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REVIEW ARTICLE

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Molecular insights of NADPH oxidases and its pathological consequences

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Reactive oxygen species (ROS), formed by the partial reduction of oxygen, were for a long time considered to be a byproduct of cellular metabolism. Since, increase in cellular levels of ROS results in oxidative stress leading to damage of nucleic acids, proteins, and lipids resulting in numerous pathological conditions; ROS was considered a bane for aerobic species. Hence, the discovery of NADPH oxidases (NOX), an enzyme family that specifically generates ROS as its prime product came as a surprise to redox biologists. NOX family proteins participate in various cellular functions including cell proliferation and differentiation, regulation of genes and protein expression, apoptosis, and host defence immunological response. Balanced expression and activation of NOX with subsequent production of ROS are critically important to regulate various genes and proteins to maintain homeostasis of the cell. However, dysregulation of NOX activation leading to enhanced ROS levels is associated with various pathophysiologies including diabetes, cardiovascular diseases, neurodegenerative diseases, ageing, atherosclerosis, and cancer. Although our current knowledge on NOX signifies its importance in the normal functioning of various cellular pathways; yet the choice of ROS producing enzymes which can tip the scale from homeostasis toward damage, as mediators of biological functions remain an oddity. Though the role of NOX in maintaining normal cellular functions is now deemed essential, yet its dysregulation leading to catastrophic events cannot be denied. Hence, this review focuses on the involvement of NOX enzymes in various pathological conditions imploring them as possible targets for therapies.

Significance of the study: The NOXs are multi-subunit enzymes that generate ROS as a prime product. NOX generated ROS are usually regulated by various molecular factors and play a vital role in different physiological processes. The dysregulation of NOX activity is associated with pathological consequences. Recently, the dynamic proximity of NOX enzymes with different molecular signatures of pathologies has been studied extensively. It is essential to identify the precise role of NOX machinery in its niche during the progression of pathology. Although inhibition of NOX could be a promising approach for therapeutic interventions, it is critical to expand the current

Abbreviations: AP-1, activator protein-1; DUOX, dual oxidase; ERK 1/2, extracellular signal-regulated kinase 1/2; FAD, flavin adenine dinucleotide; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NADPH, nicotinamide-adenine dinucleotide phosphate; NF-κB, nuclear factor kappa B; NOX, NADPH oxidase; PKC, protein kinase C; ROS, reactive oxygen species; STAT, signal transducer and activator of transcription; TNF-α, tumour necrosis factor alpha.

understanding of NOX's dynamicity and shed light on their molecular partners and regulators.

KEYWORDS
Cancer, Inflammation, NADPH oxidase, Oxidative stress, Reactive oxygen species

1 | INTRODUCTION

The process of natural selection within a cell provides us with the most energy-efficient pathways for cellular processes and the proficiency of these myriad processes mystifies the biologists even today. Each new discovery solves a part of this mega puzzle but leaves a long list of new questions in its wake. The discovery of NADPH oxidases had brought about a revolution that challenged the existing theories on cellular ROS and the subsequent years saw an explosion in the field of redox biology. The structural and functional breakthroughs have now established that NOX is a family of transmembrane proteins, which generates ROS as a prime product and not a byproduct.[1] NOXs are multi-subunit enzymes, comprising membrane subunits and cytosolic subunits. Under the resting condition, the cytosolic components remain dispersed in the cytosol, but upon activation, these cytosolic components transduce and assemble with transmembrane subunits to form an active enzyme component.[2] The activated NOX enzyme catalyses the reaction and transfers a single electron from NADPH to FAD, to the first and second heme and subsequently to molecular oxygen, resulting in the generation of free oxygen radicals, which is further dismutated to H_2O_2 in the cells.[1] This H_2O_2 radical reacts with a wide variety of targets and regulates many signalling events and cellular functions.[3] The expression of NOX has been found in various cell types and tissues and is essential for maintaining their normal physiology and activity. Dysregulation in expression and activation of NOX is related to oxidative stress and altered cellular responses which may leads to various pathological consequences including cardiovascular diseases, cancer, inflammation diabetes, and neurodegenerative diseases.[4] The massive impact created by NOX and ROS when they deviate from their normal path makes their study a necessity for cell biologists.

Different isoforms of the NOX enzyme and their tissue distribution have been related to their functions in maintaining redox homeostasis.[5] The first member of the NOX family enzymes was discovered from the plasma membrane of phagocytes, gp91phox or NOX-2, which has a pivotal role in eradicating the infected microorganism by the generation of ROS.[6] Later on, different isoforms were identified from non-phagocytic cells, but their specific cellular functions remain elusive.

Structurally, NOX family proteins comprise of seven members including five NOX members (NOX-1, NOX-2, NOX-3, NOX-4, and NOX-5) and two dual oxidase (DUOX) members (DUOX-1 and DUOX-2) as shown in Figure 1. All these isoforms share structural and functional resemblance and are widely expressed in a variety of tissues.[7,8] The NOX-1-4 enzymes consist of two transmembrane subunits with a NOX subunit and an active site containing p22 subunit. Upon stimulation, various cytosolic components form a complex with these transmembrane subunits and transduce the biochemical functions. NOX-1 and NOX-3 have NOXO1 (Nox organiser 1), NOXA1 (NOX activator 1) and Rac1/2 cytosolic components and NOX-2 has $p67^{phox}$, $p47^{phox}$, $p40^{phox}$, and Rac1/2.[9] NOX-4 does not require any cytosolic component for activation, whereas NOX-5 consists of a single transmembrane subunit and a cytosolic calcium-binding domain. The DUOX-1/2 consist of three transmembrane subunits, which include DUOX-1/2, DUOXA1/2 and has a peroxidase-like domain subunit. The DUOX-1/2 contains a cytosolic calcium-binding domain.[8] All the NOX enzyme systems contain C-terminal flavin adenine dinucleotide (FAD) and NADPH binding domains. Based on the predicted topology, the structures of different isoforms of NOX and their domain organisations have been shown in Figures 2 & 3.

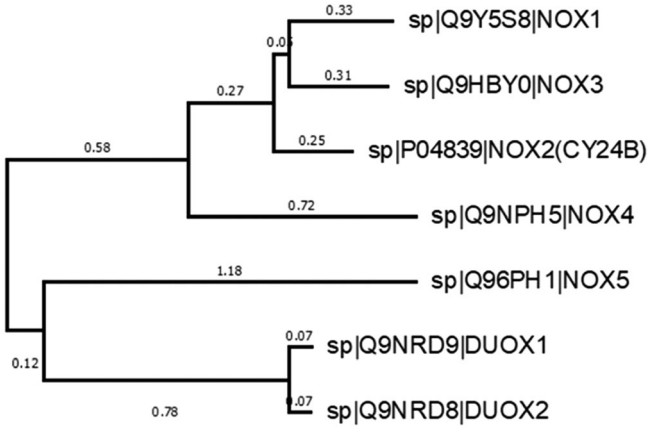

FIGURE 1 Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model.[10] The tree with the highest log likelihood (−7222.8979) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pair wise distances estimated using a JTT model, and then selecting the topology with superior log-likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved 7 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 513 positions in the final dataset. Evolutionary analyses were conducted in MEGA7[11]

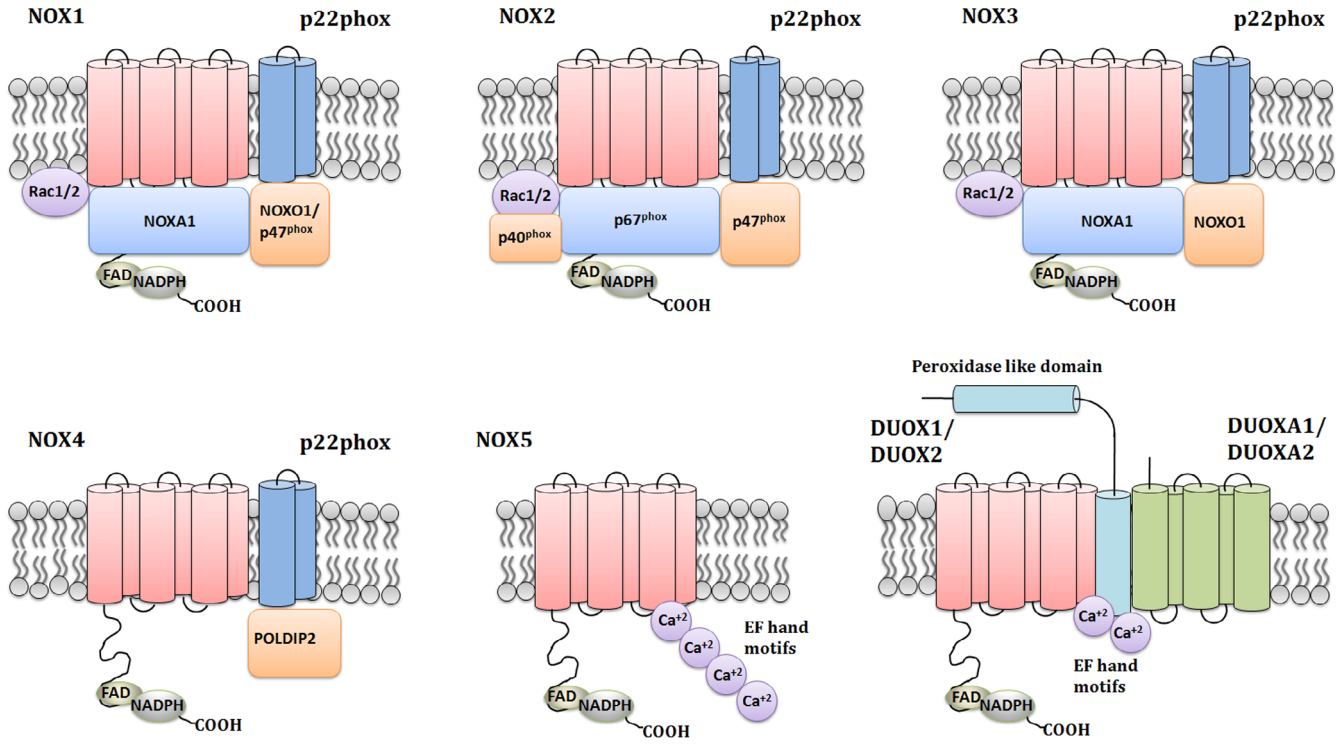

FIGURE 2 2The structural illustration of mammalian NADPH oxidase family enzymes. The catalytic site of the NOX enzymes, NOX-1-4 comprises NOX subunit (shown in pink) and $p22^{phox}$ (shown in blue); NOX-5 comprises only NOX subunit (shown in pink). DUOX-1and DUOX-2 comprises DUOX-1/2 subunits (shown in pink), DUOXA1/2 subunits (shown in green) and amino-terminal transmembrane domain with the peroxidase-like domain (shown in aqua blue). The Cytosolic components include NOXA1, $p40^{phox}$, NOXO1, $p47^{phox}$, $p67^{phox}$, NOXA1, small GTPases; RAC1 and RAC2 and polymerase δ-interacting protein 2 (POLDIP2) are shown in respect to their NOX subunits. The Ca^{2+} binding EF-hand motifs are also shown with the close proximity of NOX-5, DUOX-1, and DUOX-2 oxidases. The DUOX-1 and DUOX-2 contain additional and extracellular peroxidase-like region

2 | PHYSIOLOGICAL ROLE OF NOX

A balanced oxidation-reduction (redox) system is essential for regular cellular activity, but consequences with any perturbation leading to alteration of cellular functions. The redox system comprises of various pro-oxidant and anti-oxidant systems. It is well documented that in normal physiological conditions, an adequate level of ROS is vital for cell signalling and the regulation of numerous physiological processes. The intracellular level of ROS directs the cellular signalling network and defines the fate of cells.[15] The previous report discarded the theory of ROS as a byproduct of metabolism, rather they have defined its role in various cellular functions including immune response, developmental process, cell death, and survival.[16] The major ROS types, superoxide anion ($O2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (·OH) are known to be involved in several biochemical reactions. ROS are known to activate various signal transduction pathways including the mitogen-activated protein kinases (MAPK), Extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), NF-κB, Nrf-2/Keap-1/ARE, and PI3K/Akt pathway.[17] ROS target redox-sensitive proteins and regulate immune response, cell proliferation, cell differentiation, and cell death signalling.[18-20] Various cellular anti-oxidant systems such as superoxide dismutase (SOD), glutathione reductase, glutathione peroxidase, and catalase metabolise excessive ROS and maintain the threshold level of ROS.[21] ROS also plays a decisive role in the regulation of immune cell functions including antibacterial, antiviral, antiparasitic, and antitumor responses.[22] Under certain conditions, the harmonised ROS and anti-oxidants balance gets interrupted, resulting in the perpetuation of oxidative stress.[23] Prolonged oxidative stress may cause various pathophysiological consequences including cardiovascular diseases, cancer, inflammation, diabetes, neurodegenerative disorders, metabolic dysfunction, and premature ageing.[24-26]

A growing body of evidence indicates that NOX mediated ROS generation is associated with vital cellular functions including the differentiation, proliferation, growth, invasion, migration, apoptosis, and immune response in different cell types.[27-30] NOXs are also associated with the host-pathogen immunological response.[7,31] NOX activation can be modulated by a variety of molecules including DAMP, PAMP, inflammatory cytokines, and growth factors. An elevated NOX activity may cause oxidative stress and can alter the function of many transcription factors and cellular responses. The recent evidences revealed that NOX and ROS collectively influence various vital cellular functions and its dysregulation is associated with various pathological consequences. The NOX mediated ROS generation and its association in pathophysiological conditions has been shown in Figure 4 and the predicted association scores of NOX in the occurrence of various pathologies were identified by in silico analysis using the open target tool and indicated in Table S1.[32]

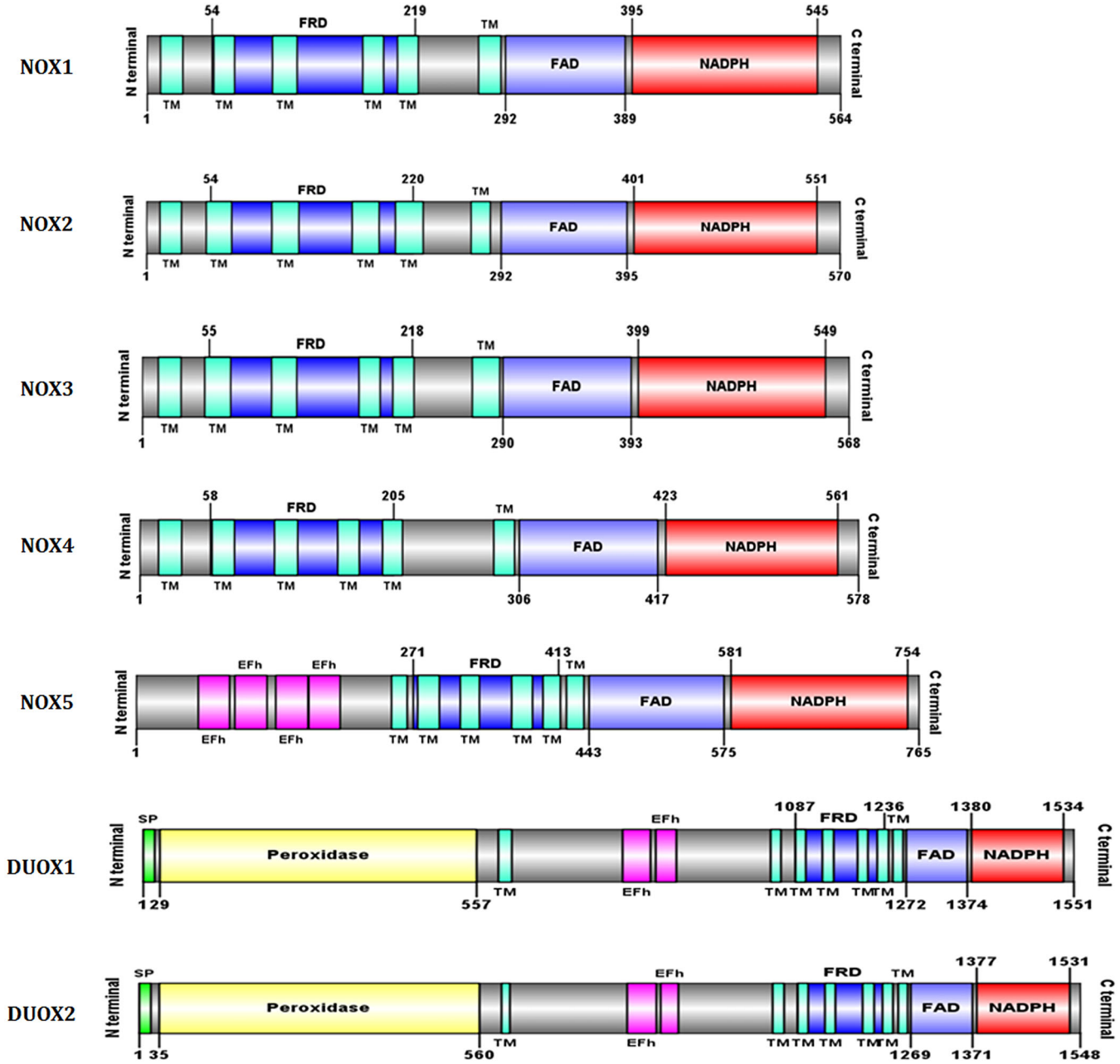

FIGURE 3 The domain organisation of mammalian NADPH oxidase family proteins. The domains were predicted with the help of Simple Modular Architecture Research Tool(SMART)[12] and PredictProtein[13] and created by Illustrator for Biological Sequences (IBS).[14] EFh, EF-hand domain (Calcium-binding domain); FAD, flavin adenine dinucleotide domain; FRD, ferric reductase domain; NADPH, nicotinamide-adenine dinucleotide phosphate domain; Peroxidase, peroxidase domain; SP, signal peptide; TM, transmembrane

3 | HUMAN PATHOLOGIES & NOX CONNECTIONS

3.1 | NOXs and cardiovascular disease

The cardiac system relies on continuous oxygen supply for the maintenance of cardiac homeostasis. Interruption in the oxygen supply leads to chronic conditions such as hypoxia and ischaemia.[33] The redox signalling plays a central role in cardiac physiology and pathologies. Elevated ROS generation and oxidative stress-induced cell damage results in structural and functional alterations in cardiac cells leading to cardiac arrest that is a major cause of mortality worldwide.[34] The NOX isoforms are abundantly expressed in cardiomyocytes, which act as a primary source of ROS and play an essential role in cardiac physiology and the progression of cardiovascular diseases.[35] Physiologically, NOX-derived ROS exhibits an essential role in angiotensin II-mediated redox signalling, cardiac mechanosensing, and endothelium-dependent relaxation[36] and maintains blood pressure homeostasis. Although, angiotensin II (Ang II) is a peptide hormone that implicates in intracellular signalling of cardiac

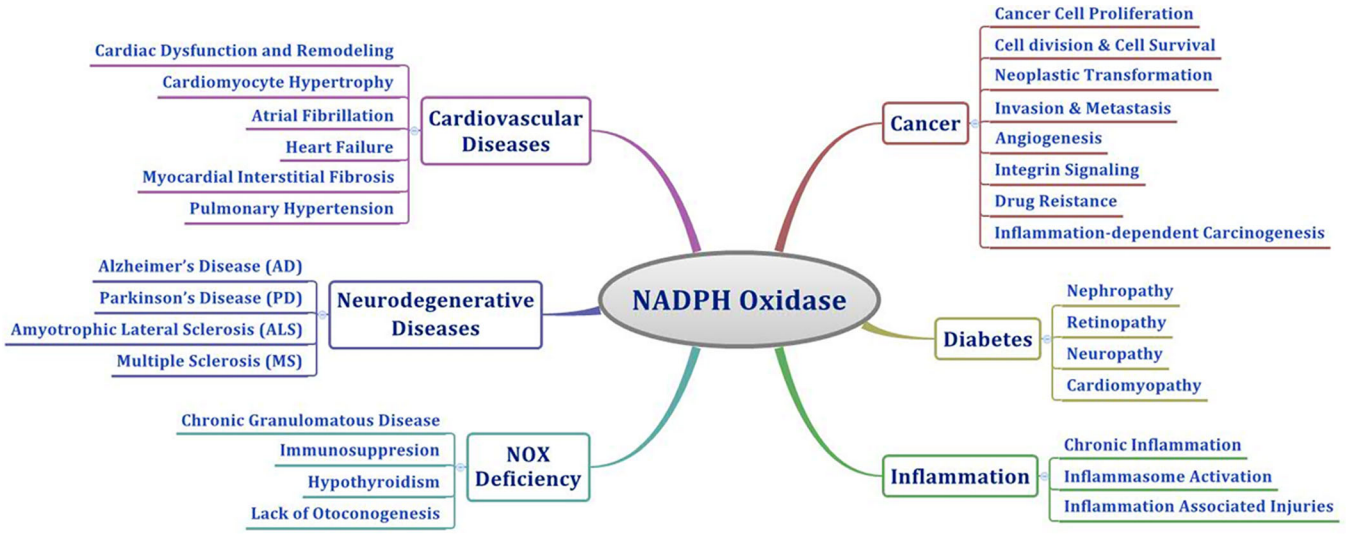

FIGURE 4 Involvement of NADPH oxidase (NOX) in various pathophysiological consequences. NADPH oxidase (NOX) mediated ROS generation contributes to cellular dysfunction and leading to multiple pathologies

cells and regulates blood pressure; it also stimulates redox signalling in two phases: In the first phase, Ang II rapidly stimulates NOX activity, while in the later phase, it sustains the expression of NOX isoforms (NOX-1, NOX-2, and NOX-4).[37] Angiotensin II also regulates protein kinase C (PKC) that has been reported to activate the NOX machinery.[38,39] NOX-2 and NOX-4 modulate physiological excitation-contraction coupling and also maintain myocardial capillary density.

Active forms of NOX-2 and NOX-4 isoforms were found to be constitutively expressed in the cardiac cells.[40] Excessive ROS generation through NOX-2 and NOX-4 has been associated with apoptotic cell death of cardiac cells resulting in cardiac dysfunction and pathogenesis. It has been found that hyperactivation of NOX elevates ROS generation that further activates the MAPK pathway and promotes apoptosis in the myocardium that may cause cardiac injury in severe acute pancreatitis (SAP).[41] Moreover, the deregulation of NOX-2 contributes to angiotensin II-induced cardiomyocyte hypertrophy, atrial fibrillation, and the development of interstitial fibrosis. Additionally, NOX-2 modulates matrix metalloprotease (MMP) activity and promotes myocyte death under stress conditions. A higher level of NOX-4 also causes detrimental effects in the cardiac system. NOX-4 mediated ROS promotes apoptosis, and mitochondrial dysfunction in cardiac myocytes causes cardiac dysfunction.[43] A report by Qin et al, suggests that angiotensin II induces NOX mediated ROS generation and apoptosis, which is linked with elevated p38/MAPK pathway and caspase-3 activity in H9C2 cells. Supportively, the inhibition of this pathway by apocynin reduces apoptosis in H9C2 cells. in vitro pharmacological inhibition of NOX using apocynin demonstrated the reduction in angiotensin II-mediated apoptosis in rat embryonic cardiomyocytes.[44] Another report showed that the over activation and expression of NOX-2 and its subunits p22phox, p67phox, and p47phox was observed in the pressure-overload LV hypertrophy group. The NOX mediated ROS generation activates several redox-sensitive kinases, including ERK 1/2/5, JNK 1/2, and p38 MAPK in cardiomyocytes as the major pathways of pressure-overload LV hypertrophy and may contribute to pathological changes causing heart failure.[45] It has been noticed that NOX-1 mediated ROS elevates Akt oxidation and also promotes subsequent Akt dephosphorylation by modulating PP2A during endotoxin-induced apoptosis in cardiomyocytes.[46] However, NOX-1 has also been reported to play a protective role against hypoxia-induced SAN dysfunction. The recent report revealed that NOX-1-deficient (Nox1−/Y) mice are more sensitive to hypoxia and bradycardia than the WT mice, suggesting the physiological role of NOX-1 in cardiac diseases.[47] Moreover, an elevated NOX-2 expression and p47phox translocation was associated with increased activity of NOX enzymes, which resulted in elevated lipid peroxidation and the activation of ERK and JNK signalling leading to cell death after ischaemia-reperfusion (I/R).[48] In addition, the silencing of NOX-2 using ADV-NOX2-AS transduction in rat cardiomyocytes representing reduced hypoxia-induced oxidative stress and apoptosis.[49]

The NOX-4 is primarily expressed in the mitochondria of cardiomyocytes. A study using cardiac-specific NOX-4 knockout (c-Nox4−/−) mice showed a reduced level of ROS, reduced mitochondrial swelling and cytochrome c release and attenuated apoptosis, cardiac hypertrophy and interstitial fibrosis in response to pressure overload (PO). Additionally, NOX-4 overexpression promoted apoptosis and exacerbated cardiac dysfunction, fibrosis in response to PO.[50] The functional association of NOX-4 has been found with vascular remodelling and pulmonary hypertension[51] and is also involved in cardiac adaptation upon chronic stress. It promotes HIF-1 and the release of VEGF under stress conditions leading to myocardial angiogenesis.[52] Hypoxia is a major condition that plays a key role in the dysfunction and apoptosis of cardiac cells. During the hypoxic condition, down regulation of the survival protein,p-AKTser473 and anti-apoptotic protein BCL2 with upregulation of pro-apoptotic proteins, Bax and caspase-3 expressions promote apoptosis of cardiac cells. It has been found that NOX mediated ROS is responsible for apoptosis in cardiomyocytes under hypoxia conditions through AT1 and PKC

activation.[53] Obstructive sleep apnea (OSA) mediated chronic intermittent hypoxia (CIH) is also involved in NOX-2 mediated ROS generation.[54] Previous report revealed that Statins, a class of drugs, which has been used for treatment of cardiovascular diseases, inhibit isoprenylation of small GTP-binding proteins including, Rac protein, which further inhibits NOX mediated ROS generation.[55] NOX-1, NOX-2 and NOX-4 could be the key isoforms mediating oxidative stress in cardiovascular pathologies. Taken together, NOX enzymes may prove to be promising therapeutic targets for cardiovascular diseases. The connection of NOX and cardiovascular pathologies has been shown in Figure 5.

3.2 | NOXs and diabetes

Compelling evidences indicate that NOX enzymes remain activated during the hyperglycemic or hyperlipidemic conditions.[17,37,56] Different isoforms of NOX and their regulatory subunits, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ play important roles in the pathogenesis of macro- and microvascular complications of diabetes.[57] Oxidative stress is one of the major catalytic factors behind the manifestation of diabetes. The chronic elevation of glucose levels (hyperglycemia) with (or without) the insulin insensitivity is the prime hallmark of the diabetic condition. Moreover, in the later phase, diabetes leads to other complex conditions that most importantly include retinopathy, nephropathy, neuropathy, and cardiomyopathy. The past decade has seen extensive research focused on understanding the underlying molecular mechanism and cellular signalling that functions during the diabetic condition. Largely, it has been considered as a stress-associated disease apart from affecting multiple individuals bearing genetic predisposition to diabetes. In the present discussion, we restricted our explanation of the mechanistic pathways that are regulated by NOX enzymes and its association with diabetic complications. It has been assumed that oxidative stress and accumulation of ROS

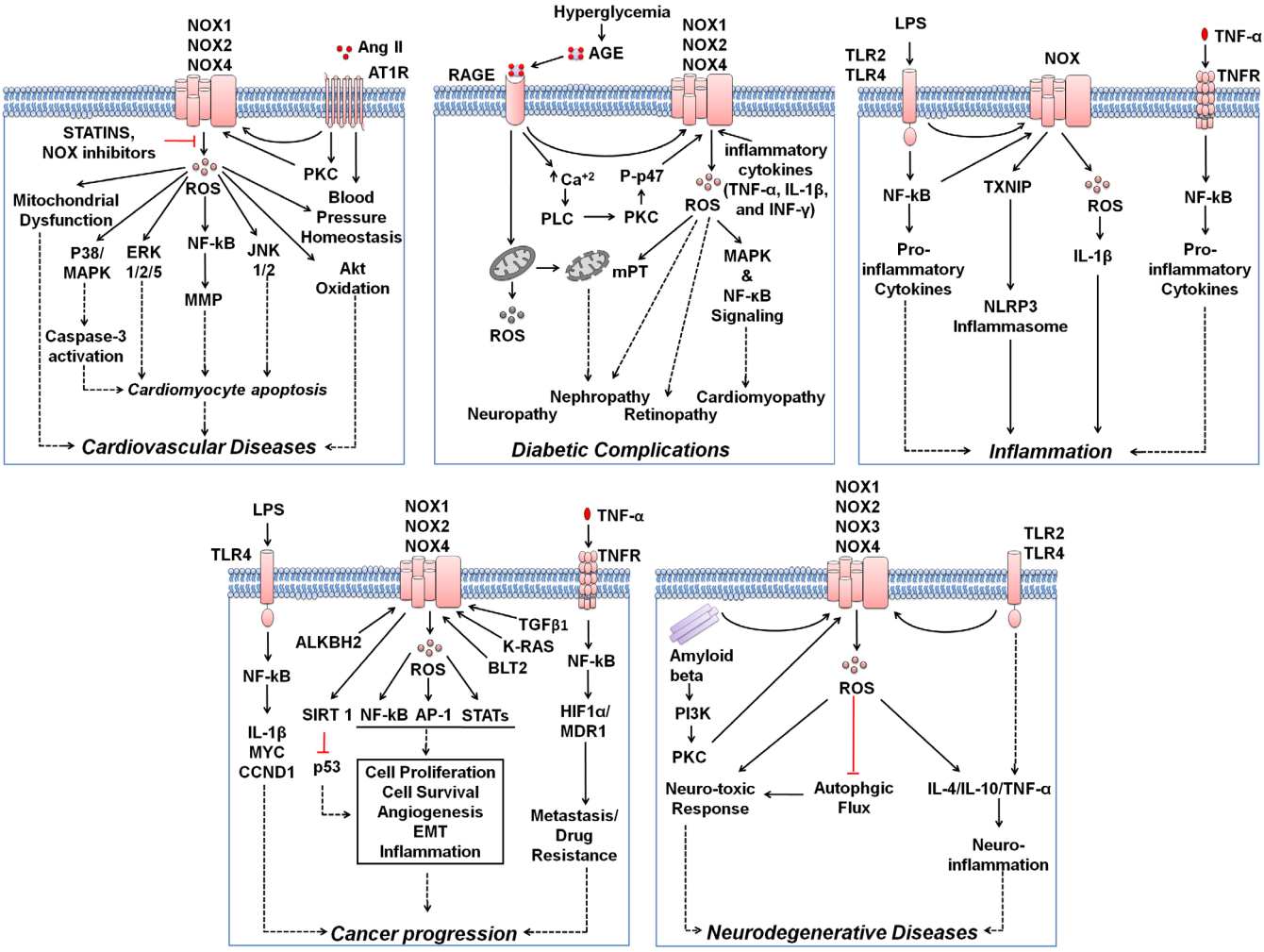

FIGURE 5 The association of NADPH oxidase (NOX) mediated ROS generation with molecular mechanisms involved during the progression of various pathophysiological consequences. ROS are mainly generated by NOX family enzymes and play an important role in maintaining homeostasis. Dysregulation of NOX activity results in various pathological conditions. The images represent the involvement of NOX isoforms mediated ROS generation in different signalling pathways during cardiovascular diseases, Diabetic complications, inflammation, cancer progression, and neurodegenerative diseases

influence diabetes by augmenting insulin insensitivity and metabolic dysregulation. An elevated ROS generation and low ATP levels has been noticed in Type 2 diabetes due to the mitochondrial dysfunction.[58] It has been revealed that higher level of glucose promotes ROS generation and tissue damage.[59] NOX is the key enzyme responsible for ROS production in different types of the cells. Increased expression of NOX isoforms and its components (NOX-1, NOX-2, NOX-4, NOXO1, and NOXA1) have been found with ROS generation and beta-cell dysfunction.[60] The NOX activity has also been linked to the regulation of insulin secretion. A previous report highlighted that pancreatic beta cells are associated with elevated NOX activity and ROS generation in diabetic patients.[61] In particular, NOX-2 mediated ROS generation exhibits insulin resistance in endothelial cells and deteriorates vascular function during diabetes. The NOX-2 deficient transgenic mice was met with improved vascular function because of reduced ROS production.[62] During the hyperglycemic (elevated glucose) state, various physiological changes occur, which promote the synthesis of advanced glycation end-products (AGEs), which binds to its receptors RAGE and promotes various pathologies including diabetes, cardiovascular, inflammation, and cancer. In addition, elevated levels of glucose may affect non-oxidative pathways including the polyolhexosamine biosynthetic pathway, PKC activation and production of AGEs.[63] An experimental data revealed that during the hyperglycemic condition, the AGE/RAGE signalling induces NOX mediated ROS generation and leads to the mitochondrial permeability transition (mPT) and mitochondrial superoxide generation that progresses diabetic nephropathy.[64] It also activates NOX-1 with simultaneous reduction of SOD-1 activity to induce intracellular ROS production that promotes diabetes-mediated vascular calcification.[65] A growing body of evidences support that diabetes mellitus is associated with impaired mitochondrial function, lipid oxidation, and excessive ROS production.[66] Moreover, chronic hyperglycemia, certain hormones, cytokines as well as growth factors induce NOX mediated ROS that alters renal function during the diabetic condition.[67] Apart from diabetic nephropathy, the NOX mediated ROS generation also contributes to diabetic cardiomyopathy.[68] A previous report mentioned that hyperglycemia stimulates the NOX-1 mediated ROS, which promotes atherosclerosis during diabetes mellitus.[69] It has been evident that inflammatory cytokines (TNF-α, IL-1β, and INF-γ) induce NOX-1 gene expression, ROS generation and insulin insensitivity. Interestingly, selective inhibitor of NOX-1/4 inhibits ROS production and cytokines mediated monocyte chemoattractant protein-1 (MCP-1) induction in the beta cells.[70] Multiple existing reports that delineate the expression of NOX and subsequent ROS generation are directly linked with diabetic complications. The involvement of NOX during diabetic conditions has been shown in Figure 5.

3.3 | NOXs and inflammation

Inflammation is one of the important defence mechanisms of the immune system. ROS plays an important role in the progression of inflammation. During tissue injury, ROS are generated by polymorphonuclear neutrophils (PMNs) at the site of inflammation and activate protective inflammatory signalling for evading pathogens.[71] NOX-2 was the first NOX identified in phagocytes involved in bacterial killing suggesting the close association of ROS and NOX with the innate immune response.[72,73] The innate immune system provides the first line of defence against both the exogenous infection (PAMPs) and the endogenous molecules (DAMPs).[74] Toll-like receptors (TLRs) present on the cell surface play an essential role in the recognition of PAMPs and DAMPs. These danger signalling molecules are then recognised by the cell surface receptor RAGE. The RAGE receptor is a transmembrane protein, which synergises with extracellular and intracellular ligand during infection, chronic inflammation or physiological stress to activate discrete signalling cascades.[75] RAGE and TLRs are reported to have cooperative interaction and share common ligands including HMGB1, S100A8/A9, and LPS that activate the NF-κB mediated inflammatory response and signal transduction pathway.[76] Both RAGE and TLRs activate the NOX machinery and ROS generation in various physiological and pathophysiological conditions.[77,78] An earlier report advocated that HMGB1-TLR-4 synergy encourages NOX activation mediated haemorrhagic shock in neutrophils. It has also been demonstrated that the binding of HMGB1 to TLR4 initiates the TLR4-MyD88-IRAK4 signalling axis, which activates p38/MAPK and Akt pathways and further activates NOX. However, in response to haemorrhagic shock/resuscitation: TLR4, HMGB1, and Rac1 activate NOX machinery, which is independent of p38/MAPK in endothelial cells (ECs).[79]

It has also been shown that NOX enzymes are involved in immunological response and inflammation-associated tissue injury.[71,80] A report by Hsieh et al talks about the proteoglycans triggering TLR-2 and TLR-4 mediated activation of NOX isoforms (NOX-1, NOX-2, and NOX-4), which in turn promotes the ROS generation and enhance IL-1β expression and ultimately induces inflammatory diseases.[81] The NOX-1 and NOX-4 mediated ROS play a decisive role in the liver pathogenesis.[82] The role of NOX isoforms in liver injury and fibrosis was revealed by using the carbon tetrachloride (CCl_4) treated mice model system wherein CCl_4 induced liver fibrosis was attenuated by depletion of NOX-1 and NOX-4 expression.[83] Inhibition of NOX-1/4 activity by using the inhibitor GKT137831 suppresses chronic inflammation.[84] It also attenuates inflammation in the neuroglial cells of the retina.[85] Furthermore, NOX-1 and NOX-4 depletion have been shown to reduce mRNA expression of proliferative and pro-fibrotic genes in hepatic stellate cells (HSCs). The overexpression of NOX-1 and NOX-4 induces liver cirrhosis.[83] The NOX isoforms are also involved in influenza A virus related pathologies. The expression of NOX-2 isoform was found to be upregulated in influenza A virus-induced lung pathogenesis. In contrast, NOX-1 reduces influenzaAvirus-induced lung inflammation in mice, notably at the initial phases of the infection. The negative regulatory role of NOX-1 was shown in the moderately pathogenic HkX-31 influenza Avirus-infected NOX-1-deficient (Nox1−/y) mice, which confers the higher expression of pro-inflammatory cytokines. These studies showed the dual role of NOX isoforms, NOX-1 and NOX-2, in influenza A viruses mediated inflammation.[86] The role of NOX-4 and NOX-1 was shown in the aortic

sinus atherosclerotic plaque in the diabetic ApoE (−/−) mice model. It has been noticed that NOX-4 and NOX-1 derived ROS contribute to atherosclerosis in the aortic sinus. In addition, NOX-4 depletion reduces pro-inflammatory gene expression in the aortic sinus and NOX-1 promotes macrophage accumulation.[87] These studies indicate the distinct roles of NOX-1 and NOX-4 in inflammation-associated atherosclerosis in the aortic sinus of diabetic mice. Importantly, NOX mediated ROS also regulates gut homeostasis. It has been found that missense mutations in NOX-1 and DUOX-2 genes lead to loss of function, subsequently resulting in defective ROS production and increased susceptibility toward the very early onset of inflammatory bowel disease (VEOIBD).[88] Various reports advocate that the generation of ROS and NF-κB activation is the most common phenomenon during infection and inflammation.[89] It is also noticed that TNF-α induced NF-κB activation modulates NOX-1 and NOX-4 expression and ROS in human aortic smooth muscle cells (SMCs). The relationship between inflammatory regulators and NOX enzymes was shown by in silico analysis to decipher the existence of typical NF-κB elements in promoters of NOX-1 and NOX-4, which were activated by transient expression of p65/NF-κB.[90] NOX mediated ROS generation and subsequent activation of NF-κB signalling is associated with the progression of inflammation and various pathological conditions.[91] It has been shown that TNF-α evokes NOX mediated ROS generation and NF-κB dependant RAGE expression in HUVEC cells.[92]

Apart from pro-inflammatory conditions, NOX isoforms also play a pivotal role in inflammasome activation, which leads to the inflammation-associated tissue injury. Under certain circumstances, cells undergo the synthesis of certain specialised cytokines, which are produced in an inactive form and converted into the active form by inflammatory caspases like caspase-1.[93] The activity of caspase-1 is regulated by the large multi-molecular protein complexes, which are termed as inflammasomes. Among all, NLRP3 inflammasome has been extensively studied and reported to be implicated in numerous inflammatory diseases.[94] The regulation of NOX enzymes during such inflammatory conditions is poorly understood. NOX may have an association with NLRP3 inflammasome and immune response.

Phagocytosis is a fundamental process that plays an important role in the microbicidal activity, immune system activation, and antigen presentation. It has been reported that the acidification of phagosomes is regulated by the NLRP3inflammasome and caspase-1 protein. NOX-2 modulates phagosomal pH during the activation of caspase-1 on phagosomes.[95] This process of phagosome acidification is involved in both innate and adaptive immunity. The inflammasome complex was also found to be hyperactivated during chronic granulomatous disease (CGD) condition, a disease related to mutations in NOX machinery components.[96] Besides this, ROS has been found to attenuate the inflammasome activation.[97] Moreover, the involvement of NOX-2 has been reported in oxidative stress-mediated injury and neuroinflammation during traumatic brain injury (TBI). For a long time, the NOX-2 mediated NLRP3 inflammasome regulation remained undetermined. However, a recent study agrees that NOX-2 regulates NLRP3 inflammasome activity through TXNIP (Thioredoxin Interacting Protein) in TBI pathology.[98] TXNIP also regulates NOX-2 mediated NLRP3 inflammasome during diabetic nephropathy. Studies also advocate that the mice lacking $p22^{phox}$ and $p47^{phox}$, show reduced cathepsin B release and IL-1β production, indicating the involvement of NOX activity during inflammasome inactivation.[99] Thus, the role of the NOX in the inflammatory signalling is highly pleiotropic and remains to be explored. The association of NOX during inflammation and associated pathologies has been shown in Figure 5.

3.4 | NOXs and cancer

The distinct characteristics of cancer cells acknowledged as "hallmarks of cancer," are all associated with dysregulated cellular signalling cascades that lead to the neoplastic transformation of a normal cell.[100] NOX family proteins and ROS either directly or indirectly modulate the cell death and survival signalling during the progression of cancer. NOX, ROS, and oxidative stress play a vital role in cancer initiation, promotion, and progression. Due to the rapid growth as well as higher metabolism of the cancer cells, they display alteration in the cellular redox system.[101] The enhanced level of ROS may cause genomic instability by triggering double-strand breaks or oxidation of nitrogenous base. The damaged macromolecules help in progression of oncogenic transformation and activation of oncogenes such as Ras, Bcr-Abl, and c-Myc. Moreover, endogenous and exogenous risk factors of cancer share the common phenomenon of ROS generation in their respective signalling pathways. A recent report suggests that the redox imbalance occurs mainly through defective mitochondria, elevated NOX activity and uncoupled nitric oxide synthase-3 (NOS3) during tumorigenesis.[102] The cancer-associated mutations and tumour microenvironment can elevate the level of mitochondrial ROS and promote tumorigenic signalling and metabolic reprogramming. A recent report revealed that generation of mitochondrial ROS and activation of hypoxia-inducible factors (HIFs) play essential roles in cancer cell proliferation and survival.[103] Thus, an extra-mitochondrial ROS is also involved in the reprogramming of cancer cell signalling.

The discovery of NOX enzymes provided better insight into the role of ROS signalling in cancer progression. Understanding the roles of various NOX isoforms in tumour development and its progression in various types of cancers is an emerging interest in the field of cancer research. A recent report suggested that the NOX-1 generated ROS plays a vital role in the proliferation of colon cancer cells. It was also observed that the knockdown of NOX-1 using shRNA in HT-29 cells (human colon cancer cells) blocked the cell cycle in the G1/S phase due to impaired cyclin D1 expression and inhibited MAPK signalling.[104] The previous report revealed that the activating mutations in K-Ras contribute to colon cancer by promoting NOX-1 expression at mRNA and protein levels. in vivo study in transgenic mice showed the elevated expression of mutated K-Ras, "K-Ras (G12V)," and NOX-1 in the epithelium of both small and large intestines.[105] The NOX-1 is also reported to participate in Ras-induced VEGF expression and angiogenesis. The growth factors such as EGF or platelet-derived growth factor (PDGF) stimulate tyrosine kinase (TRK), which activates

Ras-Raf-MEK-ERK-GATA6 pathway and induces NOX-1 expression.[106] Indeed, knockdown of NOX-1 using siRNA has been found to suppress an active ERK level and transcription factor Sp1which ultimately down-regulates VEGF expression.[107] It has been found that NOX plays a key role in colon cancer invasion by modulating the EGFR-PI3K-AKT signalling pathway. EGF activates NOX-1 by the phosphorylation of NOXO1.[108] An interesting report revealed that Memo, a Copper-Dependent Redox Protein which has an important role in cell migration and metastasis in breast cancer, is required for the sustained activation of NOX-1 mediated ROS production.[109] An another report discussed that NOX-1 interacts with metalloprotease domain 17 (ADAM17) and prevents its ubiquitin-mediated degradation to promote metastasis.[17] Furthermore, 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP)-induced colon tumours represent the overexpression of NOX-1 and NOX-4 Isoforms. NOX-1 knockdown also attenuates the lipopolysaccharide-induced NF-κB signalling and consequently reduces the expression of downstream targets including IL-1β, MYC, and CCND1.[110] Additionally, NOX-1 upregulation is reported to activate SIRT1 and inhibit p53 function during malignancy.[111]

Another study demonstrated the correlation between expressions of the NOX regulatory subunits and BRCA1 gene in the breast invasive carcinoma, ovarian cystadenocarcinoma and lung adenocarcinoma and suggested that the elevated expression of regulatory subunits of NOX-1-NOX-4 are associated with the down regulation of BRCA1 gene expression and may support the progression of malignancy.[102] NOX-1 and its activating protein p67phox were found to be upregulated in E2-induced tumours in ACI rats and in oestrogen receptor-positive (ER+) human breast Tumours. NOX-1 induced oxidative stress down-regulates survivin (anti-apoptotic protein) and initiated the ER+ breast tumour formation.[112] An earlier study reported that NOX-1 was overexpressed in the early stage of prostate cancer and modulated the ROS generation, c-fos-induced growth factor, interleukin-8, and Cav-1.[113]

The stromal oxidative status is a permissive element of the tumour microenvironment and promotes tumour metastasis. In the tumour microenvironment, cancer cells acquire the proximal relation to inflammatory mediators, which facilitates tumour progression. NOX mediated ROS generation directly or indirectly activates the transcription factors such as NF-κB, AP-1, and STATs, which then promote the inflammation and cancer.[30,114-116] A recent report stated that NOX-4 mediated ROS production is involved in TGFβ1-mediated induction of CAF-like phenotype in the tumour microenvironment. The study utilised in situ hybridisation to reveal the predominant expression of NOX-4 mRNA in the peri-tumoral stroma of prostate cancer.[117] Transforming growth factor-beta 1 (TGF-β1) induced the stromal NOX-4 expression and intracellular ROS generation stimulating the migratory properties of breast epithelial cells MCF-7.[118] Previous report highlighted that NOX-4 localises to the mitochondria and promotes ROS generation, which favours the oncogenic events.[116] Another report revealed that the expression of BLT2 (leukotriene B [4] receptor) increased manifold and promoted the survival of bladder cancer cells. The underlying mechanism involved the BLT2-dependent up-regulation of NOX-1 and NOX-4and subsequent NF-κB activation that promotes cell survival signalling, invasion and metastasis in bladder cancer cells.[119,120] Another protein, ALKBH8 (a human AlkB homologue) was shown to promote NOX-1-dependent ROS signalling and was implicated in human bladder cancer progression.[121] An interesting finding revealed that the phorbol acetyl myristate (PMA), a potent carcinogen promotes NOX-2 expression. It is mainly responsible for switching of NOX-1 to NOX-2 and is associated with invasion of colon cancer through elevated expression of MMP-7.[122] Moreover, the current compelling evidences indicate that NOX isoforms promote neoplastic transformation and are implicated in various cancer phenotypes such as cell division, cell survival, angiogenesis, and integrin signalling.[27,123,124]

Tumour metastasis has been closely related to chemoresistance and the phenomenon of drug resistance is increasing in cancer patients during chemotherapy. The elevated expression of *p*-glycoprotein (P-gp) is the major event involved in the drug resistance. A research proposed that the altered redox homeostasis may be one of the hallmarks of the drug resistance phenotype.[125] The elevated expression of NOX-1 promotes intracellular ROS generation, which activates HIF-1α/MDR1 signalling pathway to accelerate the chemoresistance in gallbladder cancer cells (GBC).[126] The expression of NOX-1 isoform has been altered in cisplatin resistance in GBC. Moreover, it has been found that HIF-1α induces the expression of P-gp, which provokes chemoresistance in prostate cancer cells.[127] These findings, therefore, show that NOX-1 is intimately associated with the development of drug resistance in cancer cells. The association of NOX with cancer has been shown in Figure 5. Therefore, it is undoubtedly perceived that NOX and its signalling pathways have pivotal roles in pathogenesis of cancer.

3.5 | NOXs and neurodegenerative diseases

Various NOX isoforms have been identified in the central nervous system (CNS). The involvement of different isoforms of NOX has been found in neuronal development, nerve cell signalling as well as in memory formation. Under physiological conditions, the brain requires a higher amount of oxygen for glucose metabolism and energy production. Efficient usage of oxygen is essential to maintain homeostasis of neuronal cells in the brain. Dysregulation of glucose and oxidative metabolism promotes the oxidative stress of neuronal brain cells which is associated with the pathologies of neurodegenerative diseases.[128] The expression and activation of NOX isoforms play a vital role in inflammatory and degenerative pathologies of the brain such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS).[129-131] During the progression of AD, NOX activation, and subsequent ROS generation are associated with astrogliosis, neuro-toxic response, and neuroinflammation in microglia. It is now well established that the accumulation of amyloid beta (Aβ) is a hallmark of the progression of AD. Different forms of amyloid beta (Aβ) such as oligomeric amyloid-β (oAβ) and Fibrillar Aβ promote NOX activity, which elevates the

generation of extracellular ROS (that directly kills the neurons) or intracellular ROS (which promotes neuroinflammation).[132] The NOX isoforms, NOX-1, NOX-2, NOX-3, and their cytosolic components including Rac1, p47-phox, and p67-phox are involved in ROS generation and are linked with astrogliosis, neuro-toxic response and neuroinflammation in AD[133,134] Besides this, oligomeric amyloid-β (oAβ) peptide activates PI3K and PKC, which leads to NOX activation. The Tg2576 mice lacking the catalytic subunit NOX-2 have exhibited attenuated cerebrovascular dysfunction even in the presence of an adequate level of the amyloid-β peptide (Aβ).[135] Thus, NOX may be a potential target for prevention and therapeutic intervention of AD.[136] Commonly known NOX inhibitors including several peptides like the pituitary adenylate cyclase-activating polypeptide, dynorphin, glycine-glycine-phenylalanine, leucine enkephalin, and des-tyrosine leucine enkephalin[137-139]; antibiotics (minocycline)[140] and small molecules such as dextromethorphan, statins, naloxone, and sinolimine[138,141,142] have been used for the treatment of AD. In continuation, PD, a progressive neurodegenerative disease, which is associated with several biological processes such as oxidative stress, mitochondrial dysfunction, impairment of proteasomal function and protein aggregation in the substantia nigra pars compacta (SNpc) has also been linked with ROS.[143] The expression of NOX-1, NOX-2, and NOX-4 were found elevated during the progression of PD and the resulting oxidative stress contributed to motor and memory deficits. The activation of NOX-1 in dopaminergic neurons was reported to promote neuronal death by oxidative DNA damage,[144] while activation of NOX-2 impaired autophagic flux which simultaneously promoted the apoptotic cell death and contributed to neurodegeneration.[145] Extensive research in the past has also concluded that inhibition of NOX and ROS generation may provide neuroprotective effects in PD. Some selective inhibitors of NOX such as DPI, apocynin and simvastatin had been found to induce neuroprotective effects by promoting anti-inflammatory response and the survival of nigrostriatal dopaminergic neurons in various PD models.[146-149]

ALS is another neurodegenerative disease that is mainly associated with gliosis and degeneration of motor neurons in the spinal cord. Oxidative stress accelerates the events associated with ALS.[150,151] Activation of NOX has been frequently observed in ALS and its inactivation was found to be neuroprotective.[152] NOX mediated ROS generation is linked with the IGF-1/Akt pathway for cell survival in neurons and promotes death of motor neurons.[153] Higher NOX-2 expression was evident in the microglia cells isolated from spinal cord.[150] NOX-2 mediated ROS generation plays a mutual role with ERK1/2 pathways in the ATP-P2X7 signalling axis during ALS.[154] Upon TLR2 stimulation, enhanced NOX activation leads to the secretion of TNF-α, which facilitates microglial neuro-toxic inflammation.[155] MS, another disease affecting the CNS, is associated with inflammation in the central nervous system leading to demyelination and neurodegeneration. The up-regulation of NOX-2 and its subunits p22phox and p47phox was observed in the initial lesions in microglia cells during MS. An experimental model of Cognitive impairment during the remission phase of MS has shown impaired long-term potentiation (LTP) in CA1 hippocampal Synapses. NOX mediated ROS also plays an important role in LTP blockade and progression of synaptic dysfunctions in MS.[156] Experimental autoimmune encephalomyelitis (EAE) model exhibits that either the deletion of NOX-2 or treatment of apocynin inhibits the expression of pro-inflammatory cytokines and concurrently stimulates the expression of the anti-inflammatory cytokines IL-4 and IL-10 in MS.[157,158] Collectively, these reports suggest that multiple NOX isoforms contribute to the pathogenesis of NDs and can be explored as potential therapeutic targets. The involvement of NOX during the progression of various NDs has been shown in Figure 5.

3.6 | Pathologies associated with NOX deficiency

NOX mediated dysregulation of ROS generation and cellular dysfunction plays a pivotal role in the initiation and progression of various pathologies as described earlier in this review. The deficient NOX condition or its dysregulated activity leads to pathological consequences such as CGD, immunosuppression, hypothyroidism, and lack of otoconogenesis. The CGD is an immunodeficiency condition mainly caused by the mutation in the NOX-2 gene, which results in defective oxidative stress-mediated antimicrobial activity of phagocytes. The phagocytic NOX-2 is involved in eradication of microbial infection thus demonstrating its role in immunity. Other NOX isoforms (NOX-1, NOX-4, and DUOX-2) play an important role in the PRRs including TLRs and NLRs mediated immunomodulation. They are also involved in viral pathogenesis and the chemokine mediated inflammatory signalling.[7] The inhibition or genetic deletion studies demonstrated that NOX deficiency abrogates various functions of the innate immune system. Furthermore, the synthesis of the thyroid hormone also requires an adequate level of ROS, mainly hydrogen peroxide (H_2O_2) during the synthesis of thyroxin (T4) and triiodothyronine (T3). DUOX-2 is the principle NOX involved in the synthesis of H_2O_2 which essentially underlies the proper synthesis and functioning of thyroid hormone. However, the dysfunctional NOX/DUOX impairs ROS production and is associated with pathological conditions such as hypothyroidism.[159] Mutations in DUOX2 gene leads to hereditary hypothyroidism. Moreover, NOX-4 has been found to be constitutively active at various cellular sites such as endoplasmic reticulum, mitochondria or nucleus in the thyroid gland resulting in ROS generation which is associated with the thyroid tumour formation.[160] Thus, it is evident that not only hyperactivation or dysregulation of NOX isoforms, but its deficiency is also responsible for progression of pathological conditions.

4 | NOX AS A THERAPEUTIC TARGET

In the recent past, several pathological disorders have been characterised by redox imbalance and the occurrence of oxidative stress. As described critically in the present review, NOX possesses pro-oxidant activity and modulates various signalling molecules, which ultimately leads to the development and progression of various pathological conditions. Here we have provided compelling evidences where NOX was directly or indirectly linked with cellular dysfunction

in various pathologies. The current understanding proposes that the expression of different isoforms of NOX can be considered as a biomarker of disease progression. However, the role of NOX in pathological signalling is paradoxical. The NOX isoforms may be a putative therapeutic target for various pathological disorders. It has been demonstrated that the controlled regulation of NOX generated ROS influences various cellular functions through the modulation of complex signal transduction pathways during physiological conditions, but its dysregulation is associated with various pathologies as described earlier. Several NOX inhibitors have been identified and have been demonstrated to inhibit NOX activity or expression during pathological conditions is beneficial. But the majority of NOX inhibitors under study possess various undesirable off-target effects. Therefore, exploring additional NOX inhibitors may be a promising approach for the therapeutics of various pathologies. However, it requires extensive research to explore the possible mechanisms of inhibition. The NOX activity or expression may be regulated by various strategies suggested by multiple findings including the modulation of peroxidase-dependent assays for oxidants, inhibiting the translocation of p47phox, irreversible binding to the cytosolic FAD domain of NOX, targeting NADPH binding site and regulating the interaction between p22phox and p47phox subunits. In the current scenario, the limited numbers of NOX inhibitors are reported as shown in Table 1. Collectively, to design or to discover the novel inhibitor of NOX

TABLE 1 NADPH oxidase inhibitors

Inhibitors	Targeted NOX Isoform	Mode of action	Pathology targeted	References
Apocynin	NOX-2	Impairs the translocation of p47phox (cytosolic component) to the membrane subunit of the NADPH-oxidase complex[161]	Neurodegenerative diseases, hypertension, atherosclerosis, stroke	162, 163
ML-171	NOX-1	ML-171 is a phenothiazine that specifically targets NOX1 protein[164]	Colon cancer invasion	164
VAS 2870	NOX-2 NOX-4 NOX-5	NOX-2:It interferes the association of NOX2 and its cytosolic components, NOX-4 & NOX-5: inter- or intramolecular conformational changes[165]	Liver tumour	166
DPI	Flavo-proteins	It may bind on the FAD or NADPH binding site[167]	PD, cancer	147, 168, 169
Ebselen	NOX-1 NOX-2	Interrupts the assembly of Nox-1 and NOX-2, blocks translocation of p47phox and p67phox[170]	cerebral ischaemia injury	171, 172
Histamine Dihydrochloride (HDC)	NOX-2	HDC ligates to H2R and inhibits NOX-2[173]	Acute Myeloid Leukaemia (AML), melanoma	173, 174
GSK2795039	NOX-2	Competitive inhibitor for NADPH binding[175]	Acute pancreatitis	175
Celastrol	NOX-1 NOX-2	Blocks the association between cytosolic subunit p47phox and membrane flavocytochrome subunits p22phox[176]	Inflammatory conditions, Neurodegenerative disease, cancer, and arthritis	176
NoxA1ds	NOX-1	Inhibits binding of the NOXA1 to NOX1[171]	Colon cancer, hypertension	177, 178
Nox2ds-tat	NOX-2	Block the association between NOX-2 and p47phox[179]	Ischaemia/reperfusion (I/R) injury	179
Fulvene-5	NOX-2 NOX-4	Inhibits NOX activation[180]	Cancer	180
AEBSF	NOX-2	Block the association of NOX-2 with p47phox and p67phox[181]		
GKT-136901	NOX-1 NOX-4	Inhibits NOX-1 and NOX-4 activation, scavenges peroxynitrite[171]	Diabetic nephropathy, Stroke, Neurodegeneration	182
PR-39	NOX-2	Targets p47phox, Influences SRC homology domain containing proteins[183]	Myocardial Infarction	184
S17834	NOX	Inhibition of NOX enzymes	Atherosclerosis, Diabetes	171, 185
VAS3947	Pan NOX	Interferes with oxidase assembly[186]	Hypertension	186
Plumbagin	NOX-4	Plumbagin directly interacts with NOX-4	Sepsis	187

isoforms could be substantial challenge but it is the need of the hour.

5 | NOXS: CURRENT CHALLENGES AND FUTURE PERSPECTIVES

5.1 | Challenges

- Identification of NOX isoforms involved in the physiology and respective pathology using a precise model system.
- Designing the selective inhibitors of NOX isoforms to hinder the activity.
- Exploring the cellular niche under which NOX isoforms play a pathological role.
- Identification of expression/activity pattern of NOX that regulates cell signalling.
- Apart from NOX, how its regulators play a decisive role in the regulation of pathologies.
- Identification of precise upstream signalling during disease condition.

5.2 | Possible solutions

- Highly specific system for regulation of NOX isoform expression and activity.
- Revise the current understanding of ROS and NOX.
- Understanding the kinetics of NOX expression and activation during the various stages of pathologies.
- Identification of molecular partners of NOX in pathologies.
- Inhibitors of regulators of NOX.
- Designing the PPI inhibitors for NOX system.
- Identification of upstream molecular signalling of NOX in specific pathology.

6 | CONCLUSION

The amplitude of ROS generation by NOX enzymes and the inability of cells to detoxify the generated ROS leads to the oxidative burden, which amplifies various cellular dysfunctions. The growing body of evidences acknowledged that the cumulative function of different isoforms of NOX and subsequent ROS generation is critically regulated to maintain cellular homeostasis. Dysregulation of NOX activation is associated with various pathophysiologies like diabetes, cardiovascular diseases, neurodegenerative diseases, and cancer. Based on the available reports, it may be suggested that NOX isoforms are involved in various cellular functions and coordination of multiple cellular signalling events including oxidative stress, inflammation, cell proliferation, and cell death. The precise framework of NOX mediated ROS generation and its role in signalling cascades and associated molecular mechanisms involved in different pathological conditions remains largely unexplored. It is beneficial to identify potential targets for prevention and therapeutic intervention of a number of pathophysiological conditions that appear to occur through NOX mediated downstream signalling. Despite the known inhibitors of NOX and its implications in disease biology, it is an important approach to explore additional NOX inhibitors for therapeutic intervention. Importantly, pharmacophore based drug discovery is needed to gain the attention to explore NOX inhibitors and disease-specific signalling targets to achieve promising strategies for the therapeutic intervention of NOX associated pathologies.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable - no new data generated Data sharing is not applicable to this article as no new data were created or analyzed in this study. As this is a review article.

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SUPPORTING INFORMATION

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