REVIEW

Nicotinamide adenine dinucleotide phosphate oxidase activation and neuronal death after ischemic stroke

Jiamei Shen[1, 2], Radhika Rastogi[2], Xiaokun Geng[1, 2, 3, *], Yuchuan Ding[2, *]

1 China-America Institute of Neuroscience, Beijing Luhe Hospital, Capital Medical University, Beijing, China

2 Department of Neurosurgery, Wayne State University School of Medicine, Detroit, MI, USA

3 Department of Neurology, Beijing Luhe Hospital, Capital Medical University, Beijing, China

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Abstract

Nicotinamide adenine dinucleotide phosphate oxidase (NOX) is a multisubunit enzyme complex that utilizes nicotinamide adenine dinucleotide phosphate to produce superoxide anions and other reactive oxygen species. Under normal circumstances, reactive oxygen species mediate a number of important cellular functions, including the facilitation of adaptive immunity. In pathogenic circumstances, however, excess reactive oxygen species generated by NOX promotes apoptotic cell death. In ischemic stroke, in particular, it has been shown that both NOX activation and derangements in glucose metabolism result in increased apoptosis. Moreover, recent studies have established that glucose, as a NOX substrate, plays a vital role in the pathogenesis of reperfusion injury. Thus, NOX inhibition has the potential to mitigate the deleterious impact of hyperglycemia on stroke. In this paper, we provide an overview of this research, coupled with a discussion of its implications for the development of NOX inhibition as a strategy for the treatment of ischemic stroke. Both inhibition using apocynin, as well as the prospect of developing more specific inhibitors based on what is now understood of the biology of NOX assembly and activation, will be highlighted in the course of our discussion.

Key Words: *nicotinamide adenine dinucleotide phosphate oxidase; stroke; nicotinamide adenine dinucleotide phosphate oxidase inhibitors; reactive oxygen species; ischemia/reperfusion; neuroprotection; hyperglycolysis; NADPH; NOX*

***Correspondence to:**
Xiaokun Geng, MD, xgeng@ccmu.edu.cn;
Yuchuan Ding, MD, PhD, yding@med.wayne.edu.

orcid:
0000-0001-5358-1660 (Yuchuan Ding)

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Introduction

Ischemic stroke remains the second leading cause of death worldwide, despite significant efforts to better understand its biology and to intervene in its pathogenesis (Chandra et al., 2017; Li et al., 2017). Even so, mortality alone hardly accounts for the total burden imposed by this disease. Leaving survivors with profound neurological deficits, stroke is the leading cause of major disability worldwide. Moreover, the acute therapies currently available are limited to recannualization procedures (mechanical or pharmacological) that are too time-sensitive to be a viable option for most patients (Ji, 2015; Kim et al., 2017). It is therefore crucial that research aimed at elucidating the mechanisms of ischemia-mediated damage continues to be pursued vigorously: successful interventions in these pathways possess nearly unparalleled potential to alleviate the suffering of patients.

One mechanism that has been the focus of recent attention is the cerebral hyperglycolysis induced by ischemia (Schurr, 2002) and thought to potentiate stroke-induced brain damage (Kochanski et al., 2013). Following ischemia/reperfusion, the brain significantly upregulates its glycolytic rate, its expression of glucose transporters 1 and 3 (Shen et al., 2016), and lactate levels, resulting in lactic acidosis (Schurr, 2002). The increased glucose is consumed *via* anaerobic respiration and further shunted through the hexose monophosphate pathway, increasing nicotinamide adenine dinucleotide phosphate (NADPH) levels and activating NADPH oxidase (NOX), as seen by increases in NOX activity, NOX subunit expression (p47-phox, p67-phox, and gp91-phox), and cell death found after ischemia/reperfusion (Tang et al., 2012; Yao et al., 2017).

NOX itself is a family of essential enzyme complexes expressed in many different tissues throughout the body. NOX is best-known for its involvement in the antimicrobial respiratory burst by which free radical production occurs in the cells involved in innate immunity (Carbone et al., 2015). Upon activation *via* assembly of its multiple subunits, NOX uses NADPH to catalyze the reduction of molecular oxygen to the superoxide anion ($O_2^{\bullet-}$). This production of reactive oxygen species (ROS) has been increasingly recognized as an important component of various cellular events, including bio-signaling and apoptotic regulation (Sumimoto et al., 2005; D'Autréaux and Toledano, 2007). In addition to its normal physiologic functions, NOX is intimately involved in the pathways leading to brain damage caused by ischemia/reperfusion injury in stroke (Tang et al., 2012; Zhao et al., 2016). Because of this participation in ischemia/reperfusion pathophysiology and its pervasive expression, NOX has emerged as an attractive therapeutic target. In particular, inhibition of NOX may prove to be a promising treatment for ischemic stroke.

NOX Subcellular Location, Structure and Subunit Activation

The NOX complex contains a membrane-bound component, as well as a cytosolic component. At rest, the catalytic center of NOX is comprised of the two tightly complexed membrane-integrated flavocytochromes, gp91-phox and p22-phox. In the cytosol, the cytosolic components contain p47-phox, p67-phox, and p40-phox and the small GTPase Rac1/Rac2; p40-phox and p67-phox are often complexed prior to activation (Yu et al., 1998; Sumimoto et al., 2005; Carbone et al., 2015). During NOX activation, phosphorylation unmasks a binding region on p47-phox, allowing it to definitively bind p67-phox to form a trimeric cytosolic complex (Tsunawaki and Yoshikawa, 2000; Lapouge et al., 2002). Subsequently, p47-phox mediates translocation of the cytosolic complex to the membrane, where it binds principally to p22-phox, resulting in assembly of the active NOX complex and activation of gp91-phox, the catalytic subunit (Ago et al., 2003). As the catalytic core, gp91-phox levels are measured as a surrogate for the extent of NOX complex formation. The gp91-phox NOX protein family is comprised of membrane-spanning structures with NADPH- (or NADH-) binding domains, using NADPH as electron donors for molecular oxygen to produce the superoxide anion ($O_2^{\bullet-}$, a precursor for other reactive oxygen species) (Yu et al., 1998; Cairns et al., 2012). Thus, NOX requires glucose metabolism to provide the NADPH necessary for NOX complex formation and function (Suh et al., 2008; Tang et al., 2012).

All of the major NOX subunits (p22-phox, p47-phox, p67-phox and gp91-phox) have been found in the brain (Bedard and Krause, 2007; Montezano and Touyz, 2012; Tang et al., 2012), in which, upon phosphorylation following ischemia, the active complex is assembled as described above (Bokoch and Knaus, 2003). Thus, upregulation of these subunits has been found to correlate with increased NOX activity (Takeya et al., 2003). The multiplicity of steps in this complex activation process provides the opportunity for specific modulation prior to and during activation of NOX (Groemping and Rittinger, 2005; Sumimoto et al., 2005) .

Another aspect of the NOX family is its plentiful isoforms, comprised of NOX 1–5, dual oxidase (DUOX) 1 and 2, with slight variations in its subunits. In NOX2, the gp91-phox isoform is present (Tang et al., 2012). Of these isoforms, NOX2 and NOX4 are the most involved in ischemia/reperfusion injury (Zhang et al., 2015; Lou et al., 2018). NOX2, mostly present in microglia and circulating immune cells, dominates in inflammatory driven conditions such as reperfusion and is upregulated in ischemic stroke with concurrent increases in microglial activation (Tang et al., 2012). NOX 4, usually present at very low physiologic levels in the brain, is upregulated in ischemia/reperfusion pathologies to further contribute to acute oxidative damage in the reperfusion period (Yao et al., 2017; Lou et al., 2018). To illustrate, a NOX4 knockout model has demonstrated attenuated oxidative stress, blood-brain barrier disruption, neuronal death, and mortality (Kleinschnitz et al., 2010). Isoform specificity yields another target to which therapies can be tailored to enhance treatment specificity.

NOX-Mediated Pathogenesis

Glucose metabolism

Ischemia is well-known to induce cerebral hyperglycolysis. Moreover, hyperglycemia during stroke worsens outcomes, independent of diabetes or pre-ischemic blood glucose concentrations (Ribo et al., 2005; Li et al., 2017). Several studies have findings that identify glucose as the requisite electron donor for reperfusion-induced neuronal superoxide production and establish a previously unrecognized mechanism by which hyperglycemia can exacerbate ischemic brain injury (Takeya et al., 2003; Yip et al., 2016): the glucose excess generated by ischemia is shunted into the hexose monophosphate pathway (**Figure 1**), where it reduces $NADP^+$ to NADPH (Suh et al., 2008). This newly formed NADPH is then itself utilized as an electron donor, in this case by NOX to generate the ROS (Kruyt et al., 2010) that damage the brain during post-ischemic hyperglycolysis (Kochanski et al., 2013). For instance, post-ischemic superoxide production and neuronal death were reversed in neuronal cultures when glucose was absent, NOX was inactivated or the hexose monophosphate shunt producing NADPH from glucose was inhibited within the culture (Takeya et al., 2003). Analogously, neuronal superoxide production and death were reduced by the glucose antimetabolite 2-deoxyglucose and exacerbated by increased blood glucose concentrations in a murine stroke model. Inactivating NADPH oxidase, either by use of apocynin or by deletion of the p47-phox subunit, was found to block neuronal superoxide production and to negate the detrimental effects of hyperglycemia (Suh et al., 2008). Additionally, Ding et al. found increased NOX activation, NOX subunit levels, and resulting apoptotic cell death in rat models with post-ischemic hyperglycolysis that was exacerbated by early rehabilitation (Shen et al., 2016; Tang et al., 2018). Thus, hyperglycemia provides the necessary substrates *via* the hexose monophosphate shunt to facilitate NOX-induced ROS production.

Hyperglycemia also potentiates NOX activity through a more direct mechanism – through pathological cellular signaling. Excess glucose has been found to activate protein kinase C and thus downstream, activate NOX *via* phosphorylation and facilitate ROS production to further worsen outcomes in ischemic stroke. Hyperglycemia-amplified protein kinase C expression increased NOX-induced ROS production in an *in vitro* simulation of the blood-brain barrier. The resulting increases in p47-phox phosphorylation and NOX activation caused blood-brain barrier breakdown and apoptosis. Such effects were mitigated by inhibiting protein kinase C expression, with subsequent decreases in p47-phox phosphorylation and NOX activation (Kleinschnitz et al., 2010; Shao and Bayraktutan, 2014). Thus excess glucose contributes twofold to NOX activation.

NOX activation and reactive oxygen species-mediated cell death

Within the brain, NOX has been identified as an important generator of ROS both in physiologic and pathologic conditions, particularly stroke (Kahles and Brandes, 2012).

Stroke-induced elevations in NOX expression, activity, and ROS-mediated damage have been widely observed following ischemia (Tang et al., 2012; Kahles and Brandes, 2013; Kochanski et al., 2013). Ischemia itself makes the brain particularly vulnerable to ROS damage. Upon cessation of blood flow, mitochondria in affected brain regions incur injury and cannot detoxify the influx of oxygen that results from reperfusion (Tang et al., 2014b). During reperfusion, the concurrent influx of excess glucose is increasingly shunted into the hexose monophosphate pathway, whereby glucose-6-phosphate is used to generate increased NADPH (**Figure 1**) (Brennan-Minnella et al., 2015; Thibodeau A et al., 2016). Thus, because plentiful substrates are present due to post-ischemic hyperglycemia, increased NOX activation produces ROS in pathogenic quantity. These ROS then, through lipid peroxidation, directly damage cells by compromising membrane integrity and precipitating damage to organelles (Rastogi et al., 2016).

To prevent ROS overproduction, NOX activity is regulated at the level of its activation *via* phosphorylation. Phosphorylation occurs due to stimuli such as phagocytosed particles or cellular stresses, including cerebral ischemia (Jiang et al., 2011; Kim et al., 2017). Though the pathways for activation have not been fully elucidated, multiple independent pathways are involved in physiologic and pathologic NOX phosphorylation and action. In ischemia, many studies have implicated the protein kinase C isoforms, particularly *via* the 5′-monophosphate-activated protein kinase/phosphorylated protein kinase B/protein kinase C pathway, as well as several other potential upstream factors such as transforming growth factor-β and myosin light chain kinase (Zhang et al., 2015; Cai et al., 2017; Lou et al., 2018). Multiple experiments have illustrated NOX activation in the brain, such as that of Zheng et al. (2014), in which oxygen-glucose deprivation of brain slices yielded upregulation of NOX and concurrent increased production of ROS. In another study, inhibition of NOX by casein kinase 2 led to neuronal survival. Conversely, activation of an NADPH oxidase subunit resulted in increased oxidative stress that caused the release of apoptogenic factors from mitochondria, damaged DNA, and was associated with neuronal death after ischemia and reperfusion (Kim et al., 2012).

Once generated by NOX, ROS mediate cellular damage in a variety of ways: superoxide may cause oxidative damage on its own; it can generate more radical species with its unpaired electron; it may produce peroxynitrite with nitric oxide, which can scavenge and prevent the function of nitric oxide, can induce further damage by nitration in most biomolecules, and is considered an important cause of post-stroke apoptosis (Broughton et al., 2009); alternatively, it may be enzymatically dismutated to hydrogen peroxide and oxygen, the former of which is a membrane-permeable oxidizer of cellular macromolecules and can generate DNA-damaging hydroxyl radicals in the presence of transition metals. The resulting ROS can cause direct damage to DNA and pervasive lipid peroxidation pertaining cellular membranes. Moreover, ROS sources may also damage cellular signaling cascades, causing apoptosis (Kim et al., 2016; Ma et al., 2017). Dysregulated NOX production of ROS may disrupt the signaling cascades in which NOX can be intimately involved. Cells damaged in these ways may die either of apoptosis or necrosis, depending on several factors including cell type, stage in development, and type of stimulus (*i.e.*, severity of ischemic injury) (Dirnagl et al., 1999). One mechanism of apoptosis thought to be activated by NOX-generated radicals involves apoptosis-inducing factor. Upon activation by oxidative stress, poly (ADP-ribose) polymerase-1, a DNA-repairing enzyme that acts to mitigate radical damage, generates poly (ADP-ribose), which is sensed as a death signal by neurons. Poly (ADP-ribose) binds apoptosis-inducing factor, triggering its liberation from the mitochondrial outer membrane; in general, pro-apoptotic molecules are released following oxidative augmentation of mitochondrial membrane permeability, and these converge to activate caspase-3, causing apoptosis (Kim et al., 2012) through destruction of nuclear DNA repair enzymes (Khoshnam et al., 2017).

Numerous factors influencing stroke outcomes have proven to modulate NOX activity, the most recent of which is glucose itself. These factors' newly identified function as NOX regulators suggest novel mechanisms for their effects on ischemic brain injury (Brennan-Minnella et al., 2015). It has been found, for instance, that stroke-induced brain injury in rats leads to activation of NADPH oxidase through a signaling pathway implicating adenosine 5′-monophosphate-activated protein kinase/phosphorylated protein kinase B/protein kinase C (**Figure 1**) (Tang et al., 2014a; Cai et al., 2017; Geng et al., 2017). In another study, infarct volume was attenuated in mice treated with a NOX inhibitor; this inhibition was associated with significant improvement in neurological function, as well as attenuation of neuronal apoptosis and expression of Bax (Bcl-2-associated X protein)/Bcl-2(B-cell lymphoma 2), cytochrome C and cleaved caspase 3 (Song et al., 2013). Finally, in a study that investigated its role in ischemia-reperfusion injury to the peri-infarct region, NOX was implicated in ROS-mediated damage to DNA, suggesting its involvement in peri-infarct neurodegeneration and inhibition of neurogenesis in this region; NOX knockdown was found to facilitate survival and development of neuronal progenitor cells and to improve functional recovery after stroke (Choi et al., 2015).

Therapeutic NOX Inhibition on Cell Death

Reduced NOX activity can protect cells from the oxidative stress and consequent cell death that otherwise occurs after ischemia. Excess ROS creates oxidative stress known to exacerbate ischemic damage, particularly through reperfusion. Antioxidant-focused therapies targeting ROS production and activity have demonstrated improved experimental outcomes. Neuronal superoxide production, of which NOX is the major source, contributes to cell death during both glutamate excitotoxicity and stroke; regulation of NOX activity can thus alter stroke outcomes (Brennan-Minnella et al., 2015). To illustrate this, the increased blood-brain barrier

permeability, infarct size, and hemorrhage with tissue-type plasminogen activator-induced reperfusion in a hyperglycemic rat model was prevented with apocynin-mediated NOX inhibition (Won et al., 2011). Apocynin is a nonspecific NOX inhibitor that has been shown in rat models of stroke to prevent NOX activation and to reduce cytosolic ROS production (Kleinschnitz et al., 2010). *In vitro* and *in vivo* models with both the nonspecific NOX inhibitor diphenyleneiodonium and NOX-siRNA have also been shown to attenuate cytosolic ROS levels (Carbone et al., 2015). Genetic deletion of NOX was found to significantly reduce disruption of the blood-brain barrier and infarct size after ischemia/reperfusion in mice (Kahles et al., 2007; Zhang et al., 2016). Similarly, after ischemia and reperfusion, NOX knockout mice demonstrated reduced infarct size and reduced post-infarct inflammation, implicating NOX in both inflammation and infarct progression in stroke (Chen et al., 2011). It has been demonstrated in a thromboembolic stroke model that other known neuroprotective modalities may exert their effect by NOX inhibition: after reperfusion by recombinant tissue plasminogen activator, combining normobaric oxygen with either hypothermia or ethanol conferred neuroprotection through modulation of NOX activation.

The recognition of oxidative stress as a pathological mechanism of post-stroke neurodegeneration, as well as the inhibition of NOX investigated as a consequence of this recognition, has been considered a conceptual breakthrough in stroke therapy (Radermacher et al., 2013). The advantages of a targeted central nervous system NOX inhibitor that would inhibit the production of superoxide by non-phagocytic cells are evident (Cairns et al., 2012; Kim et al., 2017), as both NOX inhibition and genetic deletion of certain NOX isoforms have sizable and demonstrable improvements in experimental stroke outcomes (Kahles and Brandes, 2012). Unfortunately, no such inhibitors have yet demonstrated clinical viability. The currently studied inhibitors possess insufficient safety and specificity for development in humans, with limited ability to target specific tissues or specific NOX isoforms and with many off-target effects. For instance, diphenyleneiodonium, in addition to impairing NOX-associated flavoprotein-mediated superoxide generation, also inhibits other metabolically important flavoenzymes (Kim et al., 2017). Another example is afforded by preclinical studies of the NOX inhibitor apocynin, which appears to inhibit superoxide generation by preventing p47-phox translocation to the membrane-bound NOX components: dose-dependent efficacy data for stroke is variable and suggests a narrow therapeutic window, outside of which there is risk of brain hemorrhage exacerbation (Kim et al., 2017). Other limitations of apocynin are that it acts on NOX1 and 2 more than NOX4 isoform and requires myeloperoxidase to become active. As a result, apocynin can only act on NOX in leukocytes, and in other locations such as vascular cells or smooth muscle cells, it acts as an antioxidant rather than an inhibitor (Heumüller et al., 2008; Kim et al., 2017). Therefore, its functionality and specificity must be determined more clearly before it can be translated clinically. VAS2870, a small molecule inhibitor of NOX isoforms that reduced lesion size and neurological deficits in a stroke model, provides one final example: in addition to inhibiting NOX, this molecule has been found to exert off-target thiol alkylation that may replicate ROS in redox sequences (Sun et al., 2012; Kim et al., 2017). However, with current studies elucidating both mechanisms of NOX action and the specific NOX isoforms involved, particularly NOX 2 and 4 in stroke, a wide range of potential specific targets for therapy are becoming apparent. Recent research using known inhibitors has substantially increased our knowledge of the role of NOX in central nervous system diseases, and it is this knowledge that will provide a framework for the development of specific, potent, and safe NOX inhibitors for clinical use in the future.

Conclusion

By generating ROS levels in excess of what the body is capable of handling through endogenous antioxidant defense mechanisms, NOX is a principal contributor to oxidative stress. Such disproportionate increases in ROS lead to increases in lipid peroxidation, membrane degeneration, matrix metalloprotease production, DNA damage, the propagation of dysfunctional and damaging signaling cascades, and, ultimately, cell death (Tang et al., 2012; Qin et al., 2017). Moreover, as the primary generator of ROS in the central nervous system, NOX is inextricably involved in the perpetuation of cellular damage in the wake of ischemic stroke. Accordingly, interventions aimed at downregulating the excessive, pathogenic function of this enzyme complex possess considerable promise in the effort to mitigate post-stroke damage, and ultimately to improve quality of life for afflicted patients.

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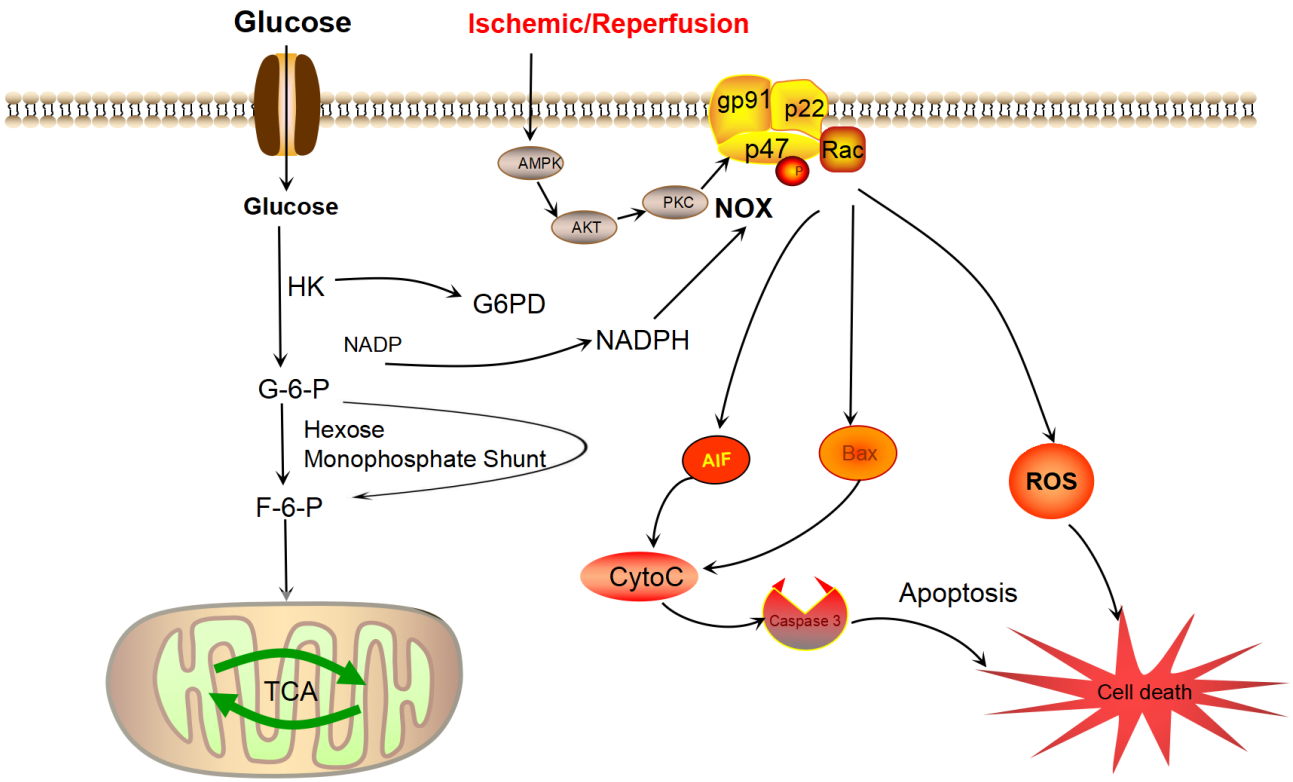

Figure 1 The nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase (NOX) complex and its subunits are shown with its activation sequence and selected downstream effects.

In the NOX complex, two membrane subunits (gp91-phox, or its homologs, and p22-phox) comprise the catalytic core of NOX, while several cytosolic subunits (p47-phox, p67-phox, p40-phox) and the G-protein Rac are all essential components for assembly and activation. Stroke induces adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK)/phosphorylated protein kinase B (Akt)/protein kinase C (PKC) activation, which can activate NOX *via* phosphorylation. When activated, NOX produces superoxide ion by transferring an electron from NADPH to molecular oxygen. NADPH is derived from glucose, which enters the cell and is converted to glucose-6-phosphate (G-6-P) by glycolytic kinases. The G-6-P is then shunted through the hexose monophosphate shunt to produce NADPH by donating an electron to $NADP^+$. The NADPH thus produced, along with molecular oxygen, acts as a substrate for NOX to produce reactive oxygen species (ROS). In disease states, ROS overproduction results in cell death, both directly and indirectly. In such cases, NOX can induce the release of apoptosis-inducing factor (AIF), cytochrome C and Bcl-2-associated X protein (BAX), resulting in the activation of caspase 3-mediated apoptotic cell death. TCA: Tricarboxylic acid cycle; F-6-P: fructose-6-phosphate; G6PD: glucose-6-phosphate 1-dehydrogenase; HK: hexokinase.

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