the apical Na^+-K^+-$2Cl^-$ cotransporter (NKCC) in the thick ascending limb (TAL) and the basolateral Na^+-K^+ ATPase along the nephron [1, 2]. In addition to the physiological importance, the renal tubular K^+ channels seem to be involved in the pathogenesis of renal cell injury or renal dysfunction. Some investigators reported that the blockade of K^+ channel activity ameliorated hypoxic renal cell injury [3–5]. Others reported that the decrease in K^+ channel activity exacerbated renal ischemia/reperfusion injury [6, 7] or endotoxemic renal failure [8].

The cytokine family comprises a variety of multifunctional proteins, which play pivotal roles in immune modulation and inflammation [9, 10]. Almost all organs are subjected to the modulatory effects of cytokines. The nervous system [11, 12], cardiovascular system [13], respiratory system [14], gastrointestinal tracts [15], and kidney [16] are also targets of cytokines. It is widely accepted that cytokines are secreted mainly by immune cells in response to microbial infection. However, other cell types are also capable of secreting cytokines [9, 10]. The kidney proximal tubule cells produce proinflammatory cytokines in response to lipopolysaccharide (LPS) [17] or albumin [18]. It is thought that the proximal tubule cells function as the proinflammatory cells or immune responders, which play roles in the pathogenesis of renal dysfunction [19, 20]. The TAL [21, 22] and collecting ducts [23] are also the sources of proinflammatory cytokines. Furthermore, renal tubule cells express specific receptors which mediate effects of individual cytokines [24–26].

Several proinflammatory cytokines, such as interferon-γ (IFN-γ), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α), have been reported to affect Na^+ reabsorption in renal tubular epithelia [27–34]. Considering that the driving force of the transepithelial Na^+ reabsorption is dependent on the K^+ channel activity, as well as Na^+-K^+-ATPase [1, 2], it is important to know whether cytokines would affect the K^+ channels. These proinflammatory cytokines were also reported to cause cell injury in various organs, including the kidney. Since the alterations in K^+ channel activity were involved in renal cell injury as described above, it is possible that the cytotoxic effects of proinflammatory cytokines would partly be mediated by their action on renal K^+ channels. To date, however, information is restricted, regarding the effects of cytokines on K^+ channel activity in renal tubule epithelia. This is in sharp contrast to the accumulating evidence for the effects of cytokines on neuronal ion channels [35]. In this review, we discuss the effects of proinflammatory cytokines on renal tubular K^+ channels and the relevance of such effects to renal cell damage.

2. Effects of Cytokines on K^+ Channels in the Proximal Tubule

In the kidney, the proximal tubule reabsorbs about 70% of Na^+ filtered in glomeruli [1, 2]. The basolateral K^+ channels provide the driving force for the apical Na^+ entry by forming the cell-negative potential and serving as the K^+ recycling pathway coupled with the Na^+-K^+-ATPase activity [1, 2]. The effects of cytokines on the K^+ channels in human proximal tubule cells have recently been reported, using the patch-clamp technique [36–38]. In cultured human proximal tubule cells (RPTECs) derived from the normal kidney, an inwardly rectifying K^+ channel with an inward conductance of 40 pS is predominantly observed under the control condition [39]. Although the molecular characteristic of this K^+ channel is still unknown, electrophysiological studies revealed various functional properties. The 40 pS K^+ channel possesses a relatively high open probability (0.8 on average) with no voltage dependence [39] and contributes to the formation of cell-negative potential [40]. Furthermore, the activity of this K^+ channel is regulated by intracellular ATP [39], pH [39], and protein phosphorylation processes mediated by protein kinase A (PKA) [39] and protein kinase C (PKC) [38, 41], all of which are consistent with the properties of the basolateral K^+ channels in animal proximal tubule cells [1, 2, 42].

It was reported that two proinflammatory cytokines, IFN-γ [36, 37] and IL-1β [36, 38], affected the activity of the 40 pS K^+ channel in RPTECs. IFN-γ possessed a time-dependent biphasic effect on the 40 pS K^+ channel: a delayed suppressive effect and an acute stimulatory one [36, 37]. Both effects were blocked by inhibitors of Janus kinase (JAK) which was closely related to the IFN-γ receptor, suggesting that the effects of IFN-γ would be receptor specific [37]. In fact, human proximal tubule cells were reported to express IFN-γ receptors [24]. The delayed suppressive effect of IFN-γ on channel activity was mediated, at least in part, by overproduction of NO in RPTECs [36, 37]. It was reported that NO stimulated activity of the 40 pS K^+ channel in RPTECs at low concentrations (micromolar level) through activation of the cGMP/protein kinase G (PKG) pathway, whereas it suppressed channel activity at higher concentrations (millimolar level) [40]. The mechanism for the delayed suppressive effect of IFN-γ is depicted in Figure 1. When RPTECs were treated with IFN-γ for 24 h, expression of inducible NO synthase (iNOS) was greatly enhanced, producing a large amount of NO. The excessive NO reacted with superoxide and generated peroxynitrite [37]. This peroxynitrite would suppress the K^+ channel activity by oxidating or nitrosylating the channel and/or its related proteins [37, 40]. Thus, the responses of the channels to NO modulators were reversed in IFN-γ-treated cells, compared to the control cells [36, 37]. A NOS inhibitor, L-NAME, stimulated channel activity and a NO donor, L-arginine, suppressed channel activity in IFN-γ-treated cells, whereas L-NAME suppressed channel activity and L-arginine stimulated channel activity in control cells [36, 37]. With regard to the acute stimulatory effect of IFN-γ, the mechanisms involved are currently obscure (Figure 1). Although the activity of the 40 pS K^+ channel was upregulated by PKA- and PKG-mediated phosphorylation processes [39, 43], inhibitors of these protein kinases failed to block the stimulatory effect of IFN-γ [37]. Phosphatidylinositol-3-kinase (PI3 K) is one of the molecules that mediate the IFN-γ signaling [44]. However, PI3 K inhibitors were also ineffective in blocking the IFN-γ-induced activation of the channel [37].

IL-1β also acutely affected the activity of the 40 pS K^+ channel in RPTECs and the mode of action was suppressive [36, 38]. The effect of IL-1β was highly likely receptor mediated, since the IL-1 receptor antagonist (IL-1RA) completely abolished it [38]. With regard to the existence of

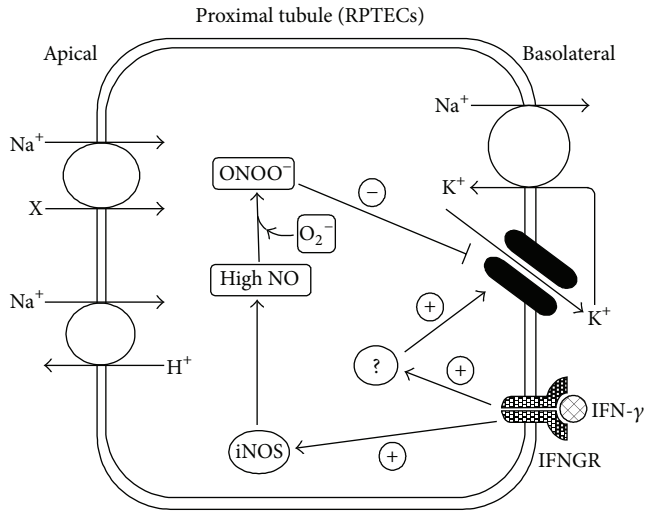

Figure 1: Illustration of the time-dependent biphasic effect of IFN-γ on the 40 pS K^+ channel in RPTECs. Prolonged treatment of cells with IFN-γ greatly enhances iNOS expression, which results in generation of a large amount of NO. The excessive NO reacts with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$), which impairs the 40 pS K^+ channel by oxidation and/or nitrosylation. IFN-γ also acutely activates the 40 pS K^+ channel by unknown mechanisms. IFNGR: IFN-γ receptor.

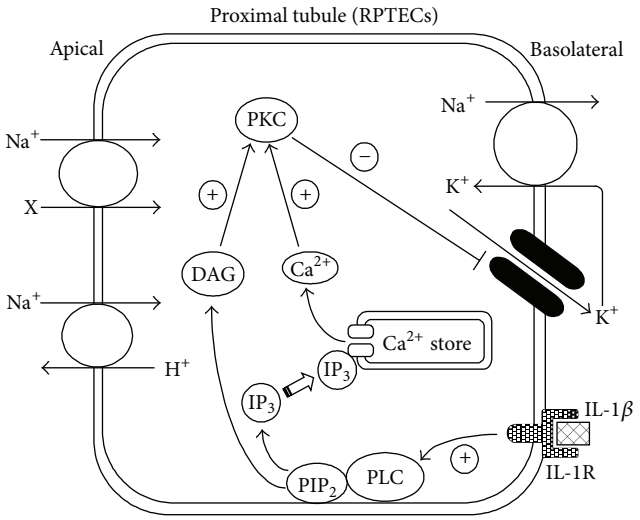

Figure 2: Illustration of the acute effect of IL-1β on the 40 pS K^+ channel in RPTECs. IL-1β activates phospholipase C (PLC), which generates diacylglycerol (DAG) and 1,4,5-trisphosphate (IP_3) from phosphatidylinositol 4,5-bisphosphate (PIP_2) in the cytoplasmic membrane. Binding of IP_3 to the IP_3 receptor of the intracellular Ca^{2+} sore releases Ca^{2+}. Both the DAG and increased intracellular Ca^{2+} activate PKC, which consequently phosphorylates the channel and/or its related proteins, suppressing the channel activity. IL-1R: IL-1 receptor.

IL-1 receptors in the kidney, it was reported that the type I IL-1 receptors were widely distributed in renal tubules, as well as glomeruli [25]. In addition to IL-1RA, the acute suppressive effect of IL-1β was blocked by a PKC inhibitor and an inhibitor of phospholipase C [38]. These observations strongly suggested that the effect of IL-1β was dependent on the PKC pathway (Figure 2). In support of this notion, PKC directly suppressed the activity of the 40 pS K^+ channel in inside-out patches [38], and fluorescent Ca^{2+} imaging using Fura 2 revealed that IL-1β increased intracellular Ca^{2+} [38], which was prerequisite for the activation of conventional PKC [45]. Furthermore, the PKC-dependent effects of IL-1β were also observed in other ion channels, including the hippocampal Ca^{2+} channel [46], the middle ear Na^+ channel [47], the intestinal Cl^- channel [48], and the K^+ channel in the cultured mouse CCD cell line, M1 cells (our unpublished observation). Although several investigators reported that IL-1β increased iNOS expression in some tissues, such effect was not observed in RPTECs [36] or the nonpassaged primary culture of human proximal tubule cells [49]. Thus, IL-1β did not possess the NO-dependent delayed suppressive effect on the 40 pS K^+ channel, which was demonstrated by IFN-γ.

Huang et al. [50] have recently reported that transforming growth factor-β1 (TGF-β1) upregulated the KCa3.1 channel in immortalized human proximal tubule cells, HK2. According to their report, the whole-cell current sensitive to TRAM34, an inhibitor of KCa3.1, was profoundly increased after treatment of HK2 cells with TGF-β1 for 48 h, while the TRAM34-sensitive current was not observed in control cells. The mRNA expression of KCa3.1 was also increased by TGF-β1. KCa3.1 is an intermediate conductance Ca^{2+}-activated K^+ channel and widely distributed throughout the body, excepting most of excitable tissues [51]. Much more attention has been paid to this Ca^{2+}-activated K^+ channel because of its pathological relevance in various diseases, including asthma, atherosclerosis, autoimmunity, and renal fibrosis [51]. Indeed, Huang et al. [50] suggested that the activation of KCa3.1 would play an important role in the TGF-β-induced renal fibrosis. TGF-β increased KCa3.1 activity, which in turn contributed to the activation of mitogen-activated protein kinase signaling and increased expression of monocyte chemoattractant protein-1 [50].

3. Effects of Cytokines on K^+ Channels in the TAL and CCD

The first report that cytokines affect K^+ channel activity was presented in 2003 by Wei et al. [52], who clearly demonstrated that TNF acutely stimulated activity of a 70 pS K^+ channel in the apical membrane of rat TAL, using the patch-clamp technique. Although the PKA- and nitric oxide- (NO-) dependent pathways had been shown to stimulate the activity of the 70 pS K^+ channel [53, 54], both a PKA inhibitor and an inhibitor of NO synthase did not affect the TNF-induced activation of the channel [52]. In contrast, an inhibitor of protein tyrosine phosphatase (PTP) blocked the stimulatory effect of TNF on channel activity [52]. Furthermore, an inhibitor of protein tyrosine kinase (PTK) increased channel activity, and the subsequent administration of TNF in the presence of the PTK inhibitor did not cause additional increase in channel activity [52]. It was also demonstrated that TNF significantly increased the PTP activity in cultured rat TAL cells [52]. Therefore, they concluded that the stimulatory effect of TNF on the 70 pS K^+ channel was dependent on tyrosine dephosphorylation processes mediated by PTP [52], as shown in Figure 3.

It is well known that a ROMK-like 30 pS K^+ channel, as well as the 70 pS K^+ channel, is present in the apical membrane of TAL [52, 53]. Both K^+ channels contribute to the K^+ recycling across the apical membrane of the TAL, which maintains the normal function of the NKCC [1, 2]. Wei et al. [52] also found that TNF did not affect the activity of this 30 pS K^+ channel.

With regard to ROMK, there are also reports demonstrating that the gene expression of this K^+ channel was modulated by cytokines [32]. Schmidt et al. [32] reported that administration of proinflammatory cytokines, such as IL-1β, IFN-γ, and TNF-α, to the mouse decreased mRNA expression of ROMK in the whole kidney. Although the systemic administration of cytokines resulted in hypotension and reduced blood flow in the kidney, renal ischemia itself had no apparent effect on ROMK gene expression [32]. In addition, IL-1β, IFN-γ, and TNF-α suppressed ROMK gene expression in the mouse CCD cell line to the same extent observed in the whole animal experiments [32]. Therefore, they strongly suggested that the proinflammatory cytokines directly suppressed the ROMK gene expression [32].

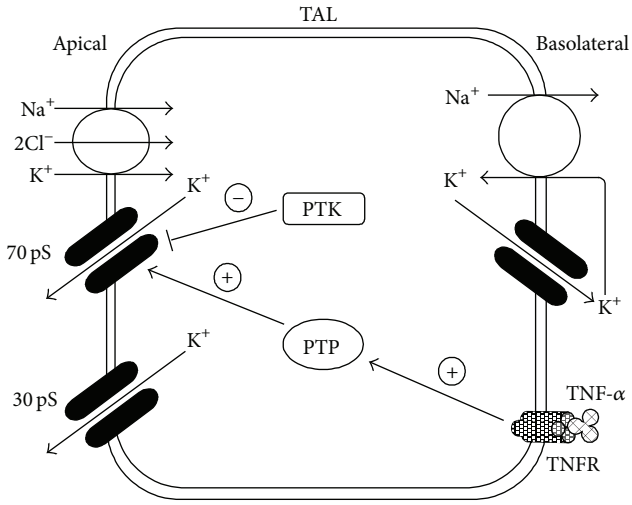

Figure 3: Illustration of the acute effect of TNF-α on the 70 pS K^+ channel in the TAL. TNF-α stimulates PTP activity, which causes tyrosine dephosphorylation of the 70 pS K^+ channel and activates it. TNFR: TNF receptor.

4. Cytokine-Induced Changes in Activities of K^+ Channels and Transporters in the Kidney

The activities of renal tubular K^+ channels are functionally coupled to various transporters [1, 2]. Basolateral K^+ channels in proximal tubule cells cooperate with the basolateral Na^+-K^+ ATPase to provide a driving force for Na^+ entry through the apical transporters, such as SGLT [1, 2]. Similar cooperation of basolateral K^+ channels with the Na^+-K^+ ATPase also exists in distal nephron segments [1, 2]. In the TAL, apical K^+ channels serve as the K^+ recycling pathways for the apical NKCC, which facilitates reabsorption of Na^+ and Cl^- [1, 2]. In principal cells of CCD, apical Na^+ entry through the ENaC is partly dependent on the cell-negative potential generated by apical and basolateral K^+ channels [1, 2]. Thus, the changes in renal K^+ channel activity would result in alterations in the transport activity of solutes and water. If the transport activity in the kidney drastically changes, the homeostasis of body fluid would be profoundly perturbed.

In contrast to the renal K^+ channels, there are relatively many reports demonstrating the effects of cytokines on renal transporters. TNF-α reduced ouabain-sensitive $^{86}Rb^+$ uptake in the TAL [21] and NKCC expression in the kidney [31, 33]. These observations are inconsistent with the stimulatory effect of TNF-α on the 70 pS K^+ channel in the TAL reported by Wei et al. [52]. The apical 70 pS K^+ channel acts as the recycling pathway for the apical NKCC [1, 2]. Therefore, the TNF-induced increase in channel activity should be related to increased Na^+ transport. Such a discrepancy would partly be accounted for by the temporal factor. TNF-α reduced $^{86}Rb^+$ uptake after 24 h [21] and NKCC expression after 4 h [31] or 7 days [33], whereas stimulation of channel activity by TNF-α occurred in a few minutes [52]. Besides the acute stimulatory effect on K^+ channel, TNF-α induces expression of cyclooxygenase (COX) which subsequently generates prostaglandin E_2 (PGE_2) [55]. It was reported that PGE_2 suppressed the activity of the 70 pS K^+ channel in the TAL [56]. The effects of TNF-α on channel activity might be time-dependently biphasic similar to the effect of IFN-γ on K^+ channel activity in RPTECs. If so, the delayed COX/PGE_2-dependent channel suppression would be consistent with the TNF-α-induced inhibition of transport activity and transporter expression. TNF-α was also reported to increase SGLT2 expression in the porcine proximal tubule cell line, LLC-PK_1 [57], which well fits with the stimulatory effect of TNF-α on K^+ channel activity. IFN-γ suppresses gene expression of various transporters. These include Na^+-K^+ ATPase [32, 58, 59], Na^+-H^+ exchanger (NHE) [32], Na^+-Ca^{2+} exchanger [60], Na^+-Cl^- cotransporter (NCC) [58], NKCC [32], SGLT [59], glucose transporters [59], and urea transporters [61]. On the other hand, IFN-γ seems to be involved in the upregulations of NKCC and NCC in the distal nephron and NHE in the proximal tubule, which would contribute to the salt and water retention during the angiotensin II-induced hypertension [34]. The stimulatory effect of IFN-γ on K^+ channel activity might play a role in such an upregulated Na^+ transport. Similar to TNF-α and IFN-γ, the effects of IL1-β on renal transporters are mainly suppressive, which is in good agreement with its suppressive effect on K^+ channel activity. IL-1β inhibits Na^+ reabsorption in the CCD [27] and expression of Na^+-K^+ ATPase in medullary and cortical kidney cells [28] or cultured LLC-PK_1 cells [29]. It also reduces many other transporters, as was observed with IFN-γ [32, 58, 59, 61].

5. Effects of Cytokines on K^+ Channel Activity and Renal Cell Injury

The effects of proinflammatory cytokines on renal K^+ channels would be implicated in renal dysfunction or renal cell injury. Proinflammatory cytokines are the key molecules, which promote cell injury in many organs during inflammatory diseases [9, 10]. They are involved in the pathogenesis of acute kidney injury and chronic kidney disease [62, 63]. The endotoxemia-induced acute renal failure is one of the clinical manifestations that highlight the detrimental effects of proinflammatory cytokines on the kidney [64]. Therapeutic use of cytokines sometimes brings about adverse effects. For example, application of IFNs in renal cell carcinoma or viral hepatitis may well result in undesirable severe renal dysfunction [65]. Furthermore, it has been reported that proinflammatory cytokines cause glomerulonephritis accompanied by proteinuria, tubulointerstitial fibrosis, and apoptosis/necrosis of tubular cells in various experimental models [66, 67]. These effects are generally thought to be mediated by activation of caspases and various transcription factors [9, 10]. The activated transcription factors initiate synthesis of many effector proteins, such as other cytokines, chemokines, matrix metalloproteases, iNOS, and adhesion molecules [9, 10]. All of these effector proteins could participate in the promotion of renal cell injury. As described below, the changes in K^+ channel activity themselves could affect

cell viability. Thus, it is possible that the cytokine-induced renal cell injury would be mediated by modulation of K^+ channel activity. In fact, TGF-β upregulates a Ca^{2+}-activated K^+ channel in HK2 cells, which in turn contributed to the generation of a chemokine crucial for the pathogenesis of renal fibrosis [50]. Modulation of K^+ channel activity by TNF-α was also reported to induce cell death in a rat liver cell line [68] and prolonged action potential duration, which was involved in the sudden cardiac death, in dog cardiomyocytes [69].

Many reports have demonstrated that changes in activity of renal tubular K^+ channels would be involved in renal cell injury. With regard to the mode of action of K^+ channel activity on renal cell injury, however, profound controversy exists. An ATP-sensitive K^+ channel (K_{ATP}) blocker, glibenclamide, was reported to reduce hypoxia- or ischemia/reperfusion-induced renal cell injury in isolated rat proximal tubules [3], perfused rat kidneys [4], and rats *in vivo* [5]. Glibenclamide also improved kidney structure and function in the rat model of chronic kidney disease [70]. Blocking the activity of KCa3.1 K^+ channel by TRAM34 inhibited production of inflammatory mediators, which contributed to renal fibrosis, in HK2 cells [50, 71] or diabetic mice [72]. It was reported that the downregulation of Kir4.1 by intracellular acidification would contribute to cell protection in the rat proximal tubule [73]. These findings suggested that increased K^+ channel activity would cause renal cell injury. In contrast, other investigators reported that maintaining or increasing K^+ channel activity is rather protective for renal cells. According to their reports, blockade of K^+ channel by glibenclamide enhanced renal cell injury in isolated perfused rat kidney [6], LLC-PK_1 cells [7], HK2 cells [8], and mouse proximal tubule cells [8]. Furthermore, a K_{ATP} opener, nicorandil, was reported to ameliorate ischemia/reperfusion injury in the rat kidney [74, 75]. Another K_{ATP} opener, levosimendan, also protects the kidney against the LPS-induced inflammatory responses [8] and ischemia/reperfusion injury [76]. The discrepant effects of altered K^+ channel activity among reports would partly be due to the differences in experimental systems and cellular conditions. The precise mechanisms by which changes in K^+ channel activity cause renal cell injury are not completely revealed. It is likely that the loss of intracellular K^+ through the activated K^+ channel facilitates cell shrinkage, which triggers apoptotic volume decrease [77]. Furthermore, changes in K^+ channel activity alter the driving force for Ca^{2+} entry through Ca^{2+}-permeable channels, such as TRP channels [78, 79]. Subsequent changes in intracellular Ca^{2+} concentration will activate or suppress various factors, including apoptotic or inflammatory molecules [50, 71, 72, 78, 79]. It also remained to be elucidated whether the cytotoxic effects of proinflammatory cytokines would actually be mediated by their modulation of renal K^+ channel activity.

6. Conclusion

Renal K^+ channels play important roles in maintaining the normal transport function of renal tubule epithelia. The kidney sometimes suffers from renal ischemia, endotoxemia, and diabetic nephropathy, where proinflammatory cytokines are produced. However, it was only during the last decade that the effects of proinflammatory cytokines on renal K^+ channels were reported. The effects of cytokines on K^+ channels may be involved in alterations of tubular transport or onset of renal cell injury. However, the physiological and pathological significances of proinflammatory cytokines in modulating renal tubular K^+ channels are not well understood. To complicate the matters, a variety of cytokines with different actions are produced during inflammatory responses. Some cytokines activate renal K^+ channels, while others suppress the same channels. The complexity of cytokine actions gives rise to difficulties in interpreting the final outcome of their effects. Additional studies are required to further clarify the effects of proinflammatory cytokines on renal K^+ channels.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] S. C. Hebert, G. Desir, G. Giebisch, and W. Wang, "Molecular diversity and regulation of renal potassium channels," *Physiological Reviews*, vol. 85, no. 1, pp. 319–371, 2005.

[2] G. H. Giebisch, "A long affair with renal tubules," *Annual Review of Physiology*, vol. 73, pp. 1–28, 2011.

[3] W. B. Reeves and S. V. Shah, "Activation of potassium channels contributes to hypoxic injury in proximal tubules," *The Journal of Clinical Investigation*, vol. 94, no. 6, pp. 2289–2294, 1994.

[4] R. Engbersen, M. M. Moons, A. C. Wouterse et al., "Sulphonylurea drugs reduce hypoxic damage in the isolated perfused rat kidney," *British Journal of Pharmacology*, vol. 130, no. 7, pp. 1678–1684, 2000.

[5] K. Pompermayer, D. G. Souza, G. G. Lara et al., "The ATP-sensitive potassium channel blocker glibenclamide prevents renal ischemia/reperfusion injury in rats," *Kidney International*, vol. 67, no. 5, pp. 1785–1796, 2005.

[6] M. Rahgozar, D. A. Willgoss, G. C. Gobé, and Z. H. Endre, "ATP-dependent K^+ channels in renal ischemia reperfusion injury," *Renal Failure*, vol. 25, no. 6, pp. 885–896, 2003.

[7] A. R. Assad, J. M. A. Delou, L. M. Fonseca et al., "The role of KATP channels on propofol preconditioning in a cellular model of renal ischemia-reperfusion," *Anesthesia & Analgesia*, vol. 109, no. 5, pp. 1486–1492, 2009.

[8] R. A. Zager, A. C. Johnson, S. Lund, S. Y. Hanson, and C. K. Abrass, "Levosimendan protects against experimental endotoxemic acute renal failure," *The American Journal of Physiology—Renal Physiology*, vol. 290, no. 6, pp. F1453–F1462, 2006.

[9] C. A. Feghali and T. M. Wright, "Cytokines in acute and chronic inflammation," *Frontiers in Bioscience*, vol. 2, pp. 12–26, 1997.

[10] C. A. Dinarello, "Historical insights into cytokines," *European Journal of Immunology*, vol. 37, supplement 1, pp. S34–S45, 2007.

[11] M. Schäfers and L. Sorkin, "Effect of cytokines on neuronal excitability," *Neuroscience Letters*, vol. 437, no. 3, pp. 188–193, 2008.

[12] S. M. Allan, P. J. Tyrrell, and N. J. Rothwell, "Interleukin-1 and neuronal injury," *Nature Reviews Immunology*, vol. 5, no. 8, pp. 629–640, 2005.

[13] V. C. Mehra, V. S. Ramgolam, and J. R. Bender, "Cytokines and cardiovascular disease," *Journal of Leukocyte Biology*, vol. 78, no. 4, pp. 805–818, 2005.

[14] H. Kimura, M. Yoshizumi, H. Ishii, K. Oishi, and A. Ryo, "Cytokine production and signaling pathways in respiratory virus infection," *Frontiers in Microbiology*, vol. 4, article 276, 2013.

[15] H. Akiho, E. Ihara, Y. Motomura, and K. Nakamura, "Cytokine-induced alterations of gastrointestinal motility in gastrointestinal disorders," *World Journal of Gastrointestinal Pathophysiology*, vol. 2, no. 5, pp. 72–81, 2011.

[16] E. D. Morrell, J. A. Kellum, K. R. Hallows, and N. M. Pastor-Soler, "Epithelial transport during septic acute kidney injury," *Nephrology Dialysis Transplantation*, vol. 29, no. 7, pp. 1312–1319, 2014.

[17] R. A. Zager, A. C. M. Johnson, and A. Geballe, "Gentamicin suppresses endotoxin-driven TNF-α production in human and mouse proximal tubule cells," *American Journal of Physiology—Renal Physiology*, vol. 293, no. 4, pp. F1373–F1380, 2007.

[18] G. K. Rangan, Y. Wang, Y.-C. Tay, and D. C. H. Harris, "Differential effects of albumin on cytokine gene expression in proximal tubular epithelial cells," *Nephrology Dialysis Transplantation*, vol. 20, no. 5, pp. 1013–1014, 2005.

[19] M. R. Daha and C. van Kooten, "Is the proximal tubular cell a proinflammatory cell?" *Nephrology Dialysis Transplantation*, vol. 15, supplement 6, pp. 41–43, 2000.

[20] N. Nakhoul and V. Batuman, "Role of proximal tubules in the pathogenesis of kidney disease," *Contributions to Nephrology*, vol. 169, pp. 37–50, 2011.

[21] B. A. Escalante, N. R. Ferreri, C. E. Dunn, and J. C. McGiff, "Cytokines affect ion transport in primary cultured thick ascending limb of Henle's loop cells," *American Journal of Physiology*, vol. 266, no. 6, pp. C1568–C1576, 1994.

[22] N. R. Ferreri, B. A. Escalante, Y. Zhao, S.-J. An, and J. C. McGiff, "Angiotensin II induces TNF production by the thick ascending limb: functional implications," *American Journal of Physiology—Renal Physiology*, vol. 274, no. 1, pp. F148–F155, 1998.

[23] I. Tikkanen, N. Uhlenius, T. Tikkanen et al., "Increased renal expression of cytokines and growth factors induced by DOCA-NaCl treatment in Heymann nephritis," *Nephrology Dialysis Transplantation*, vol. 10, no. 12, pp. 2192–2198, 1995.

[24] J. S. Gerritsma, A. F. Gerritsen, M. De Ley, L. A. van Es, and M. R. Daha, "Interferon-γ induces biosynthesis of complement components C2, C4 and factor H by human proximal tubular epithelial cells," *Cytokine*, vol. 9, no. 4, pp. 276–283, 1997.

[25] J. R. Timoshanko, A. R. Kitching, Y. Iwakura, S. R. Holdsworth, and P. G. Tipping, "Leukocyte-derived interleukin-1β interacts with renal interleukin-1 receptor I to promote renal tumor necrosis factor and glomerular injury in murine crescentic glomerulonephritis," *The American Journal of Pathology*, vol. 164, no. 6, pp. 1967–1977, 2004.

[26] A. Castillo, M. T. Islam, M. C. Prieto, and D. S. A. Majid, "Tumor necrosis factor-α receptor type 1, not type 2, mediates its acute responses in the kidney," *American Journal of Physiology—Renal Physiology*, vol. 302, no. 12, pp. F1650–F1657, 2012.

[27] Y. Sakairi, Y. Ando, K. Tabei, E. Kusano, and Y. Asano, "Interleukin-1 inhibits sodium and water transport in rabbit cortical collecting duct," *American Journal of Physiology—Renal Fluid and Electrolyte Physiology*, vol. 266, no. 4, pp. F674–F680, 1994.

[28] S. I. Kreydiyyeh and R. Al-Sadi, "Interleukin-1β increases urine flow rate and inhibits protein expression of Na^+/K^+-ATPase in the rat jejunum and kidney," *Journal of Interferon & Cytokine Research*, vol. 22, no. 10, pp. 1041–1048, 2002.

[29] S. I. Kreydiyyeh and R. Al-Sadi, "The signal transduction pathway that mediates the effect of interleukin-1 beta on the Na^+-K^+-ATPase in LLC-PK_1 cells," *Pflügers Archiv*, vol. 448, no. 2, pp. 231–238, 2004.

[30] M. Vinciguerra, U. Hasler, D. Mordasini et al., "Cytokines and sodium induce protein kinase A-dependent cell-surface Na, K-ATPase recruitment via dissociation of NF-κB/IκB/protein kinase A catalytic subunit complex in collecting duct principal cells," *Journal of the American Society of Nephrology*, vol. 16, no. 9, pp. 2576–2585, 2005.

[31] S. I. Kreydiyyeh and S. Markossian, "Tumor necrosis factor α down-regulates the Na^+-K^+ ATPase and the Na^+-K^+2Cl^- cotransporter in the kidney cortex and medulla," *Cytokine*, vol. 33, no. 3, pp. 138–144, 2006.

[32] C. Schmidt, K. Höcherl, F. Schweda, A. Kurtz, and M. Bucher, "Regulation of renal sodium transporters during severe inflammation," *Journal of the American Society of Nephrology*, vol. 18, no. 4, pp. 1072–1083, 2007.

[33] S. Battula, S. Hao, P. L. Pedraza, C. T. Stier, and N. R. Ferreri, "Tumor necrosis factor-α is an endogenous inhibitor of Na^+-K^+-$2Cl^-$ cotransporter (NKCC2) isoform a in the thick ascending limb," *American Journal of Physiology—Renal Physiology*, vol. 301, no. 1, pp. F94–F100, 2011.

[34] N. V. Kamat, S. R. Thabet, L. Xiao et al., "Renal transporter activation during angiotensin-II hypertension is blunted in interferon-$\gamma^{-/-}$ and interleukin-$17A^{-/-}$ mice," *Hypertension*, vol. 65, no. 3, pp. 569–576, 2015.

[35] B. Viviani, F. Gardoni, and M. Marinovich, "Cytokines and neuronal ion channels in health and disease," *International Review of Neurobiology*, vol. 82, pp. 247–263, 2007.

[36] K. Nakamura, Y. Komagiri, T. Kojo, and M. Kubokawa, "Effects of cytokines on activity of an inwardly rectifying K^+ channel in cultured human proximal tubule cells," *The Journal of the Iwate Medical Association*, vol. 59, no. 5, pp. 375–385, 2007.

[37] K. Nakamura, Y. Komagiri, T. Kojo, and M. Kubokawa, "Delayed and acute effects of interferon-γ on activity of an inwardly rectifying K^+ channel in cultured human proximal tubule cells," *American Journal of Physiology—Renal Physiology*, vol. 296, no. 1, pp. F46–F53, 2009.

[38] K. Nakamura, Y. Komagiri, and M. Kubokawa, "Interleukin-1β suppresses activity of an inwardly rectifying K^+ channel in human renal proximal tubule cells," *Journal of Physiological Sciences*, vol. 63, no. 5, pp. 377–387, 2013.

[39] K. Nakamura, J. Hirano, and M. Kubokawa, "An ATP-regulated and pH-sensitive inwardly rectifying K^+ channel in cultured human proximal tubule cells," *Japanese Journal of Physiology*, vol. 51, no. 4, pp. 523–530, 2001.

[40] K. Nakamura, J. Hirano, and M. Kubokawa, "Regulation of an inwardly rectifying K^+ channel by nitric oxide in cultured human proximal tubule cells," *American Journal of Physiology—Renal Physiology*, vol. 287, no. 3, pp. F411–F417, 2004.

[41] M. Kubokawa, T. Kojo, Y. Komagiri, and K. Nakamura, "Role of calcineurin-mediated dephosphorylation in modulation of an inwardly rectifying K^+ channel in human proximal tubule cells," *Journal of Membrane Biology*, vol. 231, no. 2-3, pp. 79–92, 2009.

[42] K. L. Hamilton and D. C. Devor, "Basolateral membrane K^+ channels in renal epithelial cells," *American Journal of*

Physiology—Renal Physiology, vol. 302, no. 9, pp. F1069–F1081, 2012.

[43] K. Nakamura, J. Hirano, S.-I. Itazawa, and M. Kubokawa, "Protein kinase G activates inwardly rectifying K^+ channel in cultured human proximal tubule cells," *American Journal of Physiology—Renal Physiology*, vol. 283, no. 4, pp. F784–F791, 2002.

[44] L. C. Platanias, "Mechanisms of type-I- and type-II-interferon-mediated signalling," *Nature Reviews Immunology*, vol. 5, no. 5, pp. 375–386, 2005.

[45] P. Lipp and G. Reither, "Protein kinase C: the 'masters' of calcium and lipid," *Cold Spring Harbor Perspectives in Biology*, vol. 3, no. 7, Article ID a004556, 2011.

[46] C. R. Plata-Salamán and J. M. H. Ffrench-Mullen, "Interleukin-1β inhibits Ca^{2+} channel currents in hippocampal neurons through protein kinase C," *European Journal of Pharmacology: Molecular Pharmacology*, vol. 266, no. 1, pp. 1–10, 1994.

[47] J. Y. Choi, Y.-S. Choi, S. J. Kim, E. J. Son, H. S. Choi, and J.-H. Yoon, "Interleukin-1β suppresses epithelial sodium channel β-subunit expression and ENaC-dependent fluid absorption in human middle ear epithelial cells," *European Journal of Pharmacology*, vol. 567, no. 1-2, pp. 19–25, 2007.

[48] E. G. Cafferata, A. M. González-Guerrico, L. Giordano, O. H. Pivetta, and T. A. Santa-Coloma, "Interleukin-1β regulates CFTR expression in human intestinal T84 cells," *Biochimica et Biophysica Acta*, vol. 1500, no. 2, pp. 241–248, 2000.

[49] P. K. Chatterjee, G. M. Hawksworth, and J. S. McLay, "Cytokine-stimulated nitric oxide production in the human renal proximal tubule and its modulation by natriuretic peptides: a novel immunomodulatory mechanism?" *Experimental Nephrology*, vol. 7, no. 5-6, pp. 438–448, 1999.

[50] C. Huang, M. L. Day, P. Poronnik, C. A. Pollock, and X.-M. Chen, "Inhibition of KCa3.1 suppresses TGF-β1 induced MCP-1 expression in human proximal tubular cells through Smad3, p38 and ERK1/2 signaling pathways," *The International Journal of Biochemistry & Cell Biology*, vol. 47, no. 1, pp. 1–10, 2014.

[51] H. Wulff and N. A. Castle, "Therapeutic potential of $K_{Ca}3.1$ blockers: recent advances and promising trends," *Expert Review of Clinical Pharmacology*, vol. 3, no. 3, pp. 385–396, 2010.

[52] Y. Wei, E. Babilonia, P. L. Pedraza, N. R. Ferreri, and W.-H. Wang, "Acute application of TNF stimulates apical 70-pS K^+ channels in the thick ascending limb of rat kidney," *American Journal of Physiology—Renal Physiology*, vol. 285, no. 3, pp. F491–F497, 2003.

[53] D. Li, Y. Wei, and W.-H. Wang, "Dietary K intake regulates the response of apical K channels to adenosine in the thick ascending limb," *American Journal of Physiology—Renal Physiology*, vol. 287, no. 5, pp. F954–F959, 2004.

[54] M. Lu, X. Wang, and W. Wang, "Nitric oxide increases the activity of the apical 70-pS K^+ channel in TAL of rat kidney," *American Journal of Physiology—Renal Physiology*, vol. 274, no. 5, pp. F946–F950, 1998.

[55] D. Wang, P. L. Pedraza, H. I. Abdullah, J. C. McGiff, and N. R. Ferreri, "Calcium-sensing receptor-mediated TNF production in medullary thick ascending limb cells," *American Journal of Physiology—Renal Physiology*, vol. 283, no. 5, pp. F963–F970, 2002.

[56] H. J. Liu, Y. Wei, N. R. Fererri, A. Nasjletti, and W. H. Wang, "Vasopressin and PGE2 regulate activity of apical 70 pS K^+ channel in thick ascending limb of rat kidney," *American Journal of Physiology—Cell Physiology*, vol. 278, no. 5, pp. C905–C913, 2000.

[57] M. I. Maldonado-Cervantes, O. G. Galicia, B. Moreno-Jaime et al., "Autocrine modulation of glucose transporter SGLT2 by IL-6 and TNF-α in LLC-PK_1 cells," *Journal of Physiology and Biochemistry*, vol. 68, no. 3, pp. 411–420, 2012.

[58] C. Schmidt, K. Höcherl, F. Schweda, and M. Bucher, "Proinflammatory cytokines cause down-regulation of renal chloride entry pathways during sepsis," *Critical Care Medicine*, vol. 35, no. 9, pp. 2110–2119, 2007.

[59] C. Schmidt, K. Höcherl, and M. Bucher, "Regulation of renal glucose transporters during severe inflammation," *American Journal of Physiology—Renal Physiology*, vol. 292, no. 2, pp. F804–F811, 2007.

[60] V. M. Radhakrishnan, P. Kojs, R. Ramalingam et al., "Experimental colitis is associated with transcriptional inhibition of Na^+/Ca^{2+} exchanger isoform 1 (NCX1) expression by interferon γ in the renal distal convoluted tubules," *The Journal of Biological Chemistry*, vol. 290, no. 14, pp. 8964–8974, 2015.

[61] C. Schmidt, K. Höcherl, and M. Bucher, "Cytokine-mediated regulation of urea transporters during experimental endotoxemia," *American Journal of Physiology—Renal Physiology*, vol. 292, no. 5, pp. F1479–F1489, 2007.

[62] G. Ramesh and W. B. Reeves, "Inflammatory cytokines in acute renal failure," *Kidney International, Supplement*, vol. 66, no. 91, pp. S56–S61, 2004.

[63] L. M. Ortega and A. Fornoni, "Role of cytokines in the pathogenesis of acute and chronic kidney disease, glomerulonephritis, and end-stage kidney disease," *International Journal of Interferon, Cytokine and Mediator Research*, vol. 2, no. 1, pp. 49–62, 2010.

[64] R. W. Schrier and W. Wang, "Acute renal failure and sepsis," *The New England journal of medicine*, vol. 351, no. 2, pp. 159–169, 2004.

[65] E. Jonasch and F. G. Haluska, "Interferon in oncological practice: review of interferon biology, clinical applications, and toxicities," *Oncologist*, vol. 6, no. 1, pp. 34–55, 2001.

[66] T. M. Phillips, "Interferon-alpha induces renal dysfunction and injury," *Current Opinion in Nephrology and Hypertension*, vol. 5, no. 4, pp. 380–383, 1996.

[67] D. A. Vesey, C. Cheung, Z. Endre, G. Gobé, and D. W. Johnson, "Role of protein kinase C and oxidative stress in interleukin-1β-induced human proximal tubule cell injury and fibrogenesis," *Nephrology*, vol. 10, no. 1, pp. 73–80, 2005.

[68] H. H. Nietsch, M. W. Roe, J. F. Fiekers, A. L. Moore, and S. D. Lidofsky, "Activation of potassium and chloride channels by tumor necrosis factor α. Role in liver cell death," *The Journal of Biological Chemistry*, vol. 275, no. 27, pp. 20556–20561, 2000.

[69] J. Wang, H. Wang, Y. Zhang, H. Gao, S. Nattel, and Z. Wang, "Impairment of HERG K^+ channel function by tumor necrosis factor-α: role of reactive oxygen species as a mediator," *The Journal of Biological Chemistry*, vol. 279, no. 14, pp. 13289–13292, 2004.

[70] V. Diwan, G. Gobe, and L. Brown, "Glibenclamide improves kidney and heart structure and function in the adenine-diet model of chronic kidney disease," *Pharmacological Research*, vol. 79, pp. 104–110, 2014.

[71] C. Huang, C. A. Pollock, and X.-M. Chen, "High glucose induces CCL20 in proximal tubular cells via activation of the KCa3.1 channel," *PLoS ONE*, vol. 9, no. 4, Article ID e95173, 2014.

[72] C. Huang, S. Shen, Q. Ma et al., "Blockade of KCa3.1 ameliorates renal fibrosis through the TGF-β1/smad pathway in diabetic mice," *Diabetes*, vol. 62, no. 8, pp. 2923–2934, 2013.

[73] M. A. Garcia, R. Meca, D. Leite, and M. A. Boim, "Effect of renal ischemia/reperfusion on gene expression of a pH-sensitive K^+ channel," *Nephron Physiology*, vol. 106, no. 1, pp. 1–7, 2007.

[74] S. Shimizu, M. Saito, Y. Kinoshita et al., "Nicorandil ameliorates ischaemia-reperfusion injury in the rat kidney," *British Journal of Pharmacology*, vol. 163, no. 2, pp. 272–282, 2011.

[75] Y.-J. Zhang, A.-Q. Zhang, X.-X. Zhao, Z.-L. Tian, and L. Yao, "Nicorandil protects against ischaemia-reperfusion injury in newborn rat kidney," *Pharmacology*, vol. 92, no. 5-6, pp. 245–256, 2014.

[76] E. Grossini, C. Molinari, P. Pollesello et al., "Levosimendan protection against kidney ischemia/reperfusion injuries in anesthetized pigs," *Journal of Pharmacology and Experimental Therapeutics*, vol. 342, no. 2, pp. 376–388, 2012.

[77] L. C. Penning, G. Denecker, D. Vercammen, W. Declercq, R. G. Schipper, and P. Vandenabeele, "A role for potassium in TNF-induced apoptosis and gene-induction in human and rodent tumour cell lines," *Cytokine*, vol. 12, no. 6, pp. 747–750, 2000.

[78] F. Lang, E. Shumilina, M. Ritter, E. Gulbins, A. Vereninov, and S. M. Huber, "Ion channels and cell volume in regulation of cell proliferation and apoptotic cell death," *Contributions to Nephrology*, vol. 152, pp. 142–160, 2006.

[79] G. Shapovalov, V. Lehen'kyi, R. Skryma, and N. Prevarskaya, "TRP channels in cell survival and cell death in normal and transformed cells," *Cell Calcium*, vol. 50, no. 3, pp. 295–302, 2011.

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Research Article

Modulation of Voltage-Gated Sodium Channels by Activation of Tumor Necrosis Factor Receptor-1 and Receptor-2 in Small DRG Neurons of Rats

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Tumor necrosis factor- (TNF-) α is a proinflammatory cytokine involved in the development and maintenance of inflammatory and neuropathic pain. Its effects are mediated by two receptors, TNF receptor-1 (TNFR-1) and TNF receptor-2 (TNFR-2). These receptors play a crucial role in the sensitization of voltage-gated sodium channels (VGSCs), a key mechanism in the pathogenesis of chronic pain. Using the whole-cell patch-clamp technique, we examined the influence of TNFR-1 and TNFR-2 on VGSCs and TTX-resistant NaV1.8 channels in isolated rat dorsal root ganglion neurons by using selective TNFR agonists. The TNFR-1 agonist R32W (10 pg/mL) caused an increase in the VGSC current ($I_{Na(V)}$) by 27.2 ± 5.1%, while the TNFR-2 agonist D145 (10 pg/mL) increased the current by 44.9 ± 2.6%. This effect was dose dependent. Treating isolated NaV1.8 with R32W (100 pg/mL) resulted in an increase in $I_{NaV(1.8)}$ by 18.9 ± 1.6%, while treatment with D145 (100 pg/mL) increased the current by 14.5 ± 3.7%. Based on the current-voltage relationship, 10 pg of R32W or D145 led to an increase in $I_{Na(V)}$ in a bell-shaped, voltage-dependent manner with a maximum effect at −30 mV. The effects of TNFR activation on VGSCs promote excitation in primary afferent neurons and this might explain the sensitization mechanisms associated with neuropathic and inflammatory pain.

1. Introduction

Tumor necrosis factor- (TNF-) α is a proinflammatory cytokine that is expressed by a variety of cell types, such as immune or neuronal cells. TNF is involved in the development and maintenance of inflammatory and neuropathic pain [1, 2]. However, the mechanisms by which TNF elicits neuropathic pain are not fully understood. The effects of TNF are mediated by two distinct receptor subtypes, TNFR-1 and TNFR-2, which colocalize in nucleated cells [3, 4]. Both TNF and its receptors are expressed in rat dorsal root ganglion (DRG) neurons and are upregulated after nerve injury [5, 6]. *In vivo* application of TNF to DRG neurons induces pain-related behavior in rats [6], which is accompanied by mechanical and thermal hyperalgesia. These pain-inducing effects are prevented by preemptively using TNF-neutralizing agents [7, 8] or by inhibiting the TNF-signaling pathway [6]. In addition, TNF influences neuronal excitability by increasing voltage-gated sodium channel (VGSC) currents ($I_{Na(V)}$), which promotes action potential generation and may maintain neuropathic pain [9]. Local application of TNF to nociceptive neurons evokes action potentials and increases discharge rates of nerve fibers [10]. Tetrodotoxin- (TTX-) resistant NaV1.8 channel currents ($I_{NaV(1.8)}$), which are essential in the pathogenesis of neuropathic pain, are increased by a TNF-mediated MAP kinase-dependent pathway in DRG neurons [11, 12]. Furthermore, the lack of TNFR-1 in TNFR-1$^{-/-}$ mice led to reduced mechanical hypersensitivity, which is induced by exogenous TNF or inflammation [13]. Neutralizing antibodies against TNFR-1 also reduced thermal or mechanical hypersensitivity induced by nerve injury, while antibodies against TNFR-2 were ineffective [14]. These results suggest a crucial role for TNFR-1, but not TNFR-2, in the sensitization of VGSCs [12].

In the present study, we examined the influence of TNFR-1 and TNFR-2 on the modulation of $I_{Na(V)}$ and $I_{NaV(1.8)}$ in rat DRG neurons by using selective TNFR agonists.

2. Methods

2.1. Animals. Adult male Wistar rats (3 weeks old, 80–120 g) were used. All experiments were performed in accordance with the guidelines of the Animal Care and Use Committees of the University of Duisburg-Essen, Germany. All animals were kept on a 14/10 h light/dark cycle with water and food pellets available *ad libitum*.

2.2. Selective TNF Receptor Agonists. TNF mutant proteins were used (given by the P. Vandenabeele Lab, Ghent, Belgium) for the selective stimulation of either TNFR-1 or TNFR-2. The R32W and D145 proteins contain double mutations (R32W/S86T and D143N/A145R, resp., [15, 16]) and can selectively activate rat TNFR-1 and TNFR-2, respectively [17]. Mutated TNF proteins R32W and D145 have been extensively tested and showed differential binding to purified TNF receptors. The binding studies have been confirmed by biological assays using either TNFR-1 or TNFR-2 [16]. Each receptor-specific protein was dissolved in an ACSF vehicle containing 0.1% bovine serum albumin (BSA).

2.3. Cell Culture. DRG neurons were isolated from 3-week-old Wistar rats. The animals were anaesthetized with isoflurane. In the absence of pain reflexes, the animals were decapitated. The spinal column was removed and opened from the dorsal side. After dissection of the spinal cord, the DRGs were collected and placed in ice cold F12 media (Biochrom AG, Germany). Under optic control, the spinal nerves were cut off and the ganglion capsules were opened. The capsules were transferred into a medium containing 0.9 mL of F12 and 0.1 mL of collagenase (2612.5 U/mL, Type II, Biochrom AG, Germany) and incubated for 45 min in a humidified atmosphere containing 5% CO_2 at a temperature of 37°C. To remove the collagenase, the DRGs were washed three times in 1 mL of F12 medium. Afterwards, the DRGs were trypsinized (2525 U trypsin/mL F12 medium) for 2 min under the same conditions. DRGs were washed twice, left in a final volume of 0.7 mL of F12 medium, and triturated with a pipette until the neurons were released. A total of 50 μL of this suspension was placed in the middle of each Petri dish (3 cm, Falcon Easy Grip). Cells were incubated for at least 2 h so that the neurons could adhere to the dish, and then 1 mL of medium containing F12 and 10% horse serum (Biochrom AG, Germany) was added to each dish.

2.4. Electrophysiology. $I_{Na(V)}$ and $I_{NaV(1.8)}$ were isolated by performing the whole-cell patch-clamp technique using HEKA EPC 10 amplifier with Patchmaster software (HEKA Electronics, Germany). Only cells with a small diameter < 30 μm were chosen. Microelectrodes, consisting of borosilicate glass (Biomedical Instruments), were pulled with a HEKA Pipette Puller (PIP6, HEKA Electronics, Germany) and were fire polished to a final resistance of 4-5 MΩ by using a microforge (Narishige, Japan). This relatively high resistance was intentionally chosen to guarantee the necessary stable configuration during the course of the experiments. Although it is possible that this configuration can have a negative effect on the electrodes' ability to pass sufficient current, especially at peak currents while maintaining a current-voltage relationship (*IV*-curve), this configuration allowed us to analyze the underlying mechanisms that have small effects on the current.

Before starting experiments, F12 culture medium was replaced by an external solution containing 72 mM NaCl, 72 mM choline-Cl, 2.5 mM KCl, 10 mM HEPES, 10 mM glucose, and 50 μM CdCl and adjusted to pH of 7.4 with NaOH. The internal pipette solution contained 140 mM CsCl, 5 mM NaCl, 10 mM HEPES, 10 MM EGTA, and 4 mM MgCl2 and was adjusted to pH of 7.2 with TEA-OH.

The *IV*-curve for $I_{Na(V)}$ was recorded for depolarizing steps starting at −60 mV and increased stepwise by 10 mV to maximum depolarization of +60 mV after hyperpolarizing prepulse to −120 mV for 500 ms to recover sodium channels from state of inactivation. Application of the drugs started after obtaining 2 *IV*-curves under control conditions. For time course experiments, TNFR agonists were applied after 10 control depolarization procedures. NaV1.8 currents were isolated after eliminating tetrodotoxin-sensitive currents using 500 nM tetrodotoxin in the external solution and maintaining a −80 mV potential, which efficiently eliminates the persistent tetrodotoxin-resistant NaV1.9 current as shown before [18, 19]. *IV*-curves of isolated NaV1.8 currents were similar to other curves described elsewhere [20]. The somata of the small DRG neurons were classified by their diameters (15~30 μm) and C_m (≤45 pF). Neurons were not considered for analysis if they had high leakage currents (holding current > 1.0 nA at −80 mV), membrane blebs, total sodium current < 500 pA, or access resistance > 5 MΩ. Access resistance was monitored throughout the experiment and data were not used if resistance changes of >20% occurred. The offset potential was zeroed before patching the cells and checked after each recording for drift. Data were sampled at 10 kHz, compensated for series resistance, and stored on hard disk.

2.5. Data Analysis. All currents were online corrected by using a P/4 protocol. All $I_{Na(V)}$ used for the time course and current-voltage relationships were rundown corrected assuming linear rundown. Current values were standardized to the mean current before application of the drugs (= 100%). For calculation of the mean current, 20 data points were used. The nonresponders were given a value of 0 in the calculation determining the current increase.

2.6. Statistical Analysis. All data are given as the mean +/− standard deviation. Data were analyzed by using double-sided Student's *t*-test. A difference was accepted as significant if $p < 0.05$.

3. Results

$I_{Na(V)}$ was successfully recorded from 112 DRG neurons. Cells were classified as responders if the current was changed by at least 10% ($n = 71$). Depolarization of DRG neurons from the holding potential to 0 mV led to an inwardly directed, inactivating current. Administration of the dissolving agents

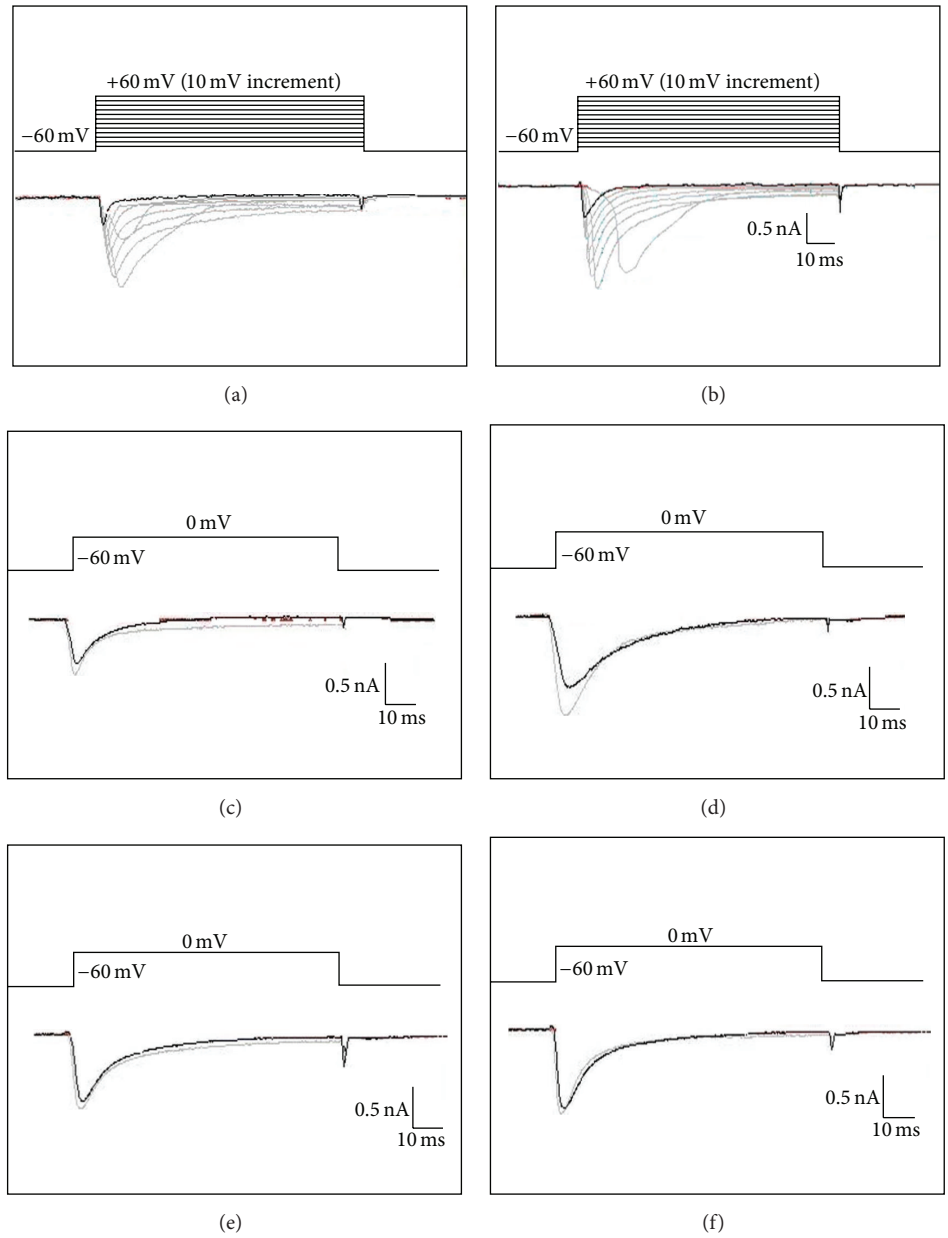

Figure 1: Representative traces of VGSCs. Cells were depolarized to a variety of membrane potentials ((a) $I_{Na(V)}$ and (b) $I_{Na(V1.8)}$). $I_{Na(V)}$ currents before (black) and after (gray) application of 10 pg R32W (c) and D145 (d). $I_{Na(V1.8)}$ currents before (black) and after (gray) application of 100 pg R32W (e) and D145 (f).

(ACSF + 0.1% BSA) does not change the currents (data not shown). Treatment of DRG neurons with either R32W or D145 led to an increase of $I_{Na(V)}$ and $I_{NaV(1.8)}$ (Figures 1(a) and 1(b)).

Application of R32W (10 pg) led to an increase of $I_{Na(V)}$ by 27.2 ± 5.1%. A steady state current was reached after 415 s ($n = 11$). Application of D145 (10 pg) increased $I_{Na(V)}$ by 44.9 ± 2.6%, reaching a steady state after 500 s ($n = 8$). During the washout, the currents return to almost baseline (111.45 ± 8.5% versus 112.87 ± 7.6%) (Figure 2(a)).

In the *IV*-curve, a maximum current was elicited at depolarization to −30 mV. R32W and D145 increased the current in a bell-shaped voltage-dependent manner in the range between −50 mV and +60 mV ($n = 10$ each). Reversal potentials were at +50 mV and were not changed by the application of D145 or R32W (Figures 2(b) and 2(c)).

During repetitive depolarization procedures to 0 mV, application of either R32W (100 pg) ($n = 8$) or D145 (100 pg) ($n = 8$) led to an increase in $I_{NaV(1.8)}$ by 18.9 ± 1.6% and 14.5 ± 3.7% 300 s after treatment, respectively (Figure 2(d)).

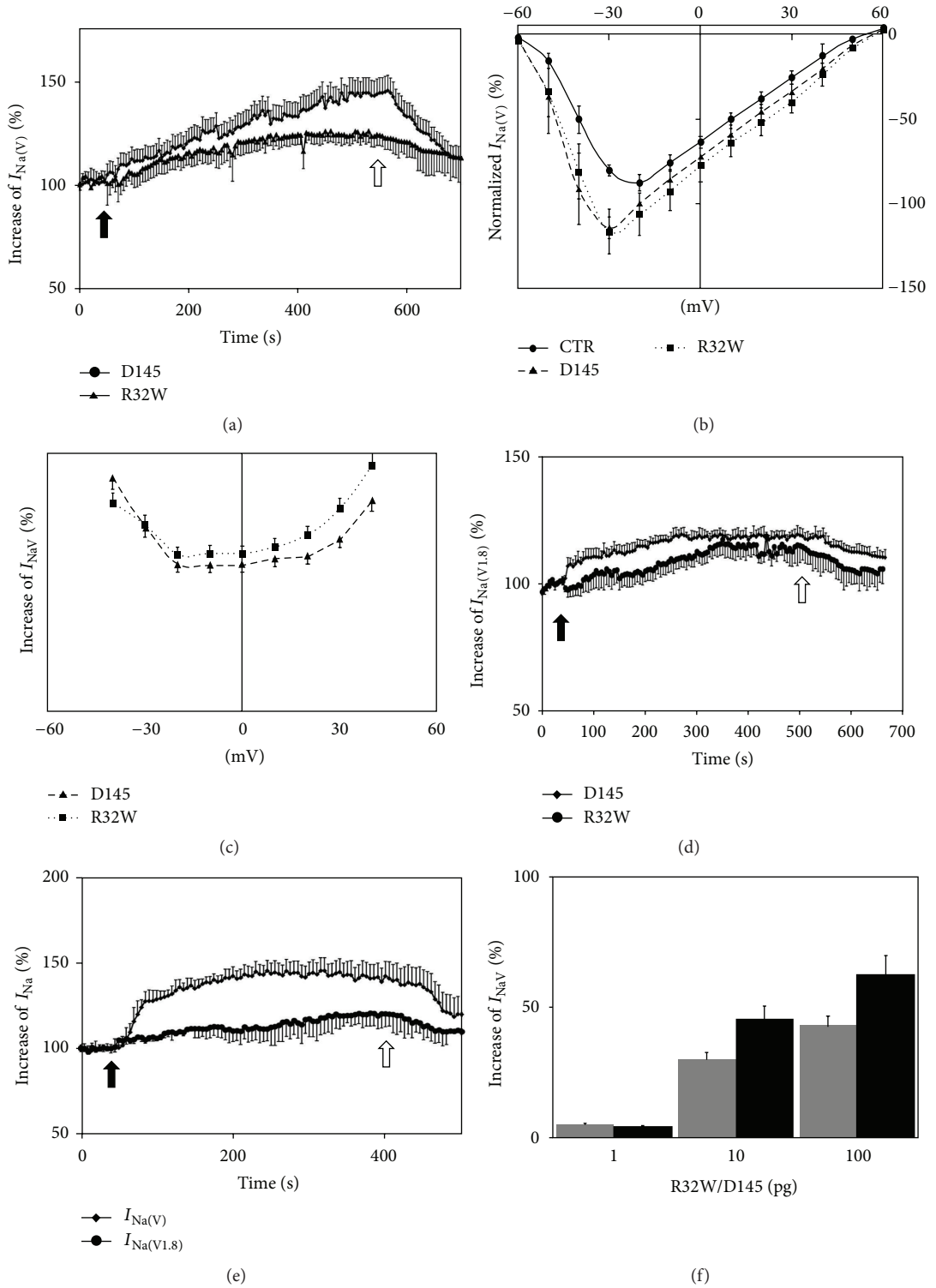

FIGURE 2: Characterization of R32W and D145 effects on $I_{Na(V)}$ and $I_{Na(V1.8)}$ currents. (a) Time course of normalized $I_{Na(V)}$ during repetitive depolarization from the holding potential to 0 mV before and after application of 10 pg R32W or 10 pg D145 (black arrow: time of application; white arrow: washout). (b) *IV*-curve of $I_{Na(V)}$ (black: control conditions; speckled line: after application of 10 pg R32W; dashed line: after application of 10 pg D145). Cells were depolarized to a variety of potentials (−60 to 60 mV) from a holding potential of –80 mV at increments of 10 mV to elicit $I_{Na(V)}$. (c) Voltage-dependent reduction of $I_{Na(V)}$ after administration of 10 pg R32W or 10 pg D145. (d) Time course of normalized $I_{Na(V1.8)}$ during repetitive depolarization from the holding potential to 0 mV before and after application of 100 pg R32W or 100 pg D145 (black arrow: time of application; white arrow: washout). (e) Time course of normalized $I_{Na(V)}$ and $I_{Na(V1.8)}$ during repetitive depolarization from the holding potential to 0 mV before and after application of 10 pg R32W *and* 10 pg D145 (black arrow: time of application; white arrow: washout). (f) R32W and D145 dose-dependent increase in $I_{Na(V)}$.

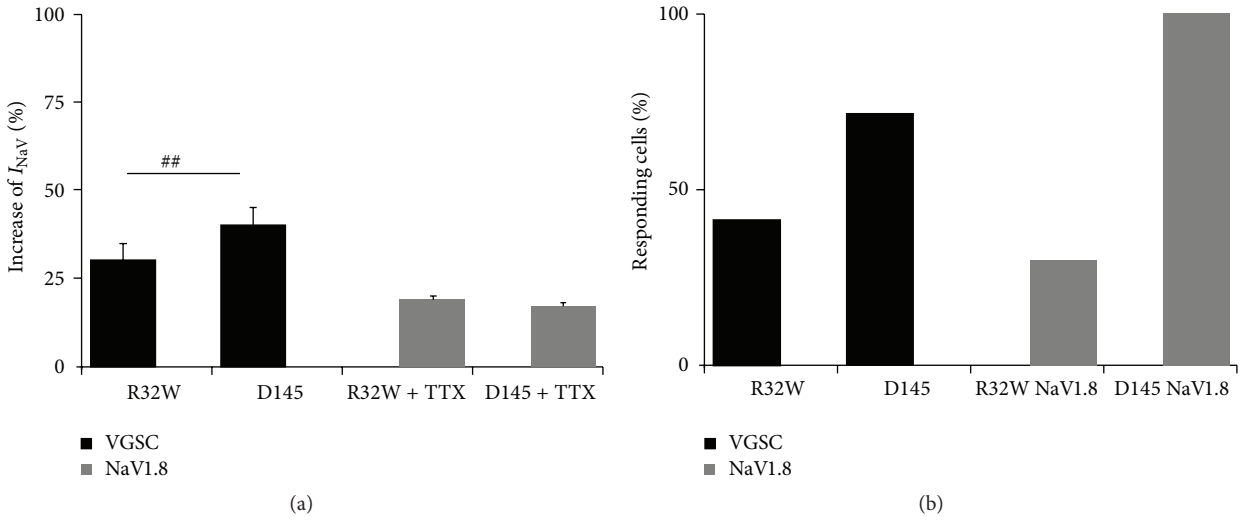

FIGURE 3: (a) Increase in $I_{Na(V)}$ (10 pg of each drug) and $I_{Na(V1.8)}$ (100 pg of each drug) currents after application of R32W or D145 during repetitive depolarization to 0 mV after 600 s when the current reaches a steady state ($^{\#\#}p < 0.01$). (b) Amount of responding cells, including $I_{Na(V)}$ or $I_{Na(V1.8)}$, in the cell population. Cells were classified as responding cells if the currents were affected by more than 10%.

The dual application of R32W and D145 (100 pg) led to an increase of $I_{Na(V)}$ by 45.2 ± 7.4% 320 s after treatment ($n = 7$), while $I_{NaV(1.8)}$ was increased by 20.6 ± 6.8% 400 s after application ($n = 7$) during repetitive depolarization to 0 mV (Figure 2(e)).

Using increasing concentrations of R32W or D145 (1–100 pg) led to an increase of $I_{Na(V)}$ in a dose-dependent manner (Figure 2(f)).

Application of D145 (10 pg) led to significantly higher increase of $I_{Na(V)}$ compared to the application of R32W (10 pg). Treatment with either 100 pg of D145 or R32W led to a significantly higher increase of $I_{Na(V)}$ compared to $I_{NaV(1.8)}$ (Figure 3(a)). In both settings, when measuring isolated $I_{Na(V)}$ and $I_{NaV(1.8)}$, the amount of responding cells was higher using D145 than R32W (Figure 3(b)).

4. Discussion

This study presents evidence for differential modulation of VGSC and the TTX-resistant subtype NaV1.8 by TNFR in small DRG neurons. TNF has been shown to regulate a variety of ion channels. It decreases potassium channel currents in retinal ganglion neurons [21] and increases calcium channel currents in hippocampal and cultured superior cervical ganglion neurons [22, 23]. In DRG neurons, a decrease of voltage-gated calcium channel currents and an increase in $I_{Na(V)}$ have been described, while voltage-gated potassium channel currents were not affected, suggesting that TNF has differential effects depending on the ion channel and cell type [9]. An increase in $I_{Na(V)}$ may promote hyperexcitability, which is a key symptom of neuropathic pain. Besides the long-lasting effects of TNF by regulating the expression of a variety of inflammatory mediators and modifying signaling proteins, the application of TNF has rapid onset effects, which suggest interactions with primary excitation proteins such as VGSC. The enhancement of TTX-resistant VGSC currents starts in <60 s and the enhancement of VGSC currents in DRG neurons begins within 20 s after TNF administration [9, 12, 24]. Using *in vitro* single-fiber recordings for isolating Aδ- and C-fiber activity in DRG neurons, perfusion with TNF increases rapid firing rates, also suggesting a direct interaction between VGSCs and TNF [25]. Variations in TNF responses exist but can be explained by technical reasons, that is, distance from application pipette to the neuron. Interestingly, the increase in $I_{Na(V)}$ is higher when using selective agonists compared to the use of TNF alone. In other systems, TNF has been shown to reduce $I_{Na(V)}$ via a PKC-dependent pathway, which may counteract effects on VGSC via other signaling pathways [26]. This can explain why TNF mediates different effects compared to the selective agonists alone. The application of TNF to DRG neurons induces mechanical allodynia and mechanical sensitivity of C-fibers [27, 28]. These results suggest an important role for the interaction of TNF and VGSC.

TNF promotes its effects via the constitutively expressed TNFR-1 and the inducible TNFR-2. Different intracellular signal pathways are affected by TNFR-1 and TNFR-2 activation. While TNFR-1 activation leads to internalization of the receptor, TNFR-2 activation is followed by shedding of the ligand-receptor complex [29]. Consequently, TNFR activation elicits distinct effects. For example, it has been shown that activation of TNFR-1 by local application of TNF to naïve DRGs induced high-frequency firing of Aβ- and Aδ-fibers, while TNFR-2 activation had no effect. In contrast, after nerve injury, both TNFR-1 and TNFR-2 activation increased discharge rates [25]. TNFR-1 seems to be very important in the pain-sensitizing actions of TNF. Mechanical hypersensitivity induced by inflammation or nerve injury is reduced in TNFR-1 knockout mice [13]. In addition, neutralizing antibodies against TNFR-1 reduced pain-associated behavior, while antibodies against TNFR-2 were noneffective [14]. These results underlie the different roles of TNFR-1 and

TNFR-2 in pathologic conditions and offer the possibility to target neuropathic pain.

In contrast to previous studies, our results showing that there is a larger increase of $I_{Na(V)}$ after activation of TNFR-2 and an increase in reacting cells provide evidence that TNFR-2 has a more important role than TNFR-1 in the modulation of nerve excitability. The higher rate of responding cells when isolating $I_{NaV(1.8)}$ is surprising. A possible reason for this higher rate may be that the membrane-bound receptors are coexpressed. It is possible that the activation of TNFR-1/TNFR-2 leads to higher response rates when coexpressed with NaV1.8 in comparison to neurons that coexpress TNFRs with other isoforms of NaV. The coexpression of TNFR and NaV isoforms has been shown for NaV1.7 in DRG neurons and chromaffin cells [30]. In addition, the expression of TNFR-2 has been shown to be closely related to NaV1.7 and NaV1.8 expression in sensory neurons, which may explain the predominant responding rates of D145 compared to R32W [31]. This effect may be important in the initial phase of inflammatory and neuropathic pain but may be superimposed by the changes in the pattern of expression of TNFR-1 and TNFR-2 after nerve injury and explain the effects of TNFR-1 and TNFR-2 seen in other studies, especially after nerve injury [6, 25]. The shift in the *IV*-curve and the increase of $I_{Na(V)}$ in the voltage above 0 mV may be explained by an increase of the available channels or an increase in channel permeability.

Several intracellular pathways have to be examined for downstream signaling of TNFR activation. VGSCs have various phosphorylation sites, on which modification may lead to changes in the biophysical properties of channel gating. Recently, chelerythrine has been shown to suppress the inhibitory effect of TNF on sodium channel currents of skeletal muscle cells, suggesting the involvement of protein kinase C (PKC) in regulation of VGSC [26, 32]. In addition, it is known that modulation of VGSCs is dependent on the special channel subtype [33]. It has been shown that the activation of TNFR-1 increases $I_{NaV(1.8)}$ rapidly in mouse DRG neurons by p38-dependent mechanisms [12]. p38 may enhance $I_{NaV(1.8)}$ by phosphorylating the NaV1.8 channel or an associated protein. In addition, p38 has been shown to directly modulate other voltage-gated channels [34]. In inflammatory responses, NF-κB has been shown to be involved in TNF-mediated ion channel regulation [35]. Pathways with common final targets are possible due to the observation that the dual application of TNFR agonists has no additive effect on $I_{Na(V)}$ or $I_{NaV(1.8)}$. The possibility of TNFR-subtype dependent signaling pathways will have to be addressed in further studies.

The limitations of our study need to be discussed. Because the mutant peptides were designed to target human TNFR, we cannot exclude the possibility that there are different effects in rat DRGs. However, the cross-reactivity of our mutants has been established before using a cell death assay [36]. The efficacy of the mutants may differ in TNFR-1 and TNFR-2. Our study only uses naïve DRG neurons. Because of the several pathways activated after nerve injury, our results concerning TNF as well as other inflammatory mediators or posttranslational modification of ion channel activity may be limited by long-lasting superimposing effects. We only used *in vitro* techniques; hence, the results may translate to *in vivo* effects. Another aspect to consider is the expression rate of TNFR-1 and TNFR-2 in nociceptive neurons. In our study, we found a responding rate of ~60% in all experiments. Other studies of mouse and rat DRG have shown that TNFR-1 or TRPV1 is expressed in up to 40% of the sensory neurons. Most of the TRPV1-expressing neurons coexpress isolectin-B4. While >30% of these neurons coexpress TNFR-1 and TRPV1, only 10% of the neurons coexpress both TNFR and IB4 [37]. In small sized DRG neurons (~30 μm), TRPV1 is expressed in ~50% of the cell population [38]. To our knowledge, the coexpression rates of TNFR-2 and TRPV1 or IB4 have not been described. This may explain the differential effects of TNFR agonists in different cell populations. Further experiments have be designed to determine whether this is the case.

In conclusion, our study provides evidence for a potential role of TNFR-2 in the generation of hyperexcitability by increasing of VGSC currents in uninjured (or peracute injured) neurons, which may be a relevant mechanism in neuropathic and inflammatory pain conditions.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] C. Sommer and M. Kress, "Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia," *Neuroscience Letters*, vol. 361, no. 1–3, pp. 184–187, 2004.

[2] E. K. Joseph and J. D. Levine, "Caspase signalling in neuropathic and inflammatory pain in the rat," *European Journal of Neuroscience*, vol. 20, no. 11, pp. 2896–2902, 2004.

[3] P. Vandenabeele, W. Declercq, R. Beyaert, and W. Fiers, "Two tumour necrosis factor receptors: structure and function," *Trends in Cell Biology*, vol. 5, no. 10, pp. 392–399, 1995.

[4] D. J. MacEwan, "TNF receptor subtype signalling: differences and cellular consequences," *Cellular Signalling*, vol. 14, no. 6, pp. 477–492, 2002.

[5] P. G. Murphy, J. Grondin, M. Altares, and P. M. Richardson, "Induction of interleukin-6 in axotomized sensory neurons," *Journal of Neuroscience*, vol. 15, no. 7, part 2, pp. 5130–5138, 1995.

[6] M. Schäfers, L. S. Sorkin, C. Geis, and V. I. Shubayev, "Spinal nerve ligation induces transient upregulation of tumor necrosis factor receptors 1 and 2 in injured and adjacent uninjured dorsal root ganglia in the rat," *Neuroscience Letters*, vol. 347, no. 3, pp. 179–182, 2003.

[7] T. Lindenlaub, P. Teuteberg, T. Hartung, and C. Sommer, "Effects of neutralizing antibodies to TNF-alpha on pain-related behavior and nerve regeneration in mice with chronic constriction injury," *Brain Research*, vol. 866, no. 1-2, pp. 15–22, 2000.

[8] S. Sweitzer, D. Martin, and J. A. DeLeo, "Intrathecal interleukin-1 receptor antagonist in combination with soluble tumor necrosis factor receptor exhibits an anti-allodynic action in a rat

model of neuropathic pain," *Neuroscience*, vol. 103, no. 2, pp. 529–539, 2001.

[9] J. C. Czeschik, T. Hagenacker, M. Schäfers, and D. Büsselberg, "TNF-α differentially modulates ion channels of nociceptive neurons," *Neuroscience Letters*, vol. 434, no. 3, pp. 293–298, 2008.

[10] L. S. Sorkin, W.-H. Xiao, R. Wagner, and R. R. Myers, "Tumour necrosis factor-α induces ectopic activity in nociceptive primary afferent fibres," *Neuroscience*, vol. 81, no. 1, pp. 255–262, 1997.

[11] G. Bhave and R. W. Gereau IV, "Posttranslational mechanisms of peripheral sensitization," *Journal of Neurobiology*, vol. 61, no. 1, pp. 88–106, 2004.

[12] X. Jin and R. W. Gereau IV, "Acute p38-mediated modulation of tetrodotoxin-resistant sodium channels in mouse sensory neurons by tumor necrosis factor-α," *Journal of Neuroscience*, vol. 26, no. 1, pp. 246–255, 2006.

[13] T. M. Cunha, W. A. Verri Jr., J. S. Silva, S. Poole, F. Q. Cunha, and S. H. Ferreira, "A cascade of cytokines mediates mechanical inflammatory hypernociception in mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 5, pp. 1755–1760, 2005.

[14] C. Sommer, C. Schmidt, and A. George, "Hyperalgesia in experimental neuropathy is dependent on the TNF receptor 1," *Experimental Neurology*, vol. 151, no. 1, pp. 138–142, 1998.

[15] H. Loetscher, D. Stueber, D. Banner, F. Mackay, and W. Lesslauer, "Human tumor necrosis factor α (TNFα) mutants with exclusive specificity for the 55-kDa or 75-kDa TNF receptors," *The Journal of Biological Chemistry*, vol. 268, no. 35, pp. 26350–26357, 1993.

[16] X. Van Ostade, P. Vandenabeele, B. Everaerdt et al., "Human TNF mutants with selective activity on the p55 receptor," *Nature*, vol. 361, no. 6409, pp. 266–269, 1993.

[17] G. T. Baxter, R. C. Kuo, O. J. Jupp, P. Vandenabeele, and D. J. MacEwan, "Tumor necrosis factor-α mediates both apoptotic cell death and cell proliferation in a human hematopoietic cell line dependent on mitotic activity and receptor subtype expression," *The Journal of Biological Chemistry*, vol. 274, no. 14, pp. 9539–9547, 1999.

[18] A. Leffler, R. I. Herzog, S. D. Dib-Hajj, S. G. Waxman, and T. R. Cummins, "Pharmacological properties of neuronal TTX-resistant sodium channels and the role of a critical serine pore residue," *Pflügers Archiv*, vol. 451, no. 3, pp. 454–463, 2005.

[19] B. T. Priest, B. A. Murphy, J. A. Lindia et al., "Contribution of the tetrodotoxin-resistant voltage-gated sodium channel Nav1.9 to sensory transmission and nociceptive behavior," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 26, pp. 9382–9387, 2005.

[20] Z.-J. Huang and X.-J. Song, "Differing alterations of sodium currents in small dorsal root ganglion neurons after ganglion compression and peripheral nerve injury," *Molecular Pain*, vol. 4, article 20, 2008.

[21] R. Diem, R. Meyer, J. H. Weishaupt, and M. Bähr, "Reduction of potassium currents and phosphatidylinositol 3-kinase-dependent Akt phosphorylation by tumor necrosis factor-(alpha) rescues axotomized retinal ganglion cells from retrograde cell death in vivo," *The Journal of Neuroscience*, vol. 21, no. 6, pp. 2058–2066, 2001.

[22] K. Furukawa and M. P. Mattson, "The transcription factor NF-κB mediates increases in calcium currents and decreases in NMDA- and AMPA/kainate-induced currents induced by tumor necrosis factor-α in hippocampal neurons," *Journal of Neurochemistry*, vol. 70, no. 5, pp. 1876–1886, 1998.

[23] B. Soliven and J. Albert, "Tumor necrosis factor modulates Ca^{2+} currents in cultured sympathetic neurons," *Journal of Neuroscience*, vol. 12, no. 7, pp. 2665–2671, 1992.

[24] S. Gudes, O. Barkai, Y. Caspi, B. Katz, S. Lev, and A. M. Binshtok, "The role of slow and persistent TTX-resistant sodium currents in acute tumor necrosis factor-α-mediated increase in nociceptors excitability," *Journal of Neurophysiology*, vol. 113, no. 2, pp. 601–619, 2015.

[25] M. Schäfers, C. Sommer, C. Geis, T. Hagenacker, P. Vandenabeele, and L. S. Sorkin, "Selective stimulation of either tumor necrosis factor receptor differentially induces pain behavior in vivo and ectopic activity in sensory neurons in vitro," *Neuroscience*, vol. 157, no. 2, pp. 414–423, 2008.

[26] M. Guillouet, G. Gueret, F. Rannou et al., "Tumor necrosis factor-α downregulates sodium current in skeletal muscle by protein kinase c activation: involvement in critical illness polyneuromyopathy," *The American Journal of Physiology—Cell Physiology*, vol. 301, no. 5, pp. C1057–C1063, 2011.

[27] Y. Homma, S. J. Brull, and J.-M. Zhang, "A comparison of chronic pain behavior following local application of tumor necrosis factor α to the normal and mechanically compressed lumbar ganglia in the rat," *Pain*, vol. 95, no. 3, pp. 239–246, 2002.

[28] H. Junger and L. S. Sorkin, "Nociceptive and inflammatory effects of subcutaneous TNFalpha," *Pain*, vol. 85, no. 1-2, pp. 145–151, 2000.

[29] E. C. Ledgerwood, J. S. Pober, and J. R. Bradley, "Recent advances in the molecular basis of TNF signal transduction," *Laboratory Investigation*, vol. 79, no. 9, pp. 1041–1050, 1999.

[30] R. Tamura, T. Nemoto, T. Maruta et al., "Up-Regulation of NaV1.7 sodium channels expression by tumor necrosis factor-alpha in cultured bovine adrenal chromaffin cells and rat dorsal root ganglion neurons," *Anesthesia and Analgesia*, vol. 118, no. 2, pp. 318–324, 2014.

[31] Z. Wu, S. Wang, S. Gruber, M. Mata, and D. J. Fink, "Full–length membrane–bound tumor necrosis factor-α acts through tumor necrosis factor receptor 2 to modify phenotype of sensory neurons," *Pain*, vol. 154, no. 9, pp. 1778–1782, 2013.

[32] M. Guillouet, G. Gueret, F. Rannou et al., "TNFα increases resting potential in isolated fibres from rat peroneus longus by a PKC mediated mechanism: involvement in ICU acquired polyneuromyopathy," *Cytokine*, vol. 56, no. 2, pp. 149–152, 2011.

[33] K. T. Murray, N. N. Hu, J. R. Daw et al., "Functional effects of protein kinase C activation on the human cardiac Na+ channel," *Circulation Research*, vol. 80, no. 3, pp. 370–376, 1997.

[34] E. K. Wittmack, A. M. Rush, A. Hudmon, S. G. Waxman, and S. D. Dib-Hajj, "Voltage-gated sodium channel Nav1.6 is modulated by p38 mitogen-activated protein kinase," *The Journal of Neuroscience*, vol. 25, no. 28, pp. 6621–6630, 2005.

[35] G. Y. Jung, J. Y. Lee, H. Rhim, T. H. Oh, and T. Y. Yune, "An increase in voltage-gated sodium channel current elicits microglial activation followed inflammatory responses in vitro and in vivo after spinal cord injury," *Glia*, vol. 61, no. 11, pp. 1807–1821, 2013.

[36] J. Pollock, S. M. McFarlane, M. C. Connell et al., "TNF-α receptors simultaneously activate Ca^{2+} mobilisation and stress kinases in cultured sensory neurones," *Neuropharmacology*, vol. 42, no. 1, pp. 93–106, 2002.

[37] S. Hensellek, P. Brell, H.-G. Schaible, R. Bräuer, and G. Segond von Banchet, "The cytokine TNFα increases the proportion

of DRG neurones expressing the TRPV1 receptor via the TNFR1 receptor and ERK activation," *Molecular and Cellular Neuroscience*, vol. 36, no. 3, pp. 381–391, 2007.

[38] L. Yu, F. Yang, H. Luo et al., "The role of TRPV1 in different subtypes of dorsal root ganglion neurons in rat chronic inflammatory nociception induced by complete Freund's adjuvant," *Molecular Pain*, vol. 4, article 61, 2008.

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Research Article

Lung Epithelial TRPA1 Transduces the Extracellular ROS into Transcriptional Regulation of Lung Inflammation Induced by Cigarette Smoke: The Role of Influxed Ca^{2+}

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The mechanism underlying the inflammatory role of TRPA1 in lung epithelial cells (LECs) remains unclear. Here, we show that cigarette smoke extract (CSE) sequentially induced several events in LECs. The Ca^{2+} influx was prevented by decreasing extracellular reactive oxygen species (ROS) with the scavenger N-acetyl-cysteine, removing extracellular Ca^{2+} with the chelator EGTA, or treating with the TRPA1 antagonist HC030031. NADPH oxidase activation was abolished by its inhibitor apocynin, EGTA, or HC030031. The increased intracellular ROS was halted by apocynin, N-acetyl-cysteine, or HC030031. The activation of the MAPKs/NF-κB signaling was suppressed by EGTA, N-acetyl-cysteine, or HC030031. IL-8 induction was inhibited by HC030031 or TRPA1 siRNA. Additionally, chronic cigarette smoke (CS) exposure in wild-type mice induced TRPA1 expression in LECs and lung tissues. In CS-exposure *trpa1* $^{-/-}$ mice, the increased BALF level of ROS was similar to that of CS-exposure wild-type mice; yet lung inflammation was lessened. Thus, in LECs, CSE may initially increase extracellular ROS, which activate TRPA1 leading to an increase in Ca^{2+} influx. The increased intracellular Ca^{2+} contributes to activation of NADPH oxidase, resulting in increased intracellular ROS, which activate the MAPKs/NF-κB signaling leading to IL-8 induction. This mechanism may possibly be at work in mice chronically exposed to CS.

1. Introduction

Inhaled cigarette smoke (CS) causes persistent lung inflammation that leads to the development of chronic obstructive pulmonary disease (COPD) in smokers [1]. CS-induced lung inflammation is well recognized as being regulated by a complex mechanism that involves various types of cells and a range of inflammatory mediators [1, 2]. For example, the chemokine interleukin-8 (IL-8), when released from lung epithelial cells, plays a vital role in the regulation of lung inflammation [3–7]. This is due to the fact that the lung epithelium is a target for direct insult by CS and that chemokines are able to promote inflammatory cell recruitment into the lungs [2, 5]. The induction of inflammatory mediators in lung epithelial cells [3–7] or other types of lung cells [8] by CS is mainly regulated by redox-sensitive signaling pathways. CS is a potent oxidant that has the ability to initially cause increases in reactive oxygen species (ROS) in extracellular fluid [7, 9] and in bronchoalveolar lavage fluid (BALF) [7, 10]. Subsequently, via activation of NADPH oxidase, CS increases intracellular ROS levels in lung epithelial cells [6, 7] and other types of lung cells [11–14]. NADPH oxidase is known to be the primary enzyme system able to generate ROS in mammalian cells [15]. This increase in intracellular ROS in epithelial cells is known to activate several ROS-sensitive signaling pathways, including the mitogen-activated protein kinases (MAPKs) and various downstream transcriptional factors, such as nuclear factor-κB (NF-κB); these effects then ultimately promote inflammatory gene expression [3, 4, 6–8]. However, the role of increased extracellular ROS in the CS-induced regulation of lung inflammation has remained largely unclear up to the present.

Transient receptor potential ankyrin 1 (TRPA1) is a type of nonselective transmembrane cation channel that is mainly involved in Ca^{2+} permeability [16]. TRPA1 was originally thought to be predominately expressed in primary sensory neurons [16]. Lung neuronal TRPA1 has been suggested to act as an oxidant sensor allowing detection of pulmonary ROS that is induced by oxidants such as CS or H_2O_2; this results in the elicitation of neural impulses in lung sensory fibers [17–19]. However, recent studies have demonstrated that TRPA1 is also expressed in various types of nonneuronal cells, including lung epithelial cells [17, 20–22]. Indeed, stimulation of lung epithelial cells by TRPA1 agonists or by CS extract (CSE) increases the production of IL-8 and this effect is able to be attenuated by TRPA1 antagonists [21, 22]. Additionally, when exposed to CS for 3 days, TRPA1 knockout ($trpa1^{-/-}$) mice display a lower level of keratinocyte chemoattractant (an IL-8 analogue) in BALF, as compared to wild-type mice [22]. These observations suggest that nonneuronal lung TRPA1 plays an important role in the CS-induced production of inflammatory mediators. Nevertheless, how nonneuronal TRPA1 in the lung participates in the CS-induced transcriptional regulation of these mediators has remained elusive.

The aims in this study were, firstly, to determine whether lung epithelial TRPA1 is able to be activated when there is an increase in extracellular ROS; this was done using an *in vitro* model involving primary human bronchial epithelial cells (HBECs) and exposure of these to CSE [6, 7]. Secondly, we wished to investigate how epithelial TRPA1 may act as a crucial regulator in the activation of NADPH oxidase and thus promote the subsequent transcriptional regulation of IL-8 in HBECs. Thirdly, we wanted to assess the importance of lung epithelial TRPA1 to CS-induced lung inflammation using a murine model that consisted of chronic CS exposure for 4 weeks [6, 7, 23].

2. Materials and Methods

2.1. Reagents. Antibodies (Abs) and ELISA kits for the detection of IL-8 and MIP-2 were purchased from R&D Systems (Minneapolis, MN, USA). Rabbit Ab against c-Jun N-terminal kinases (JNK) was obtained from Cell Signaling (Beverly, MA, USA). Mouse Ab against phospho-JNK was purchased from BD (San Jose, CA, USA). Abs against extracellular signal-regulated kinase (ERK), phospho-ERK, and p65 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse Abs against α-tubulin, HC030031, ethylene glycol tetraacetic acid (EGTA), N-acetyl-cysteine, and apocynin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse Ab against TRPA1 was obtained from Abcam (Cambridge, MA, USA) or from Calbiochem (San Diego, CA, USA). Mouse Ab against histone H1 was purchased from Millipore (Bedford, MA, USA). Mouse Ab against 4-hydroxynonenal (4-HNE) was obtained from Abcam (Cambridge, MA, USA). The Screen Quest Fluo-8 Medium Removal Calcium Assay Kit was purchased from AAT Bioquest (Sunnyvale, CA, USA). The EnzyChrom $NADP^+$/NADPH assay kit was obtained from BioAssay Systems (Hayward, CA, USA). The membrane-permeable probes hydroethidine (HE) and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Molecular Probes (Eugene, OR, USA). Scramble and TRPA1 siRNAs were obtained from Ambion (Austin, TX, USA). INTERFERin siRNA transfection reagent was purchased from Polyplus (New York, NY, USA).

2.2. Preparation of CSE. CSE was freshly prepared on the day of the experiment as previously described [6, 7] with some modifications. In brief, 1000 mL of the smoke generated from two burning cigarettes (Marlboro Red Label) without filters was sucked at a constant flow rate (8 mL/s) into a syringe and then bubbled into a tube containing 20 mL serum-free medium. The CSE solution was sterilized using a 0.22-μm filter (Millipore, Bedford, MA) and the pH was adjusted to 7.4. The optical density of the CSE solution was determined by measuring the absorbance at 302 nm [24] or 320 nm [25], which, in reality, was found to show little difference between different preparations. This CSE solution was considered 100% CSE and was further diluted with serum-free medium to the desired concentrations that were then used to treat HBECs for different durations of time.

2.3. Cell Culture. HBECs (Cascade Biologics, Portland, OR, USA) were cultured in epithelial cell growth medium (medium 200; Cascade Biologics, USA) containing 10% fetal bovine serum (FBS), 1X low serum growth supplement, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Biological Industries, Kibbutz Beit Haemek, Israel) at 37°C in an incubator with 5% CO_2.

2.4. Measurement of Intracellular Ca^{2+} Levels. Intracellular Ca^{2+} levels were determined using a Screen Quest Fluo-8 Medium Removal Calcium Assay Kit according to the manufacturer's instructions.

2.5. Determination of NADPH Oxidase Activity. The activity of NADPH oxidase was examined using an EnzyChrom $NADP^+$/NADPH assay kit according to the manufacturer's instructions. This assay kit measures the change in $NADP^+$/NADPH ratio in cellular lysate samples and reflects the relative NADPH oxidase activity in the samples tested.

2.6. Western Blot Analysis. Aliquots of cell lysates or tissue lysates were separated by 8–12% SDS-PAGE and then transblotted onto Immobilon-P membrane (Millipore). After being blocked with 5% skim milk, the blots were incubated with various primary antibodies and then appropriate secondary antibodies. The specific protein bands were detected using an enhanced chemiluminescence kit (PerkinElmer), which was followed by the quantification using ImageQuant 5.2 software (Healthcare Bio-Sciences, Philadelphia, PA, USA).

2.7. Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from cells using Tri reagent and converted into cDNA using reverse transcriptase

(Biolabs, Ipswich, New England) with oligo-dT as the primer. The cDNAs thus produced were then used as templates for the semiquantitative PCR. PCR was performed in a DNA Thermal Cycler (Biometra Tpersonal, Horsham, PA, USA) using the following program: 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min and then a final single cycle of 72°C for 10 min. The nucleotide sequences of the primers were as follows: TRPA1 sense: 5′-TCACCATGAGCTAGCAGACTATTTAATTT-3′, antisense: 5′-ATGAGAGCGTCCTTCAGAATCG-3′ and GAPDH sense: 5′-TGT TCC AGT ATG ACT CCA CTC-3′, antisense: 5′-TCC ACC ACC CTG TTG CTG TA-3′.

2.8. Small Interfering RNA (siRNA) Transfection. HBECs were transfected with scramble or TRPA1 siRNA using INTERFERin siRNA transfection reagent for 24 hours. The TRPA1 siRNA consisted of a mixture of sc-44780A, sc-44780B, and sc-44780C. The nucleotide target sequences of sc-44780A were sense, GCUAAGCCAUGUAAAUCAAtt, and antisense, UUGAUUUACAUGGCUUAGCtt. The nucleotide target sequences of sc-44780B were sense, CUGACAUAGUCCUGAACAAtt, and antisense, UUGUUCAGGACUAUGUCAGtt. The nucleotide target sequences of sc-44780C were sense, CCAUUCUCGUUGUCAAUAUtt, and antisense, AUAUUGACAACGAGAAUGGtt.

2.9. Murine Model of Chronic CS Exposure. All animal experiments were approved by the Institutional Animal Care and Use Committee of the National Yang-Ming University (Approval Number: 991220). The murine model of chronic CS exposure has been described in detail previously [6, 7, 23]. Briefly, male *trpa1*$^{-/-}$ mice (Jackson Laboratory, Maine, USA) and wild-type C57BL/6J mice (National Laboratory Animal Center, Taipei, Taiwan) at the age of 8 weeks were randomly divided into four groups for exposure to air or CS; the groups were, namely, air-wild-type, air-*trpa1*$^{-/-}$, CS-wild-type, and CS-*trpa1*$^{-/-}$. At each CS exposure, the mice were placed in an exposure chamber and 750 mL of fresh CS generated from 1.5 cigarettes (Marlboro Red Label; 10.8 mg nicotine and 10.0 mg tar per cigarette) was delivered to the chamber. The CS passed out of the chamber via four exhaust holes (1 cm) on the side panels. During the exposure, the mice were conscious and breathing spontaneously and the treatment in the chamber lasted for 10 min. After exposure, the mice were transferred to a new cage and allowed to inspire air normally. The mice were exposed at 10:00 and 16:00 each day for 4 weeks. The control animals underwent identical procedures in another chamber but were only exposed to air. For each CS exposure, the particle concentration inside the exposure chamber was about 625 mg/m^3 initially but decreased overtime due to the fact that the CS passed out of the chamber via the exhaust holes [7]. The HbCO levels immediately after the 10-minute exposure protocol for air-exposure and CS-exposure mice were 0.42% and 31.48% ($n = 6$), respectively [7].

2.10. En Face Immunostaining of the Whole Lung. Male *trpa1*$^{-/-}$ mice and wild-type C57BL/6J mice at the age of 8 weeks were euthanized with CO_2 and a middle thoracotomy was performed. The lungs were then infused with 4% paraformaldehyde (0.8 mL) overnight. After infusion with 3% H_2O_2 and being blocked with bovine serum albumin, the lungs were infused with IgG or TRPA1 Ab overnight at 4°C and then with HRP-conjugated corresponding secondary Ab for 1 hour. The antigenic sites were visualized by the addition of DAB. The lung was then soaked in xylene and its image was photographed by a digital camera (Sanyo, Osaka, Japan).

2.11. Preparation of BALF and Lung Tissues. At the end of each experiment, the mice were euthanized with CO_2 and a middle thoracotomy was performed. The left lung was ligated and the right lung was lavaged four times with 0.6 mL of warm PBS containing a complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The BALF samples were then centrifuged at 350 ×g for 5 min at 4°C, and the supernatant of the first lavage fluid was stored at −80°C for later analysis of total protein using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). The cell pellets of the BALF samples were resuspended in PBS for cell counting. Furthermore, the right lung was then stored at −80°C for subsequent analysis. The left lung was fixed with 4% paraformaldehyde and embedded in paraffin.

2.12. Histological and Immunohistochemical Assessments. Formalin-fixed, paraffin-embedded tissue blocks were cut into 8-μm sections. The sections were deparaffinized, rehydrated, and finally stained with hematoxylin and eosin (H&E) staining; they were then viewed under a microscope (Motic TYPE 102M, Xiamen, China). The histological assessments were conducted by a pathologist who was blinded to the data. Each histological characteristic was scored on a scale of 0-normal to 5-maximal. Lung inflammatory score was categorized according to the sum of the score of infiltration cell numbers and damage levels, including thickening of alveolar walls and epithelium, as well as increases in peribronchial and perivascular cuff area. For immunohistochemical assessment, sections were deparaffinized, rehydrated, and then covered with 3% H_2O_2 for 10 min. After being blocked with bovine serum albumin, each slide was first incubated with primary antibodies for 1 hour at 37°C, followed by the corresponding secondary antibodies for an additional hour. The color of all of the sections was developed with 0.1% diaminobenzidine (DAB) and then the sections were counterstained with hematoxylin; this was followed by examination under a microscope. The detected signal was digitally captured using an image analysis system (Image-Pro Plus 4.5, Media Cybernetics, Bethesda, MD, USA) as described previously [7, 26]. The intensity of the immunoreaction developed within epithelium was then assessed densitometrically. Ten epithelial cells were analyzed for each section and three different sections were analyzed for each animal. The data were averaged for each animal and are expressed in arbitrary units.

2.13. Measurement of an Oxidative Stress Biomarker. Levels of 4-HNE modified proteins, a product of lipid peroxidation, in

lung tissues were measured and this served as a biomarker of oxidative stress as described previously [27].

2.14. Measurement of Extracellular and Intracellular ROS Levels. The membrane-permeable probes HE and DCFH-DA were used to assess levels of ROS using methods that have been described previously [28, 29]. For the *in vitro* study, HBECs were incubated in culture medium containing 10 μM HE at 37°C for 30 min. After stimulation with CSE for the desired time, the culture medium was removed for the measurement of extracellular ROS levels. The cells were washed and detached with trypsin/EDTA to allow the measurement of intracellular ROS levels. For the *in vivo* study, the supernatant of the first BALF sampled from all study groups was incubated with 10 μM DCFH-DA at 37°C for 15 min. The fluorescence intensities of the culture medium, cells, and BALF samples were then analyzed using a multilabel counter (PerkinElmer, Waltham, MA, USA). Images of the cells were also obtained by examining them using a Nikon TE2000-U florescence microscope (Tokyo, Japan).

2.15. Determining the Concentration of Macrophage Inflammatory Protein 2 (MIP-2). The concentrations of MIP-2 in BALF and in lung tissue were measured using an ELISA kit according to the manufacturer's instructions.

2.16. Statistical Analysis. The results are presented as mean ± SEM. Statistical evaluations involved one-way ANOVA followed by Dunnett's test or Fisher's least significant difference procedure for multiple comparisons as appropriate. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. CSE Increases the Expression of TRPA1 in HBECs. We found that exposure of HBECs to various concentrations (0, 0.75, 1.5, and 3%) of CSE for 24 hours increased the protein level of TRPA1 in a concentration-dependent manner (Figure 1(a)). In addition, exposure of HBECs to 3% CSE for up to 24 hours time-dependently increased the protein level of TRPA1 (Figure 1(b)). Furthermore, an elevation in the mRNA level of TRPA1 was also detected at 6–18 hours after CS exposure (Figure 1(c)).

3.2. TRPA1 Is Important to the Induction of IL-8 by CSE in HBECs. Next we determined the role of TRPA1 in the induction of IL-8 by CSE. Based upon the concentration-response relationship and time-response relationship reported previously [6, 7], 3% CSE with exposure for 24 hours was chosen as the standard treatment for all subsequent experiments throughout this study. Pretreatment with HC-030031 (a TRPA1 antagonist; 3–9 μM) was found to dose-dependently attenuate the induction of IL-8 by CSE, whereas pretreatment with DMSO (the vehicle) failed to produce such an effect (Figure 2(a)). Additionally, pretreatment with siRNA (25 or 50 nM) that was designed to bring about gene knockdown of TRPA1 significantly reduced the amount of TRPA1 protein present after activation (Figure 2(b)). Furthermore, pretreatment with TRPA1 siRNA (50 nM) also attenuated the induction of IL-8 by CSE, whereas pretreatment with scramble siRNA failed to produce any such effect (Figure 2(c)). These findings suggest that TRPA1 is important to the induction of IL-8 by CSE in HBECs.

3.3. CSE Increases the Level of Intracellular Ca^{2+} via a TRPA1-Mediated Ion Influx. After exposure of HBECs to CSE, an increase in the intracellular Ca^{2+} level was found to start at 1 min after treatment initiation, to peak at 2 min after treatment initiation, and to have declined somewhat at 5 min after treatment initiation; nevertheless, at 5 min the level was still higher than the baseline level. This elevation in the intracellular Ca^{2+} level was then maintained until the end of the observation period (60 min) (Figure 3(a)). The increase in the intracellular Ca^{2+} level measured at 2 min after CSE exposure was inhibited by pretreatment with EGTA (an extracellular Ca^{2+} chelator; 500 μM), by pretreatment with N-acetyl-cysteine (a ROS scavenger; 1 mM), or by pretreatment with HC030031 (Figure 3(b)).

3.4. CSE-Induced Extracellular ROS Stimulates TRPA1 to Increase Intracellular ROS via the Ca^{2+}-Dependent Activation of NADPH Oxidase. At 2 min after exposure of HBECs to CSE, the extracellular ROS level was significantly increased in the medium; in contrast, at the same time point, the intracellular ROS level had remained unchanged (Figure 4(a)). The CSE-induced increases in extracellular ROS levels in the medium containing HBECs (1.432 ± 0.101-fold of control; $n = 5$) and in the cell-free medium (1.377 ± 0.107-fold of control; $n = 5$) were comparable. This increase in the extracellular ROS level was unaffected by pretreatment with apocynin (an inhibitor of NADPH oxidase; 150 μM) and by pretreatment with HC030031 (9 μM) but was prevented by pretreatment with N-acetyl-cysteine (Figure 4(a)). In contrast to the above results, at 30 min after CSE exposure, it was found that the intracellular ROS level had significantly increased, while the extracellular ROS level had returned to the baseline level (Figure 4(b)). This increase in the intracellular ROS level was prevented by pretreatment with apocynin, by pretreatment with N-acetyl-cysteine, and by pretreatment with HC030031 (Figure 4(b)). Further analysis revealed that, at 15 min after exposure, the presence of CSE had significantly increased the activity of NADPH oxidase and that this was inhibited by pretreatment with apocynin, by pretreatment with EGTA, and by pretreatment with HC030031 (Figure 4(c)).

3.5. CSE-Induced Activation of the MAPKs/NF-κB Signaling Is TRPA1-Mediated, Ca^{2+}-Dependent, and ROS-Sensitive. The activation of ERK, JNK, and NF-κB is known to be a signaling pathway that is central to the induction of IL-8 by CSE in HBECs [3, 5–8]. Exposure to CSE was able to increase the presence of the phosphorylated ERK (Figure 5(a)) and phosphorylated JNK (Figure 5(b)) in the cytosol and of the p65 subunit of NF-κB in the nucleus (Figure 5(c)). Such CSE-induced activation of the MAPKs/NF-κB signaling was significantly attenuated by pretreatment with EGTA (500 μM),

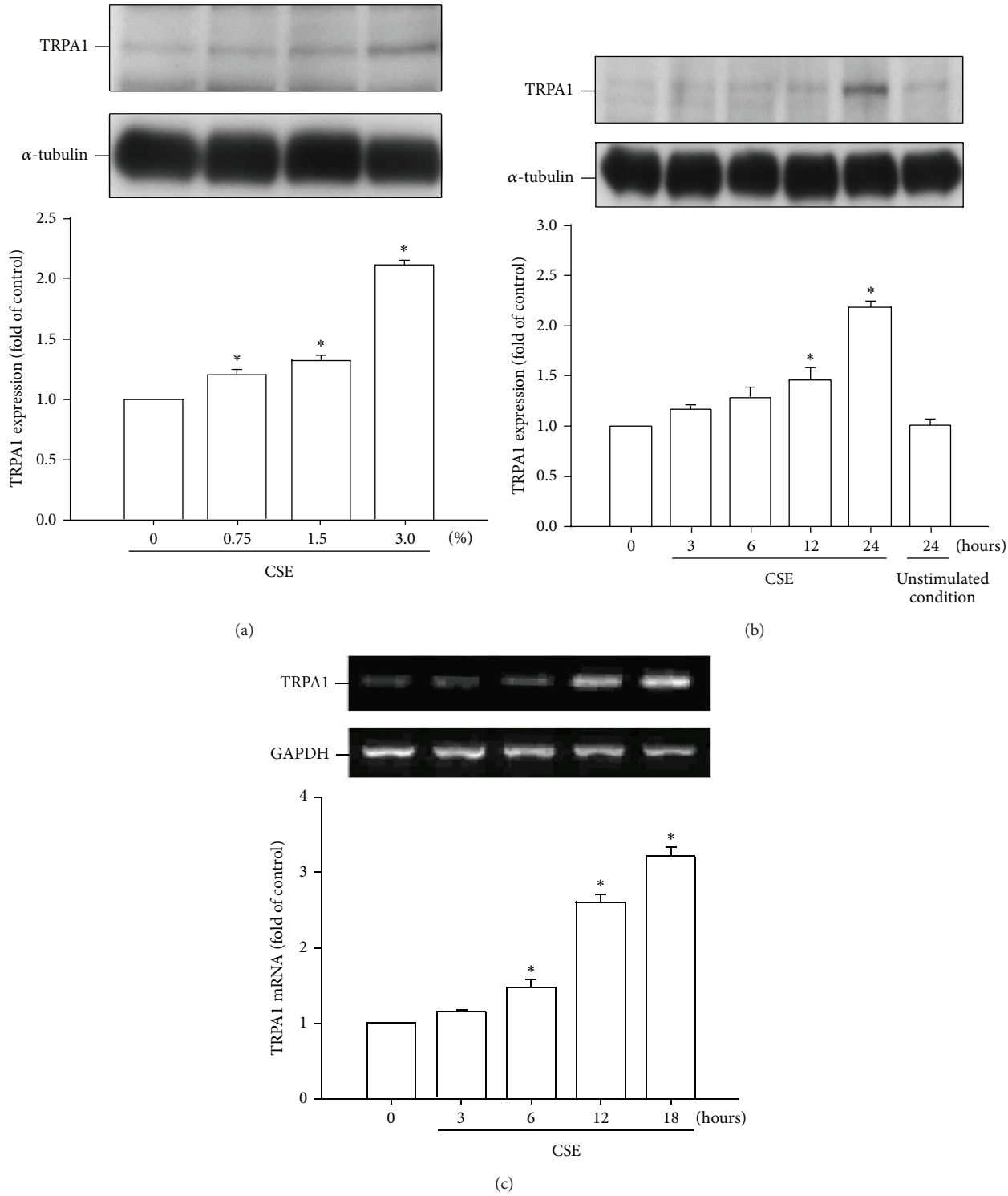

Figure 1: Cigarette smoke extract (CSE) concentration-dependently and time-dependently increases TRPA1 expression in human bronchial epithelial cells (HBECs). (a) Cells were exposed to 0–3% CSE for 24 hours. (b) Cells were incubated with medium alone at time 0 and for 24 hours or exposed to 3% CSE for the indicated times. (c) Cells were exposed to medium alone or exposed to 3% CSE for indicated times. Protein (a and b) and mRNA (c) levels in the cell lysates were analyzed by Western blotting and RT-PCR, respectively. The data in each group are mean ± SEM from five independent experiments. $^{*}p < 0.05$ versus the control or versus time zero.

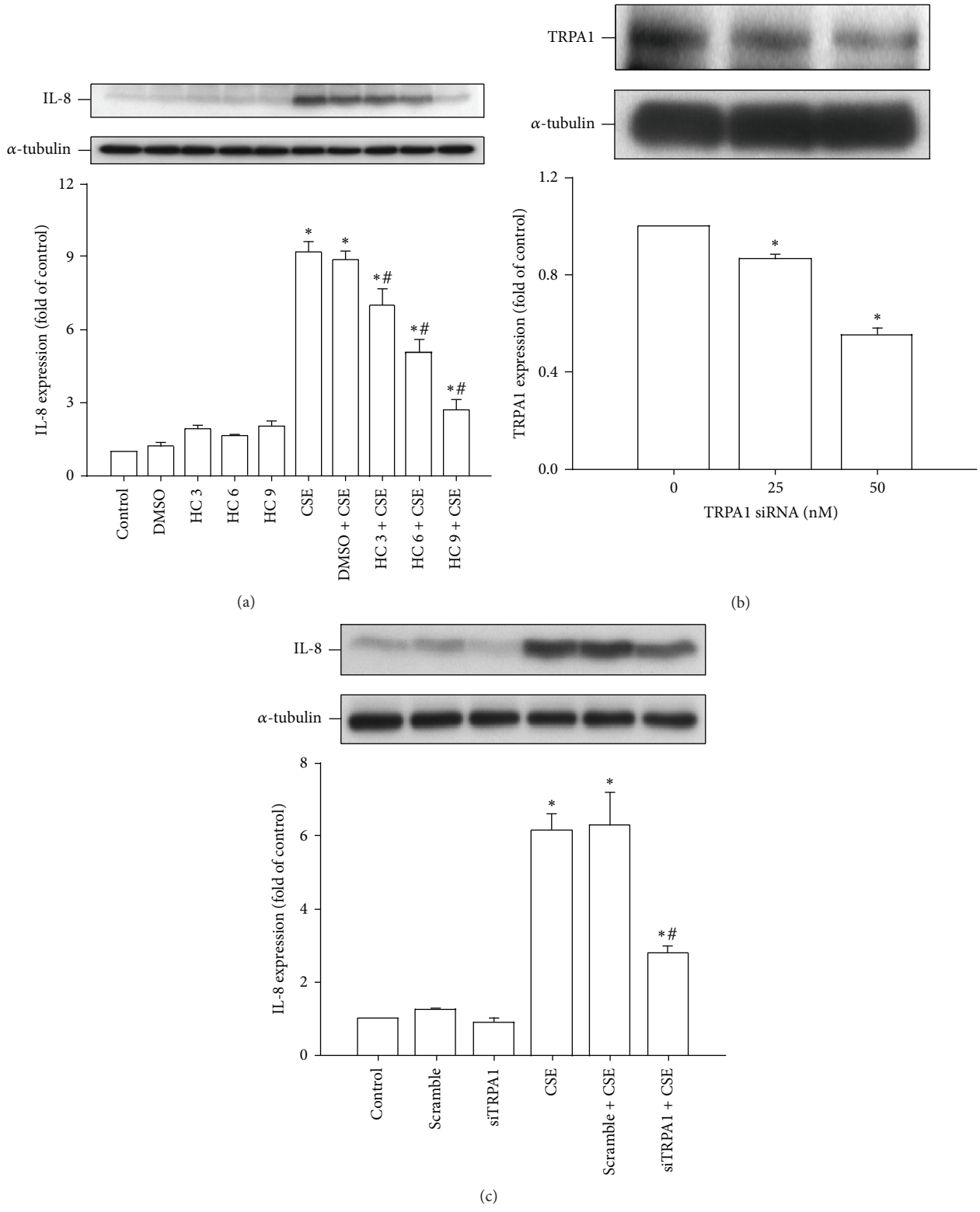

Figure 2: TRPA1 is important for the induction of IL-8 by CSE in HBECs. (a) Cells were incubated with medium alone or exposed to 3% CSE for 24 hours and pretreated with 3–9 μM HC-030031 (HC, a TRPA1 antagonist) or its vehicle DMSO. (b) Cells were incubated with or without two concentrations (25 and 50 nM) of TRPA1 siRNA (siTRPA1) for 24 hours prior to the measurement of TRPA1 expression. (c) Cells were incubated with medium alone or exposed to 3% CSE for 24 hours having been pretreated with 50 nM of either siRNA (siTRPA1) or scramble siRNA. Data in each group are mean ± SEM from five independent experiments. $^{*}p < 0.05$ versus the control or versus time zero; $^{\#}p < 0.05$ versus CSE alone.

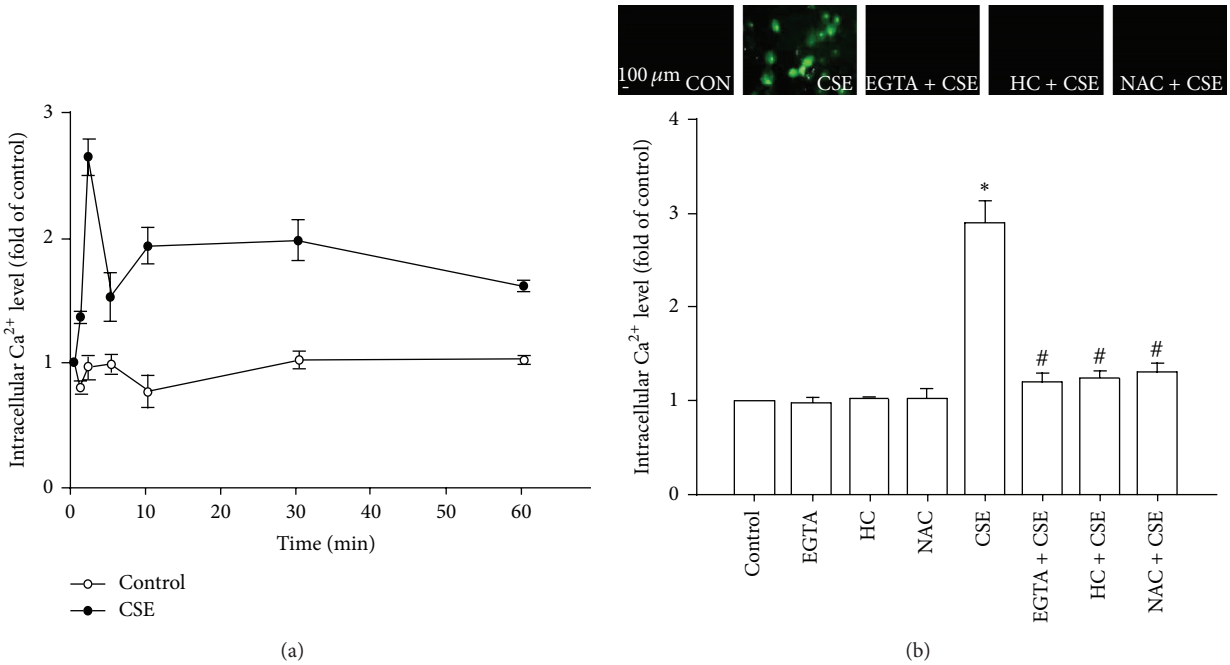

FIGURE 3: CSE increases the intracellular Ca^{2+} level via TRPA1-mediated ion influx in HBECs. Intracellular Ca^{2+} levels were measured by Fluo-8 fluorescent probe assay. (a) Cells were exposed to medium alone or 3% CSE for 1, 2, 5, 10, 30, and 60 min. (b) Cells were exposed to medium alone or 3% CSE for 2 min and pretreated with EGTA (an extracellular Ca^{2+} chelator; 500 μM), HC-030031 (HC, a TRPA1 antagonist; 9 μM), or N-acetyl-cysteine (NAC, a ROS scavenger; 1 mM). Data in each group are mean ± SEM from five independent experiments. $^{*}p < 0.05$ versus the control or time zero; $^{\#}p < 0.05$ versus CSE alone.

by pretreatment with HC030031 (9 μM), and by pretreatment with N-acetyl-cysteine (Figure 5; 1 mM).

3.6. CS Increases the Expression of TRPA1 in Lung Epithelium and Lung Tissues of Mice. In air-exposure animals, *en face* immunostaining showed strong signals across the whole of the lungs of wild-type mice (Figure 6(a), middle panel), but such signals were relatively weak when similarly treated *trpa1*$^{-/-}$ mice were examined (Figure 6(a), right panel). These results suggest that the geometry of the airways could be clearly visualized in treated wild-type mice, but not in treated *trpa1*$^{-/-}$ mice. Analysis of wild-type mouse lung sections obtained from lungs that had undergone *en face* immunostaining indicated that their lung epithelial cells (Figure 6(b), middle panel) displayed much stronger positive staining for TRPA1 than lung epithelial cells from *trpa1*$^{-/-}$ mice (Figure 6(b), right panel); these results indicate a difference in the expression levels of TRPA1 in the lung epithelium from the two types of mice. Additionally, immunohistochemical analysis showed that there was stronger positive staining for TRPA1 in the epithelial cells of lung sections from wild-type mice that had been chronically exposed to CS for 4 weeks compared to the air-exposure control mice (Figure 6(c)). The increase in relative expression level of TRPA1 measured as positive cells by immunostaining with the CS-exposure group was 2.1 ± 0.1-fold ($n = 9$), which is significantly higher than that found for the air-exposure group (1.0 ± 0.1-fold; $n = 9$). Furthermore, Western blot analysis revealed that expression of TRPA1 in the lung tissues of CS-exposure wild-type mice was also significantly higher than that of air-exposure control mice (Figure 6(d)).

3.7. CS-Induced Oxidative Stress and Lung Inflammation Are Lessened in trpa1$^{-/-}$ *Mice.* Analysis was carried out using a fluorescent probe and the results indicated that the level of ROS in the BALF, which is able to directly stimulate the airway epithelium, was increased in mice after CS exposure for 4 weeks compared to the same measurements for air-exposure mice (Figure 7(a)). However, no significant difference in the BALF levels of ROS was found between the CS-exposed wild-type and the *trpa1*$^{-/-}$ mice under the same conditions (Figure 7(a)). By way of contrast, Western blot analysis revealed that wild-type mice after exposure to CS showed increased levels of 4-HNE modified proteins (a product of lipid peroxidation) in their lungs compared to air-exposure wild-type mice (Figure 7(b)). This CS-induced increase in the level of 4-HNE, however, was greatly attenuated in the lungs of CS-exposure *trpa1*$^{-/-}$ mice relative to the wild-type mice (Figure 7(b)). A histological evaluation of the lung sections of CS-exposure wild-type mice revealed extensive infiltration of inflammatory cells, thickening of the alveolar walls, and the presence of abnormal reepithelialization and all of these changes were found to be less in the CS-exposure *trpa1*$^{-/-}$ mice (Figure 7(c)). The difference in the degree of lung inflammation between the CS-exposure wild-type and *trpa1*$^{-/-}$ mice was confirmed by comparing the group data

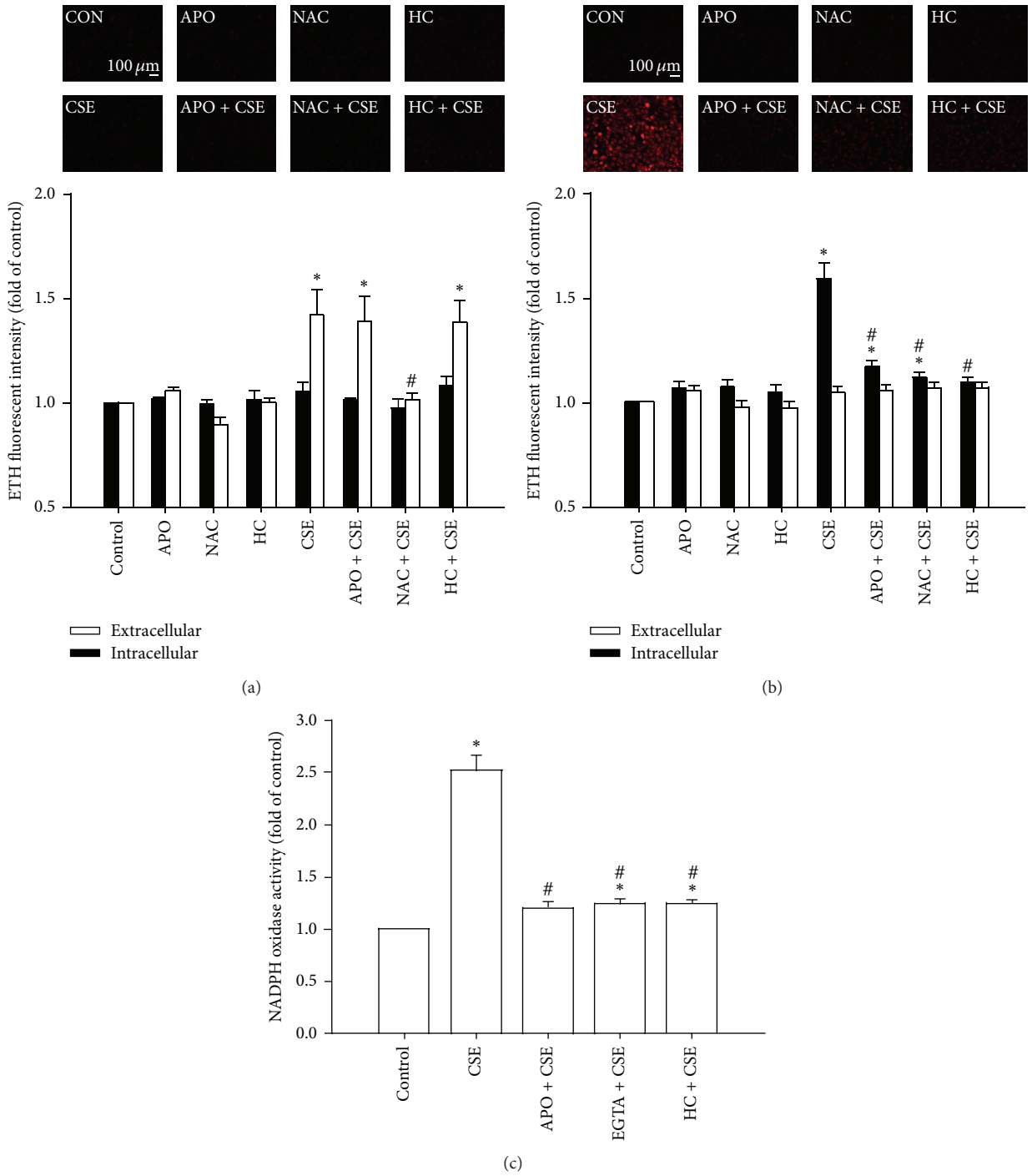

Figure 4: The CSE-induced extracellular ROS stimulate TRPA1 to Ca^{2+}-dependently increase intracellular ROS via NADPH oxidase in HBECs. (a–c) Cells were exposed to medium alone or to 3% CSE for 2, 30, and 15 min, respectively, after pretreatment with apocynin (APO; an inhibitor of NADPH oxidase; 150 μM), after pretreatment with N-acetyl-cysteine (NAC, a ROS scavenger; 1 mM), after pretreatment with HC-030031 (HC, a TRPA1 antagonist; 9 μM), or after pretreatment with EGTA (an extracellular Ca^{2+} chelator; 500 μM). Levels of ROS were measured by HE fluorescent probe assay. NADPH oxidase activity was measured by $NADP^+$/NADPH assay. Data in each group are mean ± SEM from five independent experiments. $^*p < 0.05$ versus the control; $^\#p < 0.05$ versus CSE alone.

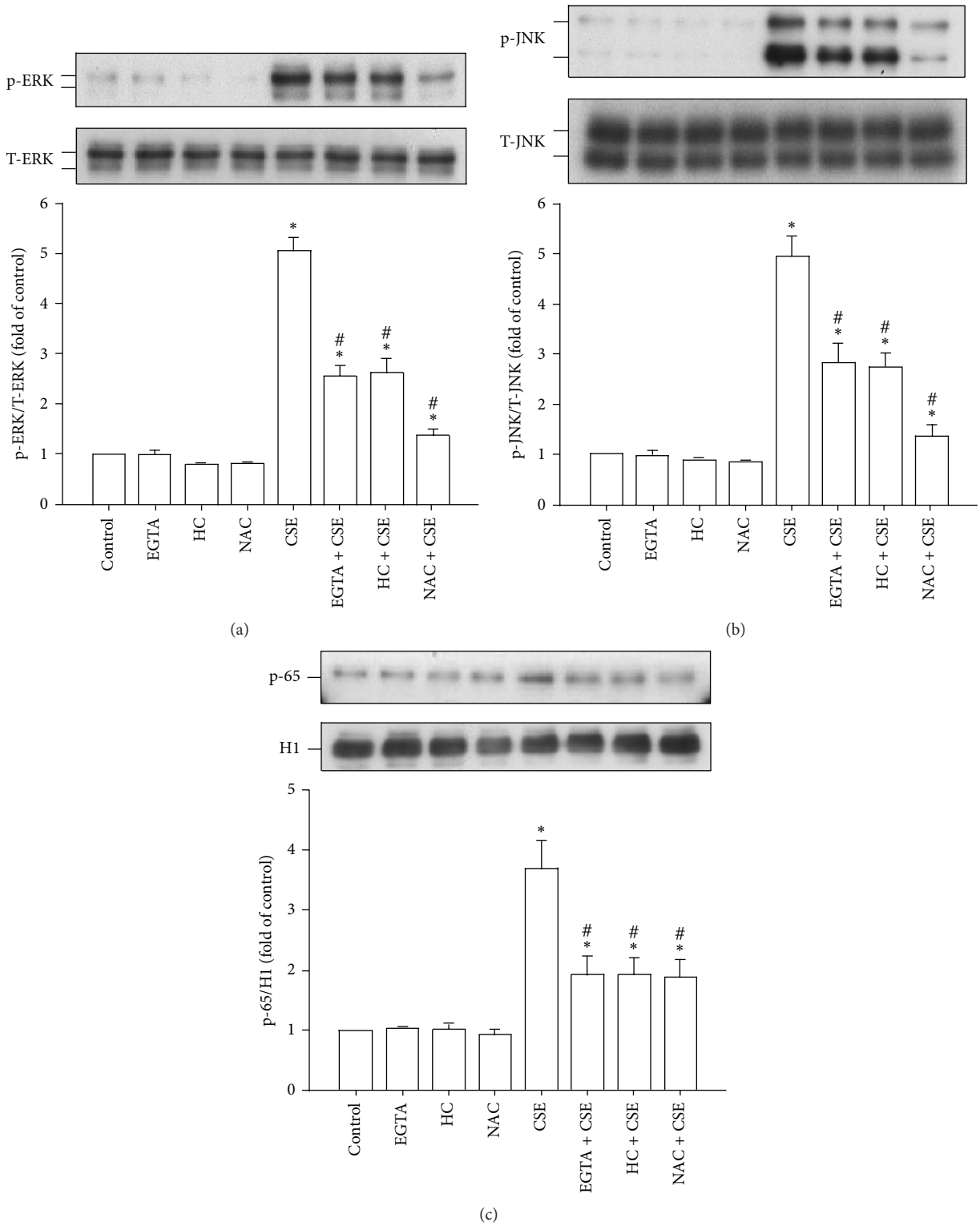

Figure 5: The CSE-induced activation of the ERK/JNK/NF-κB signaling in HBECs is TRPA1-mediated, Ca^{2+}-dependent, and ROS-sensitive. (a–c) Cells were exposed to medium alone or to 3% CSE for 6, 6, and 12 h, respectively, and pretreated with EGTA (an extracellular Ca^{2+} chelator; 500 μM), HC-030031 (HC, a TRPA1 antagonist; 9 μM), or N-acetyl-cysteine (NAC, a ROS scavenger; 1 mM). Protein expression was analyzed by Western blotting. Activation of ERK and JNK was indicated by increased phosphorylation of these kinases, whereas activation of NF-κB was indicated by the increased presence of p65 subunit in the nucleus. Data in each group are mean ± SEM from five independent experiments. $^{*}p < 0.05$ versus the control; $^{\#}p < 0.05$ versus CSE alone.

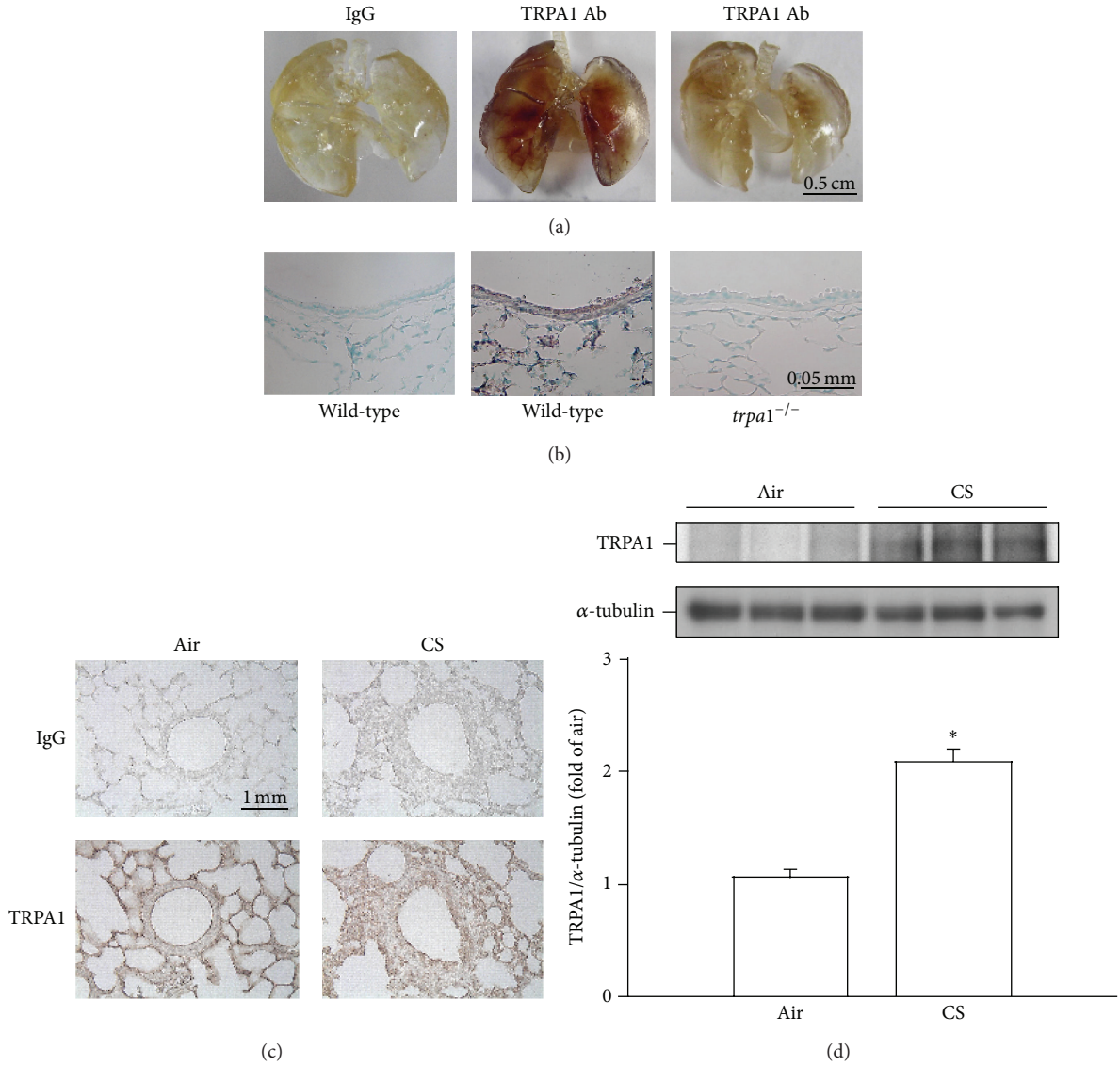

Figure 6: Cigarette smoke (CS) increases the lung expression of TRPA1 in mice. Mice were chronically exposed to air or CS for 4 weeks. (a) Representative images showing *en face* immunostaining with a TRPA1 antibody using whole lungs obtained from air-exposed wild-type mice and from *trpa1*$^{-/-}$ mice. (b) Representative images showing lung section obtained from the lungs with the *en face* TRPA1-immunostaining of air-exposed wild-type mice and *trpa1*$^{-/-}$ mice. (c) Representative images showing immunostaining with a TRPA1 antibody of representative lung sections obtained from air-exposed and CS-exposed wild-type mice. The specificity of the immunostaining was confirmed using an IgG-negative control. (d) Expression levels of TRPA1 in lung tissues were obtained from air-exposed and CS-exposed wild-type mice and were analyzed by Western blotting. $^{*}p < 0.05$ versus the air control group in both genotypes. Data in each group are mean ± SEM from nine mice.

in terms of lung inflammatory scores (Figure 7(d)). Furthermore, relative to the air-exposure wild-type mice, wild-type mice with CS exposure were found to show increases in BALF for total protein levels (Figure 8(a)), for total cell counts (Figure 8(b)), for differential cell counts (Figure 8(c)), and for MIP-2 levels (Figure 8(d)) as well as an increase in the level of MIP-2 in lung tissues samples (Figure 8(e)). All of these inflammatory indices were significantly lower in *trpa1*$^{-/-}$ mice exposed to CS (Figure 8).

4. Discussion

Our *in vitro* study confirms the important role of lung epithelial TRPA1 in the induction of IL-8 by CSE in HBECs [21, 22]. We then used this *in vitro* model to investigate how lung epithelial TRPA1 acts as a crucial regulator during the transcriptional regulation of IL-8 induction by CSE. We found that CSE sequentially caused increases in extracellular ROS, intracellular Ca^{2+} level via ion influx,

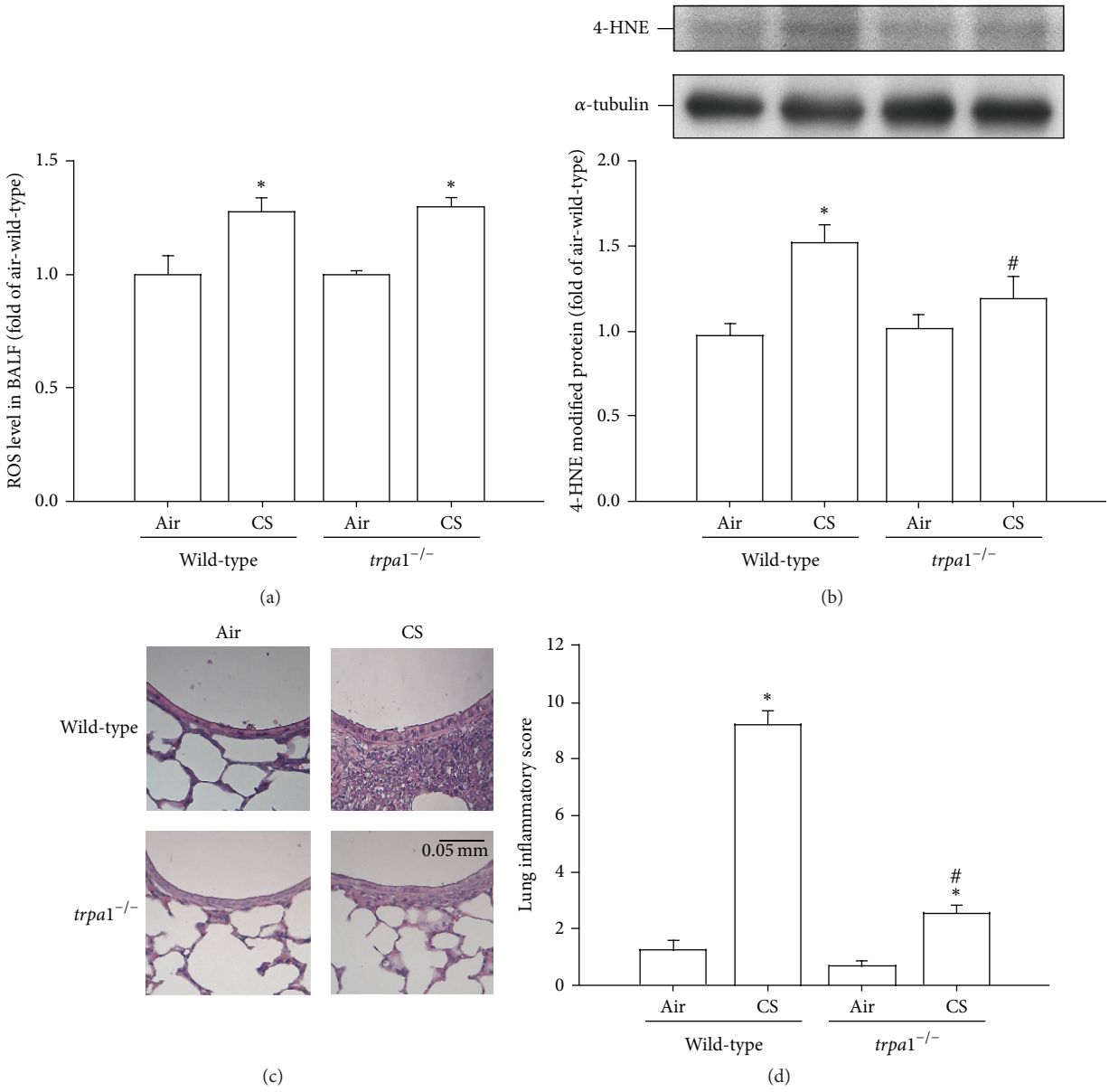

FIGURE 7: Comparisons of cigarette smoke- (CS-) induced oxidative stress and lung inflammatory score in wild-type and *trpa1*$^{-/-}$ mice. Mice were chronically exposed to air or CS for 4 weeks. (a) Levels of ROS in the bronchoalveolar lavage fluid (BALF) were sampled immediately after the last air or CS exposure across the four study groups. Levels of ROS were measured by DCFH-DA fluorescent probe assay. (b) Expression of 4-HNE modified protein, a biomarker of oxidative stress, in lung tissues was analyzed by Western blotting. (c) Representative images of H&E stained lung sections obtained from air-exposure or CS-exposure wild-type and *trpa1*$^{-/-}$ mice. (d) Lung inflammatory scores were calculated according to the sum of the levels of cell infiltration and damage levels assessed from the lung sections. Data in each group are mean ± SEM from nine mice. $^{*}p < 0.05$ versus the air-exposure group in both genotypes; $^{\#}p < 0.05$ versus the CS-exposure wild-type group.

NADPH oxidase activity, and intracellular ROS. CSE then activated the MAPKs/NF-κB signaling and induced IL-8 in HBECs. The time courses of these events are similar to those reported previously [6, 7, 21, 22]. Using various experimental interventions, we have identified the cascade of these events (Figure 9). Initially, exposure to CSE causes an increase in the extracellular level of ROS, which in turn activates lung epithelial TRPA1; this leads to the promotion of a Ca^{2+} influx. The increase in the intracellular level of Ca^{2+} of the HBECs then contributes to the activation of NADPH oxidase, which in turn results in an elevation of the intracellular level of ROS. Finally, this activates the MAPKs/NF-κB signaling pathway, which brings about the induction of IL-8. These observations suggest that lung epithelial TRPA1 may transduce the presence of extracellular ROS induced by CS into the transcriptional regulation of lung inflammation via an influx of Ca^{2+}.

Exposure of CS or CSE has been shown to increase ROS in extracellular fluid [7, 9] and in BALF [7, 10]. In

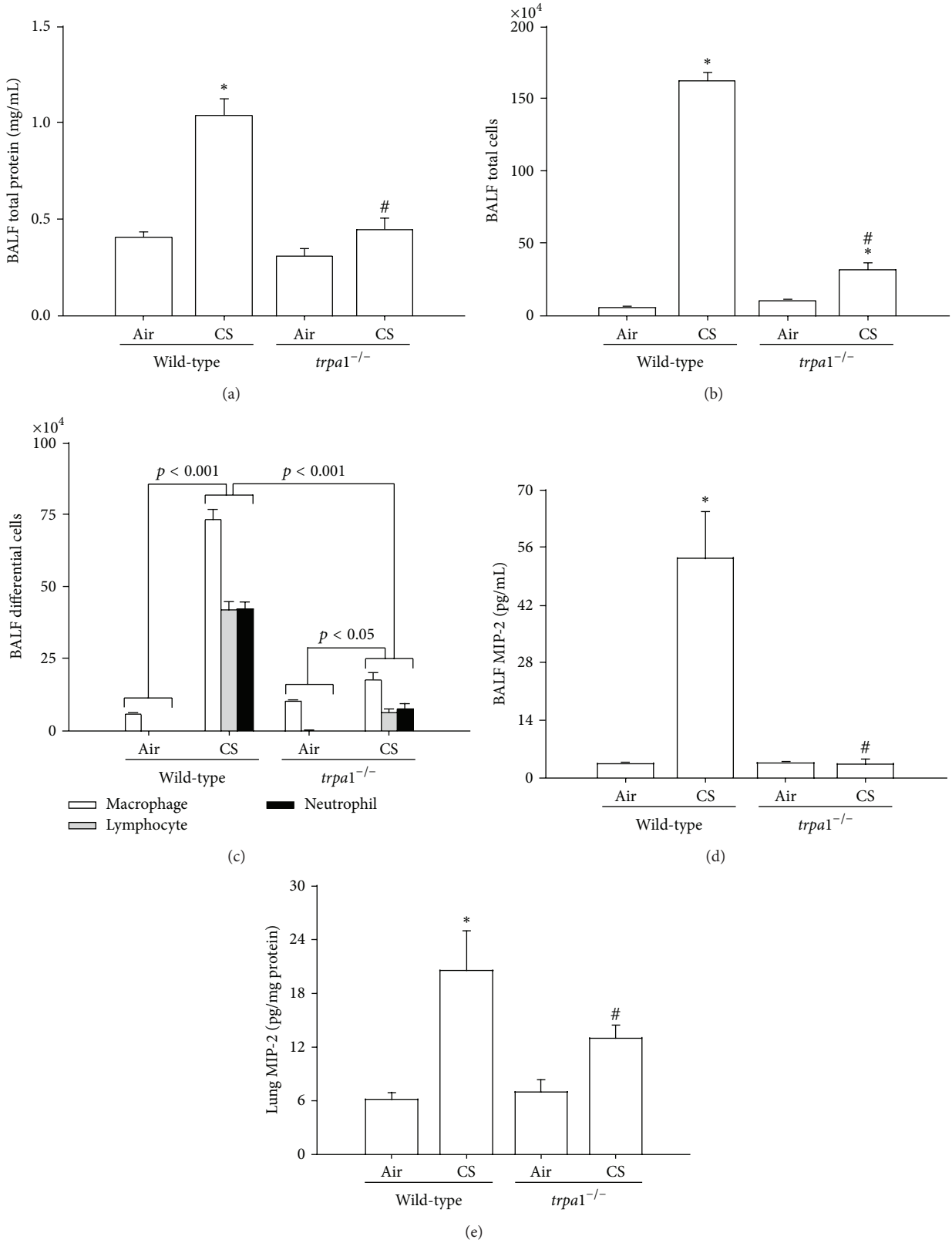

Figure 8: The cigarette smoke- (CS-) induced lung inflammation is alleviated in TRPA1 knockout mice. Mice were chronically exposed to air or CS for 4 weeks. Total protein content (a), total cell count (b), and differential cell count (c) obtained from the bronchoalveolar lavage fluid (BALF) were measured and served as indications of lung inflammation. (d, e) Levels of MIP-2 in BALF and in lung tissues were analyzed by ELISA. Data in each group are mean ± SEM from nine mice. $^{*}p < 0.05$ versus the air-exposure group in both genotypes; $^{\#}p < 0.05$ versus the CS-exposure wild-type group.

this study, the increase in extracellular ROS was evoked by CSE as early as 2 min after exposure; however, at this time point the level of intracellular ROS remained unaltered. Although CS-induced extracellular ROS are known to insult the lung epithelium, which leads to lipid peroxidation and damage of cell membranes [30], its role in the transcriptional regulation of CS-induced lung inflammation has remained largely unclear up to the present. By way of contrast, a CS-induced increase in intracellular ROS has been widely accepted as serving to trigger the activation of ROS-sensitive inflammatory signaling in lung epithelial cells [6, 7] and in other types of lung cells [11–14]. Our findings regarding the activation of TRPA1 by extracellular ROS are not totally surprising because this event has already been demonstrated for neuronal TRPA1 [16, 31, 32] and in pancreatic beta cells [33], as well as in cells that have been transfected with TRPA1 [16, 31, 34]. Since ROS is strongly electrophilic, ROS may cause the oxidation of disulfide bonds near the pore region of these channels [35] or bring about the covalent modification of cysteines within the electrophile/oxidant-sensing site, which consists of three cysteines and one lysine found at N-terminus of this type of channel [16, 31, 35, 36]; either or both of these changes may lead to activation of TRPA1. Similar ROS related regulation of other types of TRP channels has been reported elsewhere [37].

In this study, the CSE-induced and TRPA1-mediated increase in intracellular Ca^{2+} seems to form a Ca^{2+} signaling event that activates the MAPKs/NF-κB signaling pathway, which then results in the induction of IL-8. One event evidently triggered by this Ca^{2+} signaling is activation of NADPH oxidase. This requires the translocation of the $p47^{phox}$ subunit from the cytosol to the membrane compartment, which was demonstrated in two of our previous studies [6, 7]. Indeed, an elevation of the intracellular Ca^{2+} concentration is known to act as an upstream signal for the activation of NADPH oxidase during cellular stress [38, 39]. To this end, many studies have reported that activation of NADPH oxidase is responsible for the CS-induced increase in intracellular ROS in lung cells [6, 7, 11–14]. Thus, lung epithelial TRPA1 provides an important link between the initial increase in extracellular ROS and the subsequent increase in intracellular ROS, both of which occur in response to CS exposure. In vascular smooth muscle cells, an interplay between intracellular Ca^{2+} and ROS has been suggested; furthermore, the Ca^{2+} signaling mediated by various Ca^{2+} channels has been shown to regulate the activity of NADPH oxidase in these cells and to increase intracellular ROS reciprocally via the activity of these Ca^{2+} channels [39]. Perhaps, this interplay can explain why, after the initial surge of Ca^{2+} influx, the intracellular Ca^{2+} remains persistently elevated until the end of the observation period (30 min) when HBECs have been exposed to CSE. At this time, the transient increase in extracellular ROS has vanished, but nevertheless the increase in intracellular ROS remains. We, however, cannot exclude the possibility that the TRPA1-mediated influx of Ca^{2+} also serves as a Ca^{2+} signal that is able to activate downstream inflammatory signaling. For example, an increase in intracellular Ca^{2+} is known to be

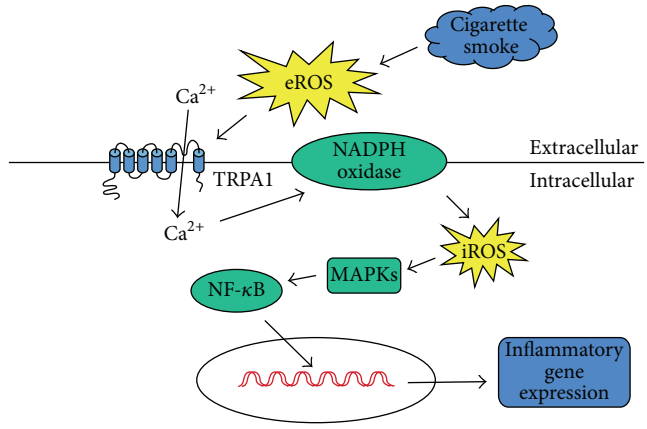

Figure 9: The proposed mechanism of TRPA1 activation for the induction of IL-8 by cigarette smoke (CS). As shown, exposure to CS initially causes an increase in the extracellular level of reactive oxygen species (ROS), which in turn activates lung epithelial TRPA1; this leads to the promotion of a Ca^{2+} influx. The increase in the intracellular level of Ca^{2+} in lung epithelial cells then contributes to the activation of NADPH oxidase, which in turn results in an elevation of the intracellular level of ROS and this then activates the mitogen-activated protein kinases (MAPKs)/nuclear factor-κB (NF-κB) signaling pathway allowing induction of IL-8.

able to activate the MAPKs/NF-κB pathway in human lung epithelial cells and bring about the release of IL-8 [40]. In this context, we have previously shown that activation of the ROS-sensitive MAPKs/NF-κB pathway is important for induction of IL-8 by CSE in HBECs [6, 7]. The present study further characterizes this pathway and shows it is a process that depends on having a functional lung epithelial TRPA1 and the presence of an influx of Ca^{2+}. This provides additional evidence that helps our understanding of the early events that are involved in the transcription regulation of IL-8 by CSE in HBECs.

To investigate the role of lung epithelial TRPA1 *in vivo*, we employed an established murine lung inflammation model involving induction by chronic CS exposure for 4 weeks [6, 7, 23]. TRPA1 antibody was then infused into the airways to bring about *en face* immunostaining, which resulted in a clear visualization of the airway geometry in wild-type mice. This showed that there is expression of TRPA1 in lung epithelium; this finding was also supported by immunohistochemical analysis of lung sections obtained from the lungs with *en face* immunostaining. We additionally showed that chronic CS exposure was able to increase the protein expression of TRPA1 in lung epithelium and in lung tissue in general, a result that is consistent with our *in vitro* findings. The mechanism underlying this increase in protein expression of TRPA1 is not clear, but it would seem to involve transcriptional regulation because the mRNA level of TRPA1 is also increased. These observations imply that the inflammatory function of lung epithelial TRPA1 may be augmented in response to CS exposure via this increased expression. We have further demonstrated that, as compared to the wild-type mice, $trpa1^{-/-}$ mice displayed a lower level of CS-induced lung

inflammation, which was identified based on an alleviation of increased vascular permeability, a reduction in inflammatory cell infiltration, and a decrease in inflammatory cytokine levels. Lung epithelium is a target for direct insult by CS and these cells play a vital role in the initiation and progression of CS-induced lung inflammation [2–4, 6, 7]; therefore we believe that the reduced lung inflammation observed in our *trpa1*$^{-/-}$ mice is, at least in part, due to a lack of epithelial TRPA1 in the *trpa1*$^{-/-}$ mice. Additionally, TRPA1 is expressed in primary sensory neurons, endothelial cells, macrophages, smooth muscle cells, and fibroblasts [17, 21, 22, 41–43], all of which may participate in the development of CS-induced lung inflammation [1, 2, 16, 22, 41]. Accordingly, the lack of TRPA1 in these cells may also contribute to the reduction in lung inflammation that was observed in our *trpa1*$^{-/-}$ mice. In this study, one intriguing finding is that the ROS levels in the BALF of our *trpa1*$^{-/-}$ mice were not different when CS-exposure wild-type mice and the *trpa1*$^{-/-}$ mice were compared, whereas the level of 4-HNE modified proteins in lung tissues was lower in the CS-exposure *trpa1*$^{-/-}$ mice compared to the wild-type mice. Since the BALF was sampled immediately after the last CS exposure, we speculate that the ROS level in BALF was directly related to the amount of CS inhaled, which indicates that the insults to the airway epithelium by ROS in BALF are similar in these two genotypes of mice. By way of contrast, the level of 4-HNE modified protein would seem to reflect the degree of lipid peroxidation caused by the total ROS present in the lung tissues [27]. This would additionally encompass the ROS generated by the infiltrated inflammatory cells such as macrophages and neutrophils [2]. Since infiltration of these inflammatory cells into the lung is alleviated in the CS-exposure *trpa1*$^{-/-}$ mice, it is reasonable to observe that there is a reduction in lung oxidative stress of the CS-exposure *trpa1*$^{-/-}$ mice compared to the wild-type mice.

In summary, our *in vitro* findings suggest that exposure to CSE initially causes an increase in the extracellular level of ROS, which in turn activates lung epithelial TRPA1. TRPA1 then transduces this stimulation induced by CS into the transcriptional regulation of lung inflammation via an influx of Ca^{2+} (Figure 9). Our *in vivo* findings indicate that, while the insults to the airway epithelium caused by the presence of ROS in the BALF after CS exposure are similar for both wild-type and *trpa1*$^{-/-}$ mice, the level of CS-induced lung inflammation is reduced in *trpa1*$^{-/-}$ mice compared to wild-type mice. Thus, a cellular mechanism may possibly be at work in mice chronically exposed to CS. Our findings provide significant novel information that will allow a better understanding of the pathogenic mechanisms associated with CS-induced lung inflammation and should help the development of potential therapies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] K. F. Chung and I. M. Adcock, "Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction," *European Respiratory Journal*, vol. 31, no. 6, pp. 1334–1356, 2008.

[2] P. J. Barnes, "Mediators of chronic obstructive pulmonary disease," *Pharmacological Reviews*, vol. 56, no. 4, pp. 515–548, 2004.

[3] T. Mio, D. J. Romberger, A. B. Thompson, R. A. Robbins, A. Heires, and S. I. Rennard, "Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells," *American Journal of Respiratory and Critical Care Medicine*, vol. 155, no. 5, pp. 1770–1776, 1997.

[4] N. Moretto, F. Facchinetti, T. Southworth, M. Civelli, D. Singh, and R. Patacchini, "α,β-Unsaturated aldehydes contained in cigarette smoke elicit IL-8 release in pulmonary cells through mitogen-activated protein kinases," *The American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 296, no. 5, pp. L839–L848, 2009.

[5] M. H. Liu, A. H. Lin, H. F. Lee, H. K. Ko, T. S. Lee, and Y. R. Kou, "Paeonol attenuates cigarette smoke-induced lung inflammation by inhibiting ROS-sensitive inflammatory signaling," *Mediators of Inflammation*, vol. 2014, Article ID 651890, 13 pages, 2014.

[6] G.-J. Tang, H.-Y. Wang, J.-Y. Wang et al., "Novel role of AMP-activated protein kinase signaling in cigarette smoke induction of IL-8 in human lung epithelial cells and lung inflammation in mice," *Free Radical Biology and Medicine*, vol. 50, no. 11, pp. 1492–1502, 2011.

[7] Y.-L. Wu, A.-H. Lin, C.-H. Chen et al., "Glucosamine attenuates cigarette smoke-induced lung inflammation by inhibiting ROS-sensitive inflammatory signaling," *Free Radical Biology and Medicine*, vol. 69, pp. 208–218, 2014.

[8] I. Rahman and I. M. Adcock, "Oxidative stress and redox regulation of lung inflammation in COPD," *European Respiratory Journal*, vol. 28, no. 1, pp. 219–242, 2006.

[9] T. Nakayama, D. F. Church, and W. A. Pryor, "Quantitative analysis of the hydrogen peroxide formed in aqueous cigarette tar extracts," *Free Radical Biology and Medicine*, vol. 7, no. 1, pp. 9–15, 1989.

[10] A. C. Toledo, R. M. Magalhaes, D. C. Hizume et al., "Aerobic exercise attenuates pulmonary injury induced by exposure to cigarette smoke," *European Respiratory Journal*, vol. 39, no. 2, pp. 254–264, 2012.

[11] G. P. Pessina, L. Paulesu, F. Corradeschi et al., "Effects of acute cigarette smoke exposure on macrophage kinetics and release of tumour necrosis factor α in rats," *Mediators of Inflammation*, vol. 2, no. 2, pp. 119–122, 1993.

[12] I. T. Lee and C. M. Yang, "Inflammatory signalings involved in airway and pulmonary diseases," *Mediators of Inflammation*, vol. 2013, Article ID 791231, 12 pages, 2013.

[13] Z. Orosz, A. Csiszar, N. Labinskyy et al., "Cigarette smoke-induced proinflammatory alterations in the endothelial phenotype: role of NAD(P)H oxidase activation," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 292, no. 1, pp. H130–H139, 2007.

[14] H. Yao, S.-R. Yang, A. Kode et al., "Redox regulation of lung inflammation: role of NADPH oxidase and NF-κB signalling," *Biochemical Society Transactions*, vol. 35, no. 5, pp. 1151–1155, 2007.

[15] K. Bedard and K.-H. Krause, "The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology," *Physiological Reviews*, vol. 87, no. 1, pp. 245–313, 2007.

[16] B. F. Bessac, M. Sivula, C. A. Von Hehn, J. Escalera, L. Cohn, and S.-E. Jordt, "TRPA1 is a major oxidant sensor in murine airway sensory neurons," *Journal of Clinical Investigation*, vol. 118, no. 5, pp. 1899–1910, 2008.

[17] E. S. Fernandes, M. A. Fernandes, and J. E. Keeble, "The functions of TRPA1 and TRPV1: moving away from sensory nerves," *British Journal of Pharmacology*, vol. 166, no. 2, pp. 510–521, 2012.

[18] Y. S. Lin, C.-C. Hsu, M.-Y. Bien, H.-C. Hsu, H.-T. Weng, and Y. R. Kou, "Activations of TRPA1 and P2X receptors are important in ROS-Mediated stimulation of capsaicin-sensitive lung vagal afferents by cigarette smoke in rats," *Journal of Applied Physiology*, vol. 108, no. 5, pp. 1293–1303, 2010.

[19] T. E. Taylor-Clark and B. J. Undem, "Sensing pulmonary oxidative stress by lung vagal afferents," *Respiratory Physiology & Neurobiology*, vol. 178, no. 3, pp. 406–413, 2011.

[20] Y.-J. Lin, H.-H. Hsu, T. Ruan, and Y. R. Kou, "Mediator mechanisms involved in TRPV1, TRPA1 and P2X receptor-mediated sensory transduction of pulmonary ROS by vagal lung C-fibers in rats," *Respiratory Physiology & Neurobiology*, vol. 189, no. 1, pp. 1–9, 2013.

[21] T. R. H. Büch, E. A. M. Schäfer, M.-T. Demmel et al., "Functional expression of the transient receptor potential channel TRPA1, a sensor for toxic lung inhalants, in pulmonary epithelial cells," *Chemico-Biological Interactions*, vol. 206, no. 3, pp. 462–471, 2013.

[22] I. Mukhopadhyay, P. Gomes, S. Aranake et al., "Expression of functional TRPA1 receptor on human lung fibroblast and epithelial cells," *Journal of Receptors and Signal Transduction*, vol. 31, no. 5, pp. 350–358, 2011.

[23] R. Nassini, P. Pedretti, N. Moretto et al., "Transient receptor potential ankyrin 1 channel localized to non-neuronal airway cells promotes non-neurogenic inflammation," *PLoS ONE*, vol. 7, no. 8, Article ID e42454, 2012.

[24] Y.-B. Yu, Y.-W. Liao, K.-H. Su et al., "Prior exercise training alleviates the lung inflammation induced by subsequent exposure to environmental cigarette smoke," *Acta Physiologica*, vol. 205, no. 4, pp. 532–540, 2012.

[25] Y. Yamaguchi, F. Nasu, A. Harada, and M. Kunitomo, "Oxidants in the gas phase of cigarette smoke pass through the lung alveolar wall and raise systemic oxidative stress," *Journal of Pharmacological Sciences*, vol. 103, no. 3, pp. 275–282, 2007.

[26] F. Facchinetti, F. Amadei, P. Geppetti et al., "α,β-unsaturated aldehydes in cigarette smoke release inflammatory mediators from human macrophages," *American Journal of Respiratory Cell and Molecular Biology*, vol. 37, no. 5, pp. 617–623, 2007.

[27] Y.-L. Yang, G.-J. Tang, Y.-L. Wu, H.-W. Yien, T.-S. Lee, and Y. R. Kou, "Exacerbation of wood smoke-induced acute lung injury by mechanical ventilation using moderately high tidal volume in mice," *Respiratory Physiology & Neurobiology*, vol. 160, no. 1, pp. 99–108, 2008.

[28] A. J. Connor, J. D. Laskin, and D. L. Laskin, "Ozone-induced lung injury and sterile inflammation. Role of toll-like receptor 4," *Experimental and Molecular Pathology*, vol. 92, no. 2, pp. 229–235, 2012.

[29] P.-L. Liu, Y.-L. Chen, Y.-H. Chen, S.-J. Lin, and Y. R. Kou, "Wood smoke extract induces oxidative stress-mediated caspase-independent apoptosis in human lung endothelial cells: role of AIF and EndoG," *The American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 289, no. 5, pp. L739–L749, 2005.

[30] L. Benov, L. Sztejnberg, and I. Fridovich, "Critical evaluation of the use of hydroethidine as a measure of superoxide anion radical," *Free Radical Biology and Medicine*, vol. 25, no. 7, pp. 826–831, 1998.

[31] A. Valavanidis, T. Vlachogianni, and K. Fiotakis, "Tobacco smoke: involvement of reactive oxygen species and stable free radicals in mechanisms of oxidative damage, carcinogenesis and synergistic effects with other respirable particles," *International Journal of Environmental Research and Public Health*, vol. 6, no. 2, pp. 445–462, 2009.

[32] D. A. Andersson, C. Gentry, S. Moss, and S. Bevan, "Transient receptor potential A1 is a sensory receptor for multiple products of oxidative stress," *Journal of Neuroscience*, vol. 28, no. 10, pp. 2485–2494, 2008.

[33] Y. Sawada, H. Hosokawa, K. Matsumura, and S. Kobayashi, "Activation of transient receptor potential ankyrin 1 by hydrogen peroxide," *European Journal of Neuroscience*, vol. 27, no. 5, pp. 1131–1142, 2008.

[34] D.-S. Cao, L. Zhong, T.-H. Hsieh et al., "Expression of transient receptor potential ankyrin 1 (TRPA1) and its role in insulin release from rat pancreatic beta cells," *PLoS ONE*, vol. 7, no. 5, Article ID e38005, 2012.

[35] N. Takahashi, Y. Mizuno, D. Kozai et al., "Molecular characterization of TRPA1 channel activation by cysteine-reactive inflammatory mediators," *Channels*, vol. 2, no. 4, pp. 287–298, 2008.

[36] M. Y. Song, A. Makino, and J. X.-J. Yuan, "Role of reactive oxygen species and redox in regulating the function of transient receptor potential channels," *Antioxidants and Redox Signaling*, vol. 15, no. 6, pp. 1549–1565, 2011.

[37] D. Shapiro, C. E. Deering-Rice, E. G. Romero et al., "Activation of transient receptor potential ankyrin-1 (TRPA1) in lung cells by wood smoke particulate material," *Chemical Research in Toxicology*, vol. 26, no. 5, pp. 750–758, 2013.

[38] M. Y. Song, A. Makino, and J. X.-J. Yuan, "Role of reactive oxygen species and redox in regulating the function of transient receptor potential channels," *Antioxidants & Redox Signaling*, vol. 15, no. 6, pp. 1549–1565, 2011.

[39] F. Jiang, Y. Zhang, and G. J. Dusting, "NADPH oxidase-mediated redox signaling: roles in cellular stress response, stress tolerance, and tissue repair," *Pharmacological Reviews*, vol. 63, no. 1, pp. 218–242, 2011.

[40] M. Trebak, R. Ginnan, H. A. Singer, and D. Jourd'Heuil, "Interplay between calcium and reactive Oxygen/Nitrogen species: an essential paradigm for vascular smooth muscle signaling," *Antioxidants & Redox Signaling*, vol. 12, no. 5, pp. 657–674, 2010.

[41] E. M. Carmona, J. D. Lamont, A. Xue, M. Wylam, and A. H. Limper, "Pneumocystis cell wall β-glucan stimulates calcium-dependent signaling of IL-8 secretion by human airway epithelial cells," *Respiratory Research*, vol. 11, article 95, 2010.

[42] E. Andrè, B. Campi, S. Materazzi et al., "Cigarette smoke-induced neurogenic inflammation is mediated by α,β-unsaturated aldehydes and the TRPA1 receptor in rodents," *Journal of Clinical Investigation*, vol. 118, no. 7, pp. 2574–2582, 2008.

[43] S. Earley, A. L. Gonzales, and R. Crnich, "Endothelium-dependent cerebral artery dilation mediated by trpa1 and CA^{2+}-activated K^+ channels," *Circulation Research*, vol. 104, no. 8, pp. 987–994, 2009.