NOX5 as a therapeutic target in cerebral ischemic injury

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In this issue of the *JCI*, Casas et al. define a previously unknown role of the NADPH oxidase catalytic subunit NOX5 in cerebral infarction. Using a mouse expressing human NOX5 in the endothelium, the investigators show that NOX5 is activated and plays a deleterious role in promoting edema, infarction, and ultimately, worsened neurological function following cerebral ischemia. They provide evidence that this is due to the breakdown of the blood-brain barrier (BBB) and that a unique pharmacological inhibitor of NOX5, ML090, if given early, around the time of reoxygenation, can maintain BBB integrity. Future studies of NOX5 inhibition in humans, particularly in the setting of thrombolysis, are warranted.

ROS and tissue damage

The NADPH oxidases are a major source of ROS in mammalian cells. These produce superoxide, which in turn yields other ROS, such as hydrogen peroxide, peroxynitrite, and the hydroxyl radical. ROS have signaling roles that influence cell growth, apoptosis, proliferation, and migration, but when produced in excess, can contribute to tissue damage and dysfunction. The catalytic subunits of the NADPH oxidases are the NOX proteins, which transfer electrons from NADPH to flavin adenine dinucleotide (FAD), a heme prosthetic group, and ultimately to molecular oxygen. There are seven such enzymes that have varying roles and cellular distribution (1). Most of these require cytoplasmic subunits that assemble with the membrane-bound NOX protein to form a functional complex. An exception to this is NOX5, which does not require cytoplasmic components, but rather has an aminoterminal cytoplasmic tail that contains calcium binding (EF-hand) domains that permit its activation upon calcium binding (Figure 1A). Another unique aspect of NOX5 is that it is present in higher primates and some larger animals, but not in rodents, and therefore has not been extensively studied in vivo to date. In humans, NOX5 is expressed in testes, fibroblasts, endothelial cells, and immune cells (2).

NOX5 and the breakdown of the blood-brain barrier

In this issue of the *JCI*, Casas et al. provide new evidence that NOX5 promotes breakdown of the blood-brain barrier (BBB), increasing infarct size and worsening functional outcome after cerebral ischemia and reperfusion injury (3). This team made a knockin mouse harboring human NOX5 driven by the Tie2 promoter, which predominantly governs endothelial cell expression. They showed that hippocampal brain slices from this mouse produced excessive amounts of ROS following simulated ischemia and reperfusion compared with brains of WT mice. They further observed that transient middle cerebral artery occlusion in the NOX5-expressing mice produced more brain edema and larger cerebral infarcts compared with that in the WT mice, ultimately leading to worsened neurological function. The investigators also found that hypoxia followed by reoxygenation enhanced permeability of human brain microvascular endothelial cells and that this was prevented by pharmacological inhibition of NOX5 at early time points and inhibition of NOX4 at later time points. Taking these data together, the authors suggest that NOX5 is a heretofore unknown mediator of BBB breakdown following stroke and that inhibition of NOX5 and possibly NOX4 might be therapeutically useful in treatment of stroke, particularly upon reperfusion.

The BBB is composed of specialized endothelial cells with uniquely tight paracellular junctions and low permeability. These properties of the BBB are critical for maintaining brain homeostasis and are perturbed in response to numerous insults, including stroke, traumatic brain injury, infection, epilepsy, and hypoxia. Loss of BBB integrity can promote neuroinflammation and, over the long term, neurodegeneration (4). Indeed, dysfunction of the BBB has been associated with myriad conditions, including Parkinson's disease, Huntington's disease, and Alzheimer's disease, and has been implicated in the progressive decline in brain function that follows stroke and traumatic brain injury (5). Given that NOX5 was predominantly expressed in endothelial cells in these animals, Casas et al. made a case for the breakdown of the BBB as being a major consequence of ROS formation upon reperfusion. This loss of BBB integrity can lead to leakage of plasma proteins, invasion of immune cells, activation of microglial cells, and increased local cytokine production (Figure 1B), which in turn could contribute to the increased cerebral infarct size in the NOX5-expressing mice. Other factors, including changes in vasomotor tone caused by oxidative inactivation of nitric oxide, enhanced platelet adhesion, and increased immune cell transmigration related to endothelial dysfunction caused by the excessive ROS, could contribute to the increase in cerebral infarct size in these animals. Further

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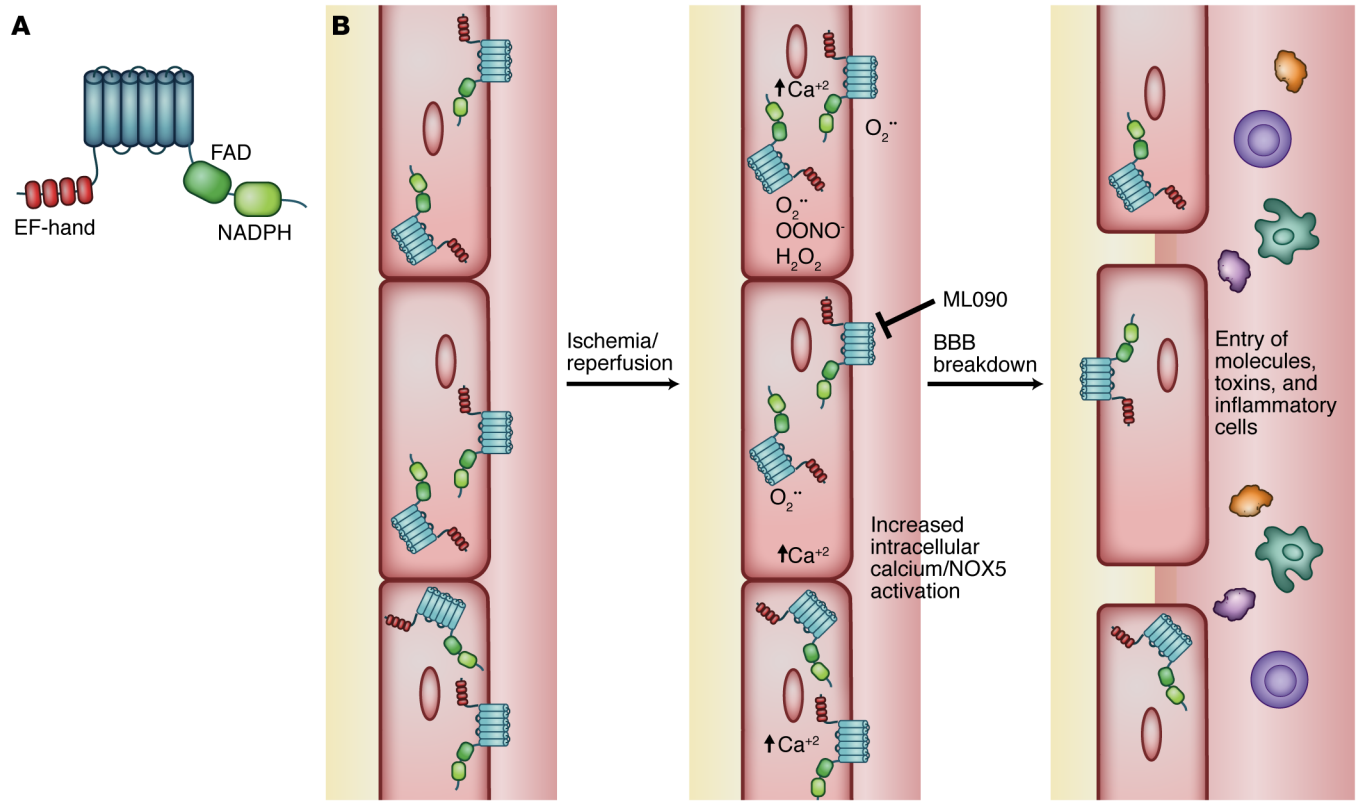

Figure 1. The role of NOX5 in BBB breakdown. (**A**) Schematic of the NOX5 protein containing calcium-binding (EF-hand) and NADPH- and FAD-binding sites. (**B**) Under normal conditions, the BBB has tight paracellular junctions and low permeability. Upon ischemia/reperfusion, intracellular calcium increases, leading to the activation of NOX5 and production of ROS including superoxide ($O_2^{\cdot-}$), peroxynitrite ($OONO^-$), and hydrogen peroxide (H_2O_2). These promote loss of BBB integrity, leading to the entry of injurious macromolecules, inflammatory cells, and other toxins that cause progressive brain injury. Illustration by Rachel Davidowitz.

studies would be useful for investigating these additional mechanisms.

Inhibiting NOX4 and NOX5

An interesting aspect of the Casas et al. study was the use of promising drugs that selectively inhibit NOX4 and NOX5. In their studies of brain microvascular endothelial cells, the authors found that the NOX5 selective agent ML090 reduced endothelial cell permeability when given immediately before and at the time of reoxygenation. Addition of this drug 20 minutes later failed to have an effect. This scenario is compatible with the immediate calcium influx known to occur in reperfusion and reoxygenation injury. An accepted mechanism that has been well characterized in the myocardium is that oxygen deprivation leads to accumulation of intracellular hydrogen ions, which are rapidly exchanged for sodium via the sodium hydrogen exchanger, which in turn leads to increased intracellular calcium via reverse action of the sodium calcium exchanger (6). Agents that interfere with this pathway may also help combat ischemic stroke (7). If it is true that NOX5 plays a predominant role very early in reperfusion, the data from Casas et al. show that it has sustained effects on infarct size and neuromotor function 24 hours later. Studies of neurological function at even later times would be useful, but this sustained effect is compatible with the notion that BBB breakdown early after reperfusion can have long-term consequences on brain function and viability.

Casas et al. also performed rather extensive studies to define the role of NOX5 in experimental models of myocardial infarction and limb ischemia. In striking contrast to the authors' findings in brain ischemia, the NOX5-expressing mice did not exhibit exacerbated responses to ischemic insults in cardiac or limb tissues. Given that NOX5 expression was present in all endothelial cells, these findings are somewhat surprising, but might reflect the specialized role of the BBB in maintaining brain integrity and its heightened sensitivity to oxidant injury. It is also possible that the NOX5-overexpressing mice have compensatory alterations in other ROS-producing enzymes or perhaps induction of antioxidant proteins, such as the superoxide dismutases in these peripheral tissues.

The findings of Casas et al. should prompt substantial further investigation. Additional studies should seek to determine the targets of the produced by NOX5. Proteomic studies of oxidative modified proteins, including those having undergone nitrosylation, nitration, and modification by lipid electrophiles, would be useful in this regard. An analysis of changes in gene expression in the brain and the BBB would also be informative. ROS are also known to activate matrix metalloproteinases (8), which in turn could degrade the BBB and promote tissue damage. Thus, studies of how various matrix metalloproteinases are altered by NOX5 activation would be of interest.

Pointing toward the lymphatic and the glymphatic systems

While the present study focused on the effects of NOX5 on the BBB, two related pathways in the brain should also be considered. First, like almost all organs, the brain has a lymphatic system, composed of endothelial cells that likely also express NOX5 (9). Second, the brain uniquely has a related physiologic pathway that is involved in the response to ischemia and reperfusion called the glymphatic system, composed of perivascular spaces, glial cells, and lymphatic vessels (10). Because this system has a major role in clearing damaged proteins, it is likely to be activated after stroke. Studies of how NOX5 affects lymphatic drainage and the glymphatic system are warranted.

It is interesting to note that antioxidants have been studied for treatment of ischemia/reperfusion injury in the heart and brain for decades with limited success. The failure of multiple trials of oral antioxidants might be due to the relatively slow rate constants for their reaction with various ROS, lack of their targeting to important subcellular structures, or unwanted scavenging of ROS that have important physiological roles. These considerations suggest that drugs that stop the production of ROS, rather than attempting to scavenge them after they are formed, might be highly preferable. The use therefore of NOX5 and possibly NOX4 inhibitors in clinical situations such as stroke and thrombolytic therapy is worthy of clinical study.

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