

How Does the Chloride/Proton Antiporter CIC-3 Control NADPH Oxidase?

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In this issue of *Circulation Research*, Miller and coworkers¹ introduce new concepts that drastically change current understanding of NADPH oxidase (Nox) regulation. Nox enzymes are important ubiquitous generators of reactive oxygen species (ROS) involved in a surprisingly varied array of functions such as immune defense and vascular pathophysiology.²

In their article, Miller et al start by demonstrating that Nox1 is localized in endosomes of vascular smooth muscle cells, and that it is stimulated by cytokines to generate ROS inside these vesicles, eventually leading to activation of NFκB. This is an important demonstration of the intracellular activity of Nox1, and it is consistent with previous observations and speculations.² The most important finding of this work, however, is that Nox1 activity and signaling require CIC-3, a chloride/proton exchanger also localized in endosomes. Indeed, the authors show that chloride channel inhibitors and knockout of CIC-3 abolish cytokine-induced generation of ROS in endosomes and ROS-dependent NFκB activation. This unexpected involvement of CIC-3 in Nox activation was first described in a recent article by the same group demonstrating that Nox2-mediated neutrophil functions are impaired in CIC-3 knockouts.³ The present study not only reveals the importance of CIC-3 in vascular tissue and its ability to control another Nox family member, but also challenges the prevalent theory of Nox activation.

The most straightforward way to explain the effect of CIC-3, which is favored by Miller and coworkers, is that this antiporter prevents Nox-induced accumulation of negative charges in the endosomal lumen. This function is required because Nox2 is readily blocked by membrane depolarization.^{4,5} Before discussing the merits and implications of Miller's model and why it is sure to raise controversy, we first need to briefly mention the molecules that control ion fluxes through endosomal membranes.

The Figure (A) shows that the Na⁺K⁺ATPase maintains endosomal membrane polarization, whereas the vacuolar proton ATPase, acidifies the lumen.^{6,7} The increase in luminal chloride concentration observed during maturation of the endosome can be explained by the presence of chloride

channels. These also favor acidification of the lumen, presumably by neutralizing the charge of protons supplied by the vacuolar ATPase. By analogy, increasing chloride and proton concentrations in the lumen were the functions attributed to the CIC-3 channel when it was first discovered in intracellular membranes.^{8–10} However, CIC-3 is now believed to be a chloride/proton exchanger, because of its high homology (close to 90% in humans) to the antiporters CIC-4 and CIC-5.^{11,12} Therefore, CIC-3 is now usually thought to transport protons out of the lumen in exchange for chloride anions going in (Figure, A). This configuration allows CIC-3 to elevate luminal chloride concentration at the expense of dissipating part of the proton gradient.¹³ This mechanism seems appealing because protons are moving down their concentration gradient. However, their driving force may not be sufficient, considering that the concentration of chloride ions is 4 orders of magnitude higher than protons. The absence of a link between the proton ATPase and CIC-3 in vascular smooth muscle is supported by an experiment of Miller and coworkers showing that inhibition of the proton pump does not alter CIC-3 function.

In Miller's model (Figure, B), ion exchange via CIC-3 is represented in the opposite orientation, to take into account the fact that CIC-3 is an almost complete outward rectifier,^{9,11} as also demonstrated for CIC-4 and CIC-5.¹¹ This means that conduction by CIC-3 is much greater when directing chloride into the cytosol. However, this would only occur when the endosomal membrane is depolarized, as expected after activation of Nox. CIC-3 thus appears to be well suited to remove an excess of negative charges from the endosome and allow sustained Nox activity.

We now face another difficulty, in the form of a large body of evidence from the literature showing that charge compensation for Nox1 and Nox2 results from proton channels, either built into the oxidase itself,⁴ or closely associated with it⁵ (Figure, C). A model of Nox2 activation in granulocytes, elaborated from electrophysiological observations and theoretical considerations,⁵ suggests that charge compensation is almost entirely provided by voltage-gated proton channels, and little or no chloride conductance. Furthermore, the intensities of currents flowing through the oxidase and proton channels appear to be equal, leaving no room for a chloride flux.^{4,5} Therefore, according to this model, the partial dependence of Nox2 on CIC-3 observed by Miller's group³ would have to be explained by other mechanisms. We will come back to this issue shortly, after briefly considering the fate of superoxide.

In nonphagocytic cells there is much evidence that superoxide triggers signaling in the cytosol.^{1,2} Since superoxide cannot readily cross lipid bilayers, it would have to be

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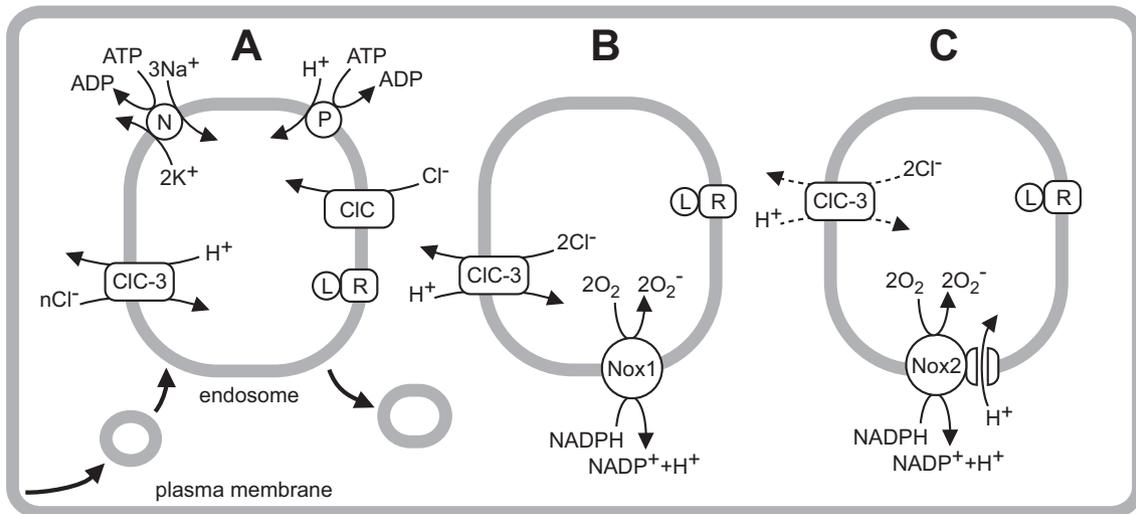


Figure. Models of endosome ion fluxes. A, Receptors (R) and their ligands (L) are transported to the endosome by incoming vesicles from the plasma membrane, and later recycled back to the plasma membrane or to lysosomes by outgoing vesicles. Endosomal membrane polarization is maintained by the Na⁺K⁺ATPase (N), acidic pH by the vacuolar ATPase (P), and luminal chloride concentration by chloride channels (CIC). If CIC-3 takes the place of CIC in this model, being a chloride/proton exchanger, it dissipates part of the proton gradient to bring chloride into the lumen. B, In this model of vascular smooth muscle endosome from Miller and coworkers,¹ stimulation of the receptor by its ligand activates NADPH oxidase 1 (Nox1). This enzyme uses electrons from cytosolic NADPH and reduces molecular oxygen to superoxide anion in the lumen. To sustain its activity, Nox1 depends on CIC-3 to provide charge compensation. This is possible because ion fluxes via CIC-3 are opposite to those depicted in panel A. C, In this model of phagocytic oxidase Nox2, charge compensation is almost entirely provided by a proton channel closely associated to the enzyme.⁵ CIC-3 could contribute to a fraction of charge compensation or interact with Nox2 by an unknown mechanism.

converted to membrane-permeable hydrogen peroxide in the endosomal lumen. Alternatively, superoxide could also become protonated in the acidic lumen and cross the membrane as an uncharged HO₂ molecule, before dissociating again at neutral pH in the cytosol. Another possibility, allowed in Miller's model, would be that CIC-3 carries superoxide to the cytosol. This would be consistent with a recent study showing that CIC-3 can transport superoxide across the plasma membrane of endothelial cells.¹⁴ However, confirmation in vascular smooth muscle will require additional experiments.

So far we have emphasized differences between models; it is now time to offer suggestions for reconciliation. It is conceivable that different mechanisms of charge compensation apply to different Nox isoforms. Indeed, in the case of vascular smooth muscle Nox1, CIC-3 appears to be a limiting factor in oxidase activity.¹ In contrast, knocking out CIC-3 only partially inhibits Nox2 in neutrophils.³ This makes sense if CIC-3 is sufficient to provide charge compensation for the low activity of Nox1, but not for Nox2 during the respiratory burst.

Furthermore, it is interesting to note that knocking out CIC-3 inhibits Nox2 activity to a greater degree in neutrophils exposed to a stimulus favoring phagocytosis (opsonized zymosan), rather than extracellular superoxide production (PMA).³ Charge compensation is more important in the small volume of the phagosome than in the virtually infinite space surrounding a neutrophil. This observation suggests that charge compensation by CIC-3 can be very significant for Nox2, when it is activated in phagosomes. This is consistent with the observation that Nox1 appears to be entirely dependent on CIC-3 in the study of Miller and coworkers, because vascular smooth muscle cells

produce superoxide essentially intracellularly. In this context it should be noted that most electrophysiological experiments are conducted at the plasma membrane, because it is difficult to isolate endosomes or phagosomes. Due to their extreme complexity, phagosomes are also less amenable to modeling than the plasma membrane. Thus, it is possible that within vesicles CIC-3 has a role in charge compensation, regardless of Nox isoform.⁵

Current evidence suggests that CIC-3 controls Nox via charge compensation. However, as mentioned for the phagocyte oxidase, it cannot be excluded that other mechanisms also intervene. One way to begin answering this question would be to demonstrate that CIC-3 interacts with a Nox subunit. In their article, Miller and coworkers show that a cell lysate without detergent can be enriched in Nox1, p22phox, and endosomal markers using an immobilized CIC-3 antibody. This suggests that CIC-3 is close to Nox1 and p22phox in lipid vesicles, but does not necessarily imply direct physical interaction. Additional experiments may establish this point.

The same question could be approached in the opposite direction by demonstrating that charge compensation alone is sufficient to explain the effect of CIC-3. For example, it may be possible to measure in which proportions cytokine-activated Nox1 is controlled by proton versus chloride currents in vascular smooth muscle cells. It may also be possible to separate proton currents in CIC-3 from those of other channels. In the same vein, perhaps charge compensation provided by artificial means, such as overexpression of other channels, may be sufficient to rescue a CIC-3 with no antiporter activity.

In conclusion, Miller and coworkers have opened new horizons before us by demonstrating that CIC-3 can control

Nox. Perhaps the underlying mechanisms are not yet fully understood, but we can expect their model of Nox activation to provide new directions for future research, and a better integration with current theories in the near future.

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Disclosures

None.

References

1. Miller F, Filali M, Huss G, Stanic B, Chamseddine A, Barna T, Lamb F. Cytokine activation of nuclear factor κ B in vascular smooth muscle cells requires signaling endosomes containing Nox1 and CIC-3. *Circ Res*. 2007;101:663–671.
2. Lassègue B, Clempus RE. Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol*. 2003;285:R277–R297.
3. Moreland J, Davis A, Bailey G, Nauseef W, Lamb F. Anion channels, including CIC-3, are required for normal neutrophil oxidative function, phagocytosis, and transendothelial migration. *J Biol Chem*. 2006;281:12277–12288.
4. Demaurex N, Petheo G. Electron and proton transport by NADPH oxidases. *Philos Trans R Soc Lond, B, Biol Sci*. 2005;360:2315–2325.
5. Murphy R, DeCoursey T. Charge compensation during the phagocyte respiratory burst. *Biochim Biophys Acta*. 2006;1757:996–1011.
6. Grabe M, Oster G. Regulation of organelle acidity. *J Gen Physiol*. 2001;117:329–344.
7. Faundez V, Hartzell H. Intracellular chloride channels: determinants of function in the endosomal pathway. *Sci STKE*. 2004;2004:re8.
8. Hara-Chikuma M, Yang B, Sonawane N, Sasaki S, Uchida S, Verkman A. CIC-3 chloride channels facilitate endosomal acidification and chloride accumulation. *J Biol Chem*. 2005;280:1241–1247.
9. Li X, Wang T, Zhao Z, Weinman S. The CIC-3 chloride channel promotes acidification of lysosomes in CHO-K1 and Huh-7 cells. *Am J Physiol Cell Physiol*. 2002;282:C1483–C1491.
10. Guan Y, Wang G, Zhou J. The CIC-3 Cl⁻ channel in cell volume regulation, proliferation and apoptosis in vascular smooth muscle cells. *Trends Pharmacol Sci*. 2006;27:290–296.
11. Picollo A, Pusch M. Chloride/proton antiporter activity of mammalian CLC proteins CIC-4 and CIC-5. *Nature*. 2005;436:420–423.
12. Scheel O, Zdebek A, Lourdel S, Jentsch T. Voltage-dependent electrogenic chloride/proton exchange by endosomal CLC proteins. *Nature*. 2005;436:424–427.
13. Jentsch T. Chloride and the endosomal-lysosomal pathway: emerging roles of CLC chloride transporters. *J Physiol (Lond)*. 2007;578:633–640.
14. Hawkins B, Madesh M, Kirkpatrick C, Fisher A. Superoxide flux in endothelial cells via the chloride channel-3 mediates intracellular signaling. *Mol Biol Cell*. 2007;18:2002–2012.

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