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Review

LRRC8/VRAC Channels and the Redox Balance: A Complex Relationship

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Abstract
More than three decades after their first biophysical description, Volume Regulated Anion Channels (VRACs) still remain challenging to understand. Initially, VRACs were identified as the main pathway for the cell to extrude Cl^- ions during the regulatory volume decrease (RVD) mechanism contributing *in fine* to the recovery of normal cell volume. For years, scientists have tried unsuccessfully to find their molecular identity, leading to controversy within the field that only ended in 2014 when two independent groups demonstrated that VRACs were formed by heteromers of LRRC8 proteins. This breakthrough gave a second breath to the research field and was followed by many publications regarding LRRC8/VRACs structure/function, physiological roles and 3D structures. Nevertheless, far from simplifying the field, these discoveries have instead exponentially increased its complexity. Indeed, the channel's biophysical properties seem to be dependent on the LRRC8 subunits composition with each heteromer showing different ion/molecule permeabilities and regulatory mechanisms. One clear example of this complexity is the intricate relationship between LRRC8/VRACs and the redox system. On one hand, VRACs appear to be directly regulated by oxidation or reduction depending on their subunit composition. On the other hand, VRACs can also impact the redox balance within the cells, through their permeability to reduced glutathione or through other as yet uncharacterized pathways. Unravelling this issue is particularly crucial as LRRC8/VRACs play an important role in a wide variety of physiological processes involving oxidative stress signaling. In this regard, we have tried to systematically identify in the literature both pre- and post-LRRC8 discovery as well as the interplay between VRACs and the redox system to provide new insights into this complex relationship.

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Introduction

Anion channels support a wide variety of physiological functions within cells including proliferation, migration, apoptosis, pH and membrane potential modulation and volume regulation [1]. Most anion channels are permeable to different anions and are therefore characterized by their limited selectivity. Due to the lack of selective inhibitors and their involvement in a lot of different processes, the role of some anion channels remains difficult to evaluate. Amongst the many anion channels, Volume Regulated Anion Channels (VRACs) have been shown to play a central role in the volume regulation following cellular swelling. In mammalian cells, regulatory volume decrease (RVD) is a classical cellular mechanism, which counteracts a decrease in extracellular osmolarity by a compensatory mechanism leading to a loss of cellular ions (mainly KCl) and osmolytes together with osmotically obliged water. This ubiquitous mechanism allows *in fine* a recovery of normal cell volume. In most cells, RVD is linked to the activation of ion channels that drive the outflow of K^+ ions and anions (predominantly Cl^- and negatively charged osmolytes). Historically, the first description of anion permeability in response to osmotic cell swelling was reported in ascini cells and lymphocytes [2, 3] while the first anion current in response to osmotic cell swelling has been independently recorded in epithelial cells [4] and in lymphoid cells [5]. Starting from this date, the VRACs were recorded in virtually all cell types: neurons [6], glial cells [7], endothelial cells [8], colon crypt cells [9] and renal cells [10]. VRACs were characterized by the biophysical properties of their current, such as outward rectification, time-dependent inactivation at positive potentials, their permeability sequence and their inhibition by non-selective anion channel blockers (DIDS, NPPB) or by the most potent inhibitor of VRACs, DCPIB [1, 11-13].

For decades, the molecular identity of VRACs has remained a mystery, and the only way of characterizing its physiological role was by the use of inhibitors. Unfortunately, such molecules were not specific enough to discriminate precisely the contributions of VRACs to different cellular or physiological mechanisms [14]. Therefore, studies that only rely on those unspecific inhibitors must be taken with caution as those molecules could target other channels as well as other proteins within the cell and could have led to misinterpretations [15, 16]. Nevertheless, in 2014, two independent groups found that Leucine-Rich Repeat Containing 8A (LRRC8A) proteins were essential to generate VRACs currents [17, 18]. LRRC8 forms a family of 5 members (LRRC8 A-E) with structural similarities to pannexins, both families sharing probably a common ancestor at the origin of the chordates [19]. Voss *et al.* [18] have shown that VRACs were composed by heteromers of LRRC8 subunits in which LRRC8A is essential for its plasma membrane expression. Cryogenic electron microscopy (cryo-EM) experiments based on monomers of LRRC8A [20, 21] or LRRC8D [22] suggest that LRRC8 subunits are organized in heterohexameric structure as suggested previously by [23, 24]. LRRC8/VRACs are permeable to chloride ions but, depending on their composition, VRACs exhibit different biophysical properties and significant permeability to various substrates that vary strongly in charge and size. For instance, LRRC8A/D has been shown to be involved in the transport of large molecules such as neurotransmitters (GABA, glutamate, etc.) as well as various amino acids (taurine, lysine, aspartate, etc.) [25], or nucleotides such as ATP [23, 26] but also antibiotics (blasticidin S, [27]) and some anticancer drugs (cisplatin and carboplatin, [28]). On the contrary, the combination of LRRC8A/C shows a significant conductance to chloride but is less permeable to bigger anions while LRRC8A/E is more permeable to aspartate and negatively charge osmolytes [25]. Recently, Zhou *et al.* and Lahey *et al.* identified heteromers containing LRRC8A/E and LRRC8A/C subunits to be permeable to cGAMP and cyclic dinucleotide [29, 30]. Interestingly, a study performed in native HEK293 found LRRC8B to be expressed in the endoplasmic reticulum where it might play a role in calcium signaling [31].

In addition to its obvious participation to RVD, VRACs have been shown to play a role in cell proliferation and migration, apoptosis, cancer drug resistance, membrane potential modulation, cell-cell communication and epithelial transport and secretion [32, 33]. In

animal models, a series of studies based on genetic manipulations of the lrrc8a gene revealed that LRRC8/VRACs play crucial physiological functions that were initially not anticipated. Global gene inactivation of LRRC8A results in strong embryonic lethality, tissue malformation and alteration of the immune functions among many other deficits [34]. Similarly, conditional gene inactivation revealed a role for LRRC8/VRACs in various tissue functions such as spermatid development [35], insulin secretion from beta cells [36, 37], myogenesis [38] and adipocytes homeostasis [39, 40], supporting the idea that the role of LRRC8/VRACs are not limited to cell volume regulation. In humans, a LRRC8A mutation was originally identified in a woman who lacked B cells in the peripheral blood and was found to have congenital agammaglobulinemia [41]. The role of LRRC8A in B and T cells maturation has been highlighted by the analysis of LRRC8 knockout mice [34].

Despite the increased findings on LRRC8/VRACs, many aspects of this anion channel are still to be discovered and characterized. One of the biggest challenges would probably be to unravel the intriguing relationship between LRRC8/VRACs and the redox balance.

Regulation of LRRC8/VRACs activity by oxidation and reduction

Oxidative stress has been studied for a long time with investigations focussing on its deleterious effects [42]. Nevertheless, it is now widely recognised that mild oxidative stress is a key element leading to cell adaptation and survival [42]. The increased presence of oxidizing agents is sensed within cells by different mechanisms that activate many signaling pathways. Oxidants directly affect proteins on cysteine and methionine residues in a process called oxidation. Mild oxidative stress produces the reversible S-Sulfenylation while severe oxidative stress leads to irreversible S-Sulfinylation and S-Sulfonylation. Those post-translational modifications alter protein functions and are partly responsible for the cellular response to oxidative stress. In addition, both nucleic acids and lipids can be modified by oxidation with dramatic effects. Redox signals can also be transmitted by the activation of more complex signaling pathways through kinases and/or phosphatases. Oxidative stress is thus an important signaling event driving cellular adaptation, but this process must be highly regulated to prevent its deleterious consequences. The antioxidant system plays a major role in the protection against oxidative stress. Beyond the diversity of cellular anti-oxidative mechanisms, reduced glutathione (GSH) is one of the major antioxidants in the cell and mediates the rapid elimination of reactive oxygen species (ROS). This tripeptide also reacts with cysteine residues of proteins to form S-Glutathionylation, a post-translational modification that can regulate their activities. Interestingly, S-Glutathionylation has been shown to elicit major changes in function of ion channels, including the Ryanodine Receptor [43] and the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) [44].

Many studies have been carried to establish the impact of redox balance on VRACs activity and the results are very divergent, likely reflecting the difference between the LRRC8 subunit composition and the cellular context.

Activation of LRRC8/VRACs by oxidants

Numerous studies have highlighted the contribution of ROS on VRACs activation and potentiation. Some describe the effect of direct application of exogenous oxidative agents on VRACs currents. Others describe the effect of inhibition of ROS producing system (eg NADPH-oxidase) or ROS scavenging on VRACs activated by hypotonic shock or by other stimuli.

In 2004, it has been described in different cellular context and by 3 different groups that exogenous hydrogen peroxide (H_2O_2) triggers a chloride conductance with VRACs properties (outward rectification, deactivation at positive potential, inhibition by DCPIB) [45-47]. Exogenous H_2O_2-activated VRACs currents were inhibited by both the antioxidant enzyme catalase and the reducing agent dithiothreitol (DTT) in HeLa and HTC cells [47] and by the oxidoreductive action of the catalase in myocytes [45]. Many publications have then reproduced the ROS-induced VRACs activation in various cell types [48-53]. Our group

has shown in renal primary cells that exogenous H_2O_2 activated VRACs current was fully inhibited by the ROS scavenger N-acetyl-L-cysteine (NAC) [54]. More recently, it has been shown that exposure of nodose ganglion neurons to H_2O_2 also induces a VRACs activation [55].

Interestingly, while the mechanism of VRACs activation triggered by hypotonic cell swelling is still not well understood, numerous studies have reported an increased ROS production during hypotonic challenge [47, 56, 57]. The involvement of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase, NOX) family members in the VRACs activation under hypotonic stimulation has also been highlighted. NOXs are key producers of ROS in many cells and their activation leads to the reduction of oxygen into superoxide (O_2^-) which is spontaneously or enzymatically converted into H_2O_2. Varela's study has shown that hypotonicity-triggered VRACs currents were inhibited by the membrane-permeable superoxide scavenger Tiron, the peroxide-scavenging enzyme catalase and DTT. This was dependent on NOX as the ROS producing protein given that the NOX inhibitor Diphenyleneiodonium (DPI) as well as $p47^{S379A}$, a dominant negative form of $p47^{PHOX}$ component of NOX, strongly inhibited VRACs current triggered by hypotonicity [47]. Using DPI and NOX4 inhibitor plumbagin, Crutzen *et al.* have implicated NOX4 in hypotonicity-induced VRACs activation [48]. They also found that a pretreatment with betulinic acid, which decrease NOX4 expression, also inhibited RVD upon hypotonic challenge. In a primary cell culture of nodose neurons, Wang *et al.* reported that the hypoosmolarity-induced VRACs currents are also strongly attenuated by NOX inhibitors, apocynin and DPI, and abrogated by the catalase [55].

If hypo-osmotic challenge is the easiest way of triggering VRACs current, various stimuli have also been reported to activate VRACs. For example, an acute exposure to the Epithelial Growth Factor (EGF), known to increase ROS within a wide variety of cells [58], has been shown to activate VRACs in a process inhibited by DPI [47]. Furthermore, this activation is also inhibited by gp91ds-tat, a dominant negative mutant of the gp91 component of NOX2 [59]. Staurosporine (STS), a powerful apoptosis inducer, was shown to induce a normotonic cell shrinkage called AVD (Apoptotic Volume Decrease) through the increase of ROS which in turn activated VRACs. It has also been shown that STS-triggered VRACs current as well as AVD and caspase-3 activation were inhibited by DPI [46] and NAC [54]. Other groups reported that other apoptosis inducers enhancing ROS production also triggered VRACs currents, including TNF-α [52], ceramide [60] and doxorubicin [51]. Okada's group also reported that bradykinin-activated VRACs in astrocyte was also dependent on the ROS increase by NOX as this activation was fully inhibited by NAC and two NOX inhibitors (DPI and apocynin) [61, 62]. Tunicamycin and angiotensin II are among the other molecules described to activate VRACs in a ROS-dependent mechanism [45, 53]. Wang *et al.* have reported in nodose neurons that low extracellular pH induces NOX-derived H_2O_2 generation involved in the activation of VRACs currents. For the first time, the authors were able to directly link their findings to LRRC8A by using silencing approaches [55]. However, the mechanism they proposed is unconventional and most probably restricted to nodose neurons since extracellular acidification seems to trigger, by a complex mechanism, cellular alkalization which then activates NOX.

Nevertheless, Deng and coworkers have shown in 2010 that ROS produced by mitochondria could actually be as important as NOX-produced ROS. In a first paper they showed that VRACs activation by endothelin required ROS produced by both NOX2 and mitochondria [63]. ROS produced by NOX2 secondarily drove the mitochondria to produce additional ROS and this induced VRACs activation. Indeed, VRACs activation by endothelin is inhibited by apocynin and the fusion peptide gp91ds-tat. Furthermore, antimycin A, reported to increase mitochondrial ROS, also activates VRACs that are not blocked by the NOX inhibition. Finally, in a follow up publication, Deng *et al.*, demonstrated that ritonavir and lopinavir, two anti-retrovirus drugs used against HIV, triggered VRACs activation through mitochondrial ROS production [64].

Other studies have focused on the effect of ROS on activated VRACs. Mongin's group has shown in microglia that exogenous H_2O_2 potentiated both VRAC-dependent hypotonicity-induced chloride currents and aspartate release. They also reported that zymosan, a NOX activator, increased ROS production and potentiated hypotonicity-induced efflux of aspartate via VRACs that was inhibited by several NOX inhibitors [49]. The same synergistic effect has been reported on taurine efflux in hypotonic condition that was inhibited by both DPI and NAC as well as the ROS scavenger butylated hydroxytoluene [65]. Pusch's group has reported in *Xenopus* oocytes the effect of oxidant agents on different LRRC8 subunit combinations that are constitutively active due to the addition of fluorescent tags. *Xenopus* oocytes expressing LRRC8A/E possess VRACs currents that are potentiated by both the ROS inducer t-BHP and the oxidizing agent chloramine-T (Chl-T) [66]. Chl-T is a specific oxidant that targets sulfur-containing residues and can oxidize both cysteine and methionine residues in proteins and thus alter their function. Furthermore, they have shown that Chl-T also potentiates the hypotonicity-triggered current of the constitutive LRRC8/VRACs in Jurkat cells expressing mainly LRRC8A/E. Using cysteine modifiers they suggest a direct oxidation of the intracellular cysteine residues of the LRRC8 subunits [66].

ROS produced by the NOX pathway play a major part in VRACs activation but the use of NOX inhibitors should be complemented by another approach as they have been shown to be not as specific as initially thought [67]. Even if exogenous ROS as well as NOX-produced ROS and mitochondrial ROS have been all shown to trigger VRACs, the ROS origin and their nature could be important, especially to know if the activation mechanism results from a direct oxidation or is mediated by other indirect pathways.

Inhibition of LRRC8/VRACs by oxidants

Only a few papers have reported an inhibitory effect of oxidants on VRACs activity. First, Gradogna *et al.*, have shown in *Xenopus* oocytes expressing different combinations of LRRC8 subunits that only the hypotonicity-induced chloride currents of LRRC8A/C and LRRC8A/D combinations were inhibited by Chl-T [66]. Nevertheless, the lack of effects of cysteine modifiers on the Chl-T response suggests that the inhibition is unlikely mediated by cysteine residues [66]. Secondly, Bach *et al.*, have found that chronic exposure to ROS inhibited VRACs activity in human alveolar carcinoma cells [68]. They have suggested that ROS inhibited VRACs through the oxidation of kinases and phosphatases that control VRACs activity such as the PI3K/AKT/mTOR pathway. They have observed that LRRC8A protein levels are increased and the VRACs inhibition could be the result of a modification of the stoichiometry of the different subunits since it has been reported that an increase of LRRC8A expression could result in an inhibition of LRRC8/VRACs current [17, 18, 24].

Modulation of LRRC8/VRACs activity by reducing agents

While most researches have focused on the effect of ROS on VRACs, little is known about the effect of reducing agents. Gradogna *et al.*, have shown that the reducing agent DTT increased constitutive LRRC8A/C and LRRC8A/D chloride currents in *Xenopus* oocytes but had no effect on LRRC8A/E [66]. Interestingly using whole-cell patch-clamp techniques, we have also observed that DTT treatment (2mM) induced a significant increase of the hypotonicity-induced VRACs chloride current in HEK293 cells (Fig. 1) that mainly express the LRRC8A/D subunits combination [14].

Modulation of Redox balance by LRRC8/VRACs

On one hand, VRACs appear to be directly modulated by oxidizing and reducing agents depending on the cellular context and probably on its LRRC8 subunits composition. On the other hand, there are also some evidences that VRACs activity can modulate the cellular redox balance. The physiological relevance of such regulation by LRRC8/VRACs should be systematically assessed, as ROS are important signaling molecules involved in numerous pathological mechanisms.

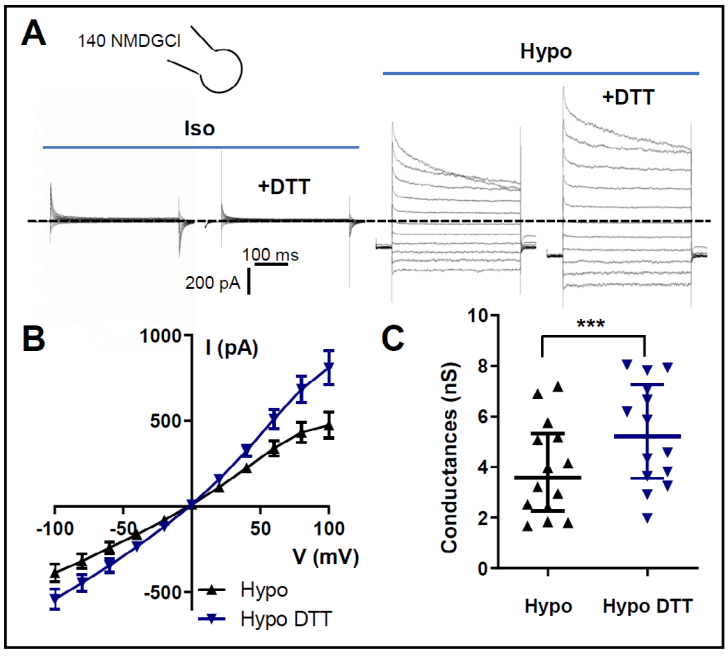

Fig. 1. Effect of DTT on VRAC chloride current measured in wild-type HEK293 cells. (A) Whole-cell chloride currents measured in control conditions (iso, 340 mOsm.l^{-1}) in the absence or presence of DTT (2mM) and after replacing the bath with a hypotonic solution (hypo, 270 mOsm.l^{-1}) containing or not DTT (2mM, 5 min). Cells were held at −50 mV, and 400 ms pulses from −100 to +100 mV were applied in 20 mV increments. Exact compositions of pipette and bath NMDGCl solutions are given in [14]. (B) I/V curve of the chloride currents (n=14) triggered by hypotonic shock recorded as in A and measured in the absence and presence of DTT (2mM). I/V relationships are expressed as the mean current amplitudes measured at all potentials at 6-10 ms after the pulse onset. (C) Comparison of the VRAC conductances slope measured after hypotonic solution exposure in the absence (hypo) or presence of DTT (2mM, hypo DTT). Conductances were calculated between +40 to +80 mV from 14 individual cells (Wilcoxon test was used, ***p< 0.0001).

Permeability to reduced glutathione

GSH is a negatively charged tripeptide that majorly contributes to the regulation of the redox state within the cell. Indeed, this peptide reacts with peroxide residues and subsequently scavenges ROS while being oxidized to glutathione disulfide (GSSG). The concentration of intracellular GSH is in the mM range which is 10 to 1000-fold higher than its extracellular concentration. GSH transporters usually support an efflux of GSH and consequently lower the cellular antioxidant potential.

Some channels have been shown to be permeable to GSH. CFTR, a cAMP sensitive chloride channel [69], and connexin hemichannels that exhibit some structural organization similarities with LRRC8/VRACs clearly contribute to GSH release outside the cell [70, 71]. In both cases, the physiological relevance of GSH efflux has been highlighted [72-74]. The first description of a GSH efflux during RVD was made in 1990 by Hussinger *et al.*, in hepatocytes [75]. Later on, Sabirov *et al.*, have shown that the osmosensitive release of GSH in rat thymocytes was inhibited by VRACs inhibitors including DCPIB [76]. They have calculated that GSH radius is ~0.54 nm which is smaller than the functional pore radius of ~0.63 nm estimated by Ternovsky *et al.* before the LRRC8 discovery [77]. We have shown very recently that (i) LRRC8/VRACs exhibit a significant permeability to GSH during an hypotonic challenge and that (ii) stimulation of renal HK2 cells with the Transforming Growth Factor β1 (TGF-β1) induces LRRC8/VRACs activation leading ultimately to a drop of intracellular GSH content and a subsequent increase of ROS production. VRACs inhibitors as well as LRRC8A knockdown significantly blunt the GSH efflux [78]. Sabirov *et al.* reported a relative permeability P_{GSH}/P_{Cl} of 0.32 for efflux and 0.10 for influx in rat thymocytes [76] while we reported a P_{GSH}/P_{Cl} of 0.11 and 0.08 for efflux in HK2 and HEK cells respectively [78]. Both HK2 and HEK cells mainly express LRRC8A/D [78] which are known to be permeable to large molecules while other subunits combinations seem to have a narrower pore. Indeed, the pore size of LRRC8A homohexamers is probably too narrow to accommodate GSH transport, even though the structure obtained by cryo-EM could be affected by the ionic strength

used during protein preparation, the lipid environment and the temperature. Nevertheless, the authors who described the LRRC8/VRACs structure suggest that assembly of LRRC8 heteromers would have larger pores, in particular heterohexamers of LRRC8A/D [20, 21, 79, 80]. A recent study performed using cryo-EM experiments with LRRC8D homohexamers highlights the larger pore of this structure [22]. It is therefore possible that LRRC8/VRACs would be permeable to GSH when considering their LRRC8 subunits expression profile. Nevertheless, we have highlighted that LRRC8/VRACs activity supports oxidative stress and that its inhibition prevents the TGF-β1-induced epithelial-mesenchymal transition in epithelial renal HK2 cells [78].

Beside the obvious role of LRRC8/VRACs in the volume regulation, its potent permeability to GSH should be taken in consideration as it could directly impact on the redox balance and subsequently activate oxidative stress signaling cascade.

Impact of VRACs activity on redox status

Interestingly, VRACs inhibition has been shown to protect against numerous pathological conditions where ROS are involved, such as hypoxia, ischemia, autophagy and apoptosis [81-86]. However, those publications, highlighting the pathological relevance of targeting VRACs, do not report any data concerning the redox state. To our knowledge, the first paper reporting a direct effect of targeting VRACs on the redox balance was published in 2012 by Crutzen and coworkers [48]. They have found in pancreatic beta cells that hypotonicity increased ROS production and secondarily increased insulin release. Interestingly, they have shown that the chloride channel inhibitor NPPB inhibited both ROS increase and insulin release. Choi *et al.* have shown that LRRC8A is a component of Nox1 signaling pathway and that its activity actually supports superoxide production. Indeed, they have shown first LRRC8A/NOX1 colocalization and interaction, and secondly that both DCPIB and knockdown of LRRC8A decrease superoxide production in response to TNF-α [87]. In another study, Wang *et al.*, have found that both DIDS (an anion channel blocker) and DCPIB block the ROS increase in response to exposure of cardiomyocytes to high glucose [88].

Nevertheless, results obtained with chloride channels inhibitors should be confirmed with gene silencing approaches to exclude non-specific effects [14]. Recently, Denton's group have highlighted that DCPIB, the most potent LRRC8/VRACs inhibitor, inhibits mitochondrial respiration, independently of the cellular expression of LRRC8A subunit [15]. Other chloride channels inhibitors such as NFA, NPPB, GlyH-101 and CFTRinh-172 have also been reported to inhibit mitochondrial respiration, which could result in dramatic changes in cellular redox state [89-91].

LRRC8/VRACs and the RedOx balance: a complex interplay

It is now possible to see that LRRC8/VRACs and the RedOx system share a complex interplay and cross regulate themselves in an intricate feedback/feedforward mechanism but only a few teams have proposed such a complex regulation. Crutzen *et al.*, have highlighted the complex co-regulation of VRACs and ROS in pancreatic beta cells [48]. In their publication, they have also shown that H_2O_2 was able to trigger VRACs current and therefore insulin release, a process that is fully inhibited by both NOX inhibitors and ROS scavengers. Curiously, VRACs inhibition reduced the oxidative stress and secondarily the insulin release. Wang *et al.*, have proposed that ROS and VRACs display a likely reciprocal regulation in cardiomyocytes exposed to high glucose [88]. In this context, NAC inhibits VRACs current triggered by high glucose whereas the use of VRACs inhibitors lower the oxidative stress. Those data highlight that the relationship between ROS and VRACs is more complex than what was initially believed, especially considering the possible VRAC-independent effects of VRACs inhibitors on ROS production.

Conclusion

After more than three decades of research and controversy on the function and the molecular identity of VRACs, the identification of LRRC8 proteins provided a breakthrough to unravel the complexity surrounding these channels, or, as we should consider them, anion-permeable channels. Within a few years, scientists were able to shed light on the molecular structure, the 3D conformation and on their roles in physiology and pathophysiology.

The VRACs regulation by oxidative stress was known years before the identification of LRRC8 proteins and only a few papers have since focused on how oxidants and antioxidants modulate LRRC8/VRACs. It appears that LRRC8/VRACs are regulated differently depending on their LRRC8 subunits composition (Fig. 2A, B). Those results could explain some of the discrepancies of the VRAC's literature, but it might also be more complex and depend on the cellular context.

On the contrary, the impact of VRACs activity on the redox balance have been mainly underestimated. Indeed, only a small number of studies have reported that VRACs activation or inhibition is linked with a change in GSH and/or ROS levels (Fig. 2C). The lack of highly specific VRACs inhibitors and their putative effects on mitochondrial function [15] appear as an important limit to overcome highlighting the need to use genetic invalidation to link LRRC8 subunits to redox status.

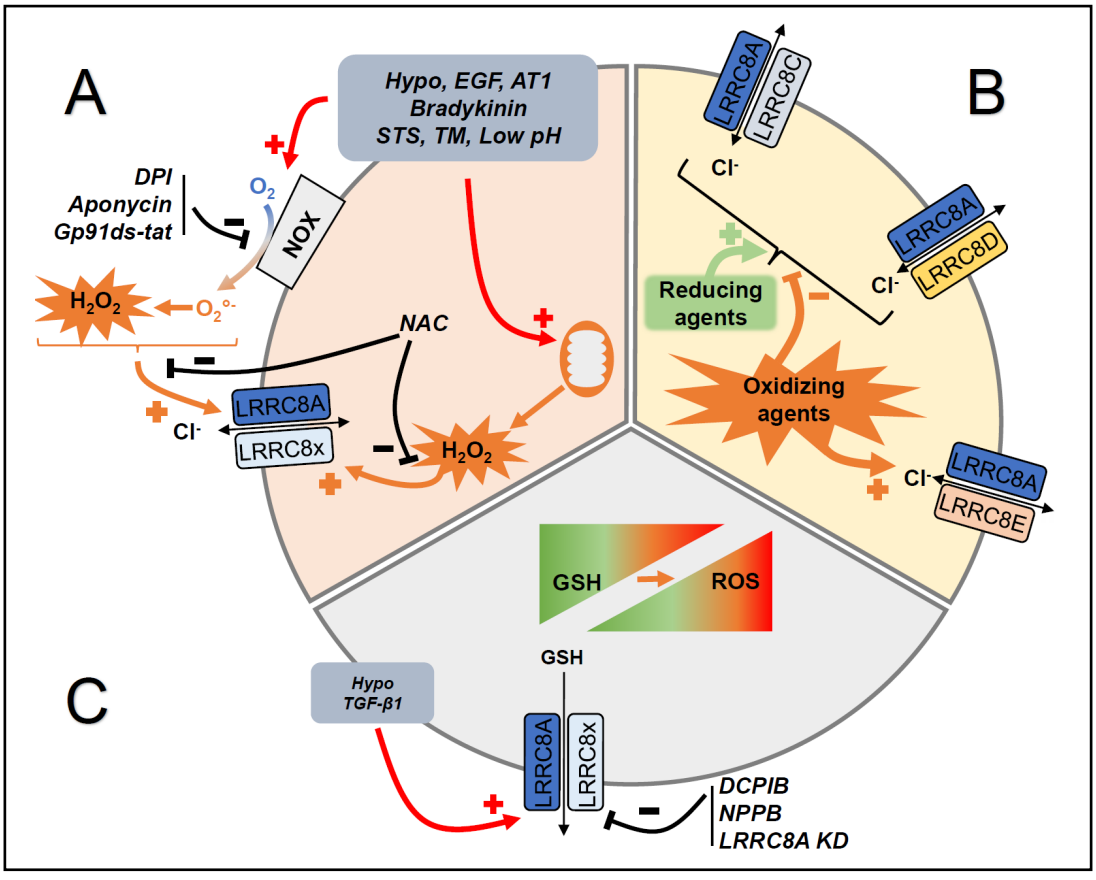

Fig. 2. LRRC8/VRACs and the cellular oxidative status. (A) Various stimuli lead to ROS increase that triggers VRACs activation. Abbreviations: AT1: Angiotensin receptor 1, DPI: Diphenyleneiodonium, EGF: Epithelial Growth Factor, NAC: *N-acetyl cysteine*, NOX: NADPH Oxidase, STS: Staurosporine; TM: Tunicamycin. (B) Regulation of LRRC8/VRACs current by oxidizing and reducing agents depending on the subunit's composition of VRAC. Adapted from [92]. (C) LRRC8/VRAC-dependent GSH efflux contributes to the cellular antioxidant system. Abbreviations: GSH: Glutathione, KD: Knockdown, TGF-β1: transforming Growth Factor.

Considering the number of pathological mechanisms involving both VRACs and oxidative stress, it appears important to focus on their complex relationship. First of all, it would be important to characterize more systematically the relationship between LRRC8 subunits composition/combination and the redox sensitivity in relation to the cellular context and the stimuli. Indeed, some effects might be caused by a direct interaction with redox agents while others might result in a more complex interaction through kinases or phosphatases. If the modulation comes from a direct interaction- which seems to be the case, at least in oocytes [66] - it is then needed to identify the interaction sites within LRRC8 subunits. Depending on those interaction sites, it would then be possible to predict if redox agents could also modulate the response of LRRC8/VRACs to other regulators such as kinases. Furthermore, the permeability of LRRC8/VRACs to GSH must be taken into consideration while investigating the physiological properties of those channels. ROS levels should be systematically measured in order to differentiate between VRACs activity and intracellular ROS effects levels. As they seem to interplay in a complex feed forward mechanism, it might be difficult to totally segregate their effects. Indeed, they might form a complex signaling platform where NOX and LRRC8/VRACs interact altogether [48, 55, 87]. Nevertheless, the identification of such signaling platforms and the linked proteins would only increase our understanding of VRACs physiology and would allow us to target more precisely the pathological mechanisms involving VRACs.

After so many years of research and controversy on VRACs, we finally have the experimental tools to address the many questions that remain unanswered. The link between LRRC8/VRACs and the redox system is about to be unravelled and we have just uncovered a part of this mystery. The coming years promise to be rich in discoveries that will illuminate the precise functions of LRRC8/VRACs in human physiology and pathophysiology.

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Disclosure Statement

All authors declare that no conflicts of interest exist.

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