Glucox Biotech's pipeline of NOX inhibitors have a large therapeutic potential

Summary: The purpose for making this document is to provide some background of the choice of target - the isoform NADPH oxidase 4 (NOX4). Different NOX isoforms (NOX1–5, DUOX1/2) are expressed in various tissues, fine-tuning ROS output based on physiological need as part of cellular metabolism, integrating its role in physiology, immunity, and redox balance.

Every second, trillions of molecules within the human body engage in an intricately choreographed network of interactions. These molecular events occur across countless cells and tissues, forming the basis of life's most essential functions. Through a precisely regulated system of feedback loops and signaling pathways, the body maintains homeostasis.

When disease arises—whether due to genetic mutations, chronic inflammation, metabolic dysfunction, autoimmune misfires, or pathogen invasion—this molecular harmony is disrupted. What makes treatment particularly challenging is the sheer complexity and interdependence of these systems. Intervening at one molecular target can ripple across pathways, unintentionally disturbing other crucial functions.

NADPH oxidase (NOX) is a membrane-bound enzyme complex that plays a unique and highly specialized role in metabolism. Unlike most enzymes that conserve energy or build biomolecules, NADPH oxidase uses energy-rich electrons to generate reactive oxygen species (ROS)—primarily superoxide (O_2^-)—from molecular oxygen. This controlled production of ROS is not a metabolic byproduct, but a deliberate and regulated metabolic function, especially critical in the immune system. Among the NADPH oxidase family, NOX4 stands out due to its unique biochemical behavior and physiological role. While most NOX enzymes (like NOX1, NOX2) generate superoxide (O_2^-) as their primary reactive oxygen species (ROS), NOX4 predominantly produces hydrogen peroxide (H_2O_2), either directly or through rapid dismutation of superoxide. NOX4 illustrates how ROS, far from being merely damaging byproducts, can serve as precise modulators of cell behavior. Its generation of H_2O_2 enables long-range redox signaling, with profound effects on gene expression, protein activity, and cell fate. As such, NOX4 sits at a critical interface between metabolism, oxidative biology, and cellular communication, making it both a fascinating research target and a potential therapeutic leverage point.

The goal of modern medicine is not simply to "fight disease," but to do so with precision—to restore balance without derailing the broader symphony of molecular life. This is the essence of precision medicine, which aims to tailor interventions to the unique molecular context of each patient, acknowledging that even small disruptions in this tightly regulated system can have widespread effects.

Thus, NOX4 represents a safe and viable therapeutic target, provided its biological functions are well understood and its inhibition is appropriately modulated. This is particularly relevant across several indications for which Glucox Biotech is actively developing targeted treatments.

Table of content

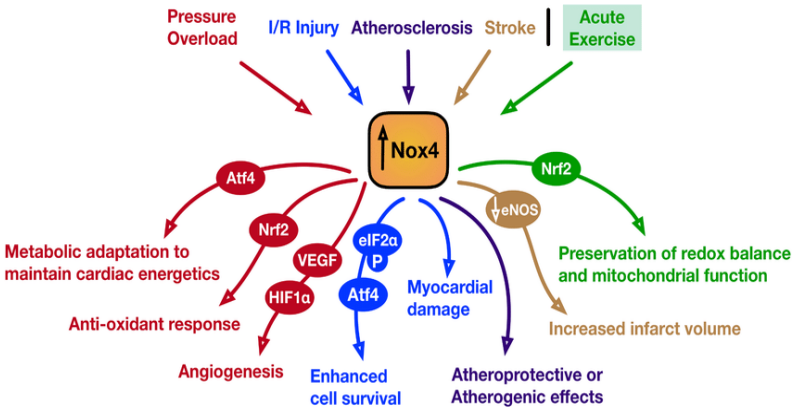
Chapter:

1. Summary of why NADPH oxidase 4 (NOX4) is central and important pharmaceutical target in various conditions

Global knockout (KO) of the NADPH oxidase 4 (NOX4) gene in mice does not adversely affect their viability or lifespan under normal conditions. Studies have shown that NOX4-deficient mice exhibit comparable median and 90% lifespans to wild-type controls, with no statistically significant differences observed. Although NOX4 is not essential for survival under baseline conditions, it plays significant roles in modulating the body's response to various stressors.

NOX4 is an enzyme that generates reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2), in a controlled physiological context. However, when NOX4 is overactive, particularly under conditions like prolonged hyperglycemia and hypoxia, its pathological effects can significantly contribute to tissue damage and disease progression.

Pathologically high NOX4 activity, driven by chronic hyperglycemia and hypoxia, contributes to progressive tissue damage, fibrosis, and vascular dysfunction. These effects are mediated through sustained oxidative stress and inflammatory signaling, highlighting NOX4 as a potential therapeutic target in chronic metabolic and hypoxic diseases.



NOX4 metabolic function in health and disease

This sketch illustrate the various conditions that will induce increased NOX4 activity. In general, NOX4 appears to play a metabolic role in responding to **diverse forms of stress** affecting the human body. Although NOX4 is clearly important for normal physiology, universal knockout (KO) mice are typically viable and develop normally under standard laboratory conditions. This observation suggests a degree of functional redundancy or compensatory mechanisms in vivo. What is abundantly clear is that excessive NOX4 activity have a large detrimental impact in health. The potential of NOX4 as a pharmaceutical target is further explored and supported throughout this document.

Understanding the balance between these protective and destructive mechanisms is crucial for developing therapeutic strategies targeting NOX4 and HIF-1α pathways.

2. Background view of metabolism regarding NOX4 and NOX2 in relation to Diabetic retinopathy

Background view of metabolism NOX4 and NOX2: Metabolism operates as a finely tuned network of signaling systems, relying on precise interactions between ligands, receptors, and second messengers. This network functions through tightly regulated on/off responses to maintain balance and avoid disruption. A powerful analogy for this complexity is the image of a pianist performing on a large keyboard: just as music is created through the careful coordination of finger movements across many keys, metabolic harmony arises from the right balance of molecular signals. Pressing a single key too strongly —or not enough—can distort the melody, just as imbalance in signaling pathways can lead to disease.

Within this network, **NOX4** and **NOX2**, members of the NADPH oxidase family, serve crucial roles as metabolic and inflammatory regulators:

- **NOX4** is activated primarily through increased expression and provides **long-term ROS (reactive oxygen species) signaling**, particularly within mitochondria. At **low levels**, NOX4 activity can be protective—supporting cell signaling and adaptation. However, **excessive NOX4 activity** becomes harmful, contributing to oxidative stress, fibrosis, and tissue damage.
- **NOX2**, in contrast, is activated through **rapid assembly of co-factors**, allowing for **acute responses**, especially in immune cells like phagocytes. NOX2 plays a vital role in the body's defense against pathogens. A deficiency in NOX2 impairs the ability to clear infections and is associated with **Chronic Granulomatous Disease (CGD)**—a rare immunodeficiency characterized by recurrent infections and granuloma formation.

Both NOX4 and NOX2 are intricately linked to inflammatory pathways and mitochondrial function. However, **timing and dosage of inhibition** are critical when targeting NOX enzymes—particularly NOX4—in therapeutic contexts. Inhibiting NOX4 activity too broadly or for too long may disrupt its protective roles, while insufficient suppression may fail to curb its harmful effects in disease.

NOX4 deficiency, whether due to genetic mutations, experimental knockdown models, or epigenetic regulation, presents variable outcomes depending on the tissue and disease context. Understanding this balance—like mastering the keys of a piano—is essential to designing effective and safe interventions. Something that Glucox Biotech have the understanding and the suitable NOX inhibitors to do.

NOX4 is a Key Detrimental Driver in Diabetic Retinopathy (DR):

- **NOX4 Overexpression** contributes significantly to early pathological changes in DR, such as vascular leakage, oxidative stress, and glial activation. This is especially emphasized in **Publication 1**, where topical administration of a selective NOX4 inhibitor (GLX7013114) improved BRB integrity and reduced gliosis and oxidative markers.
- NOX4 is **upregulated in hyperglycemic as well as hypoxic conditions**, which are present in DR, thereby increasing **ROS production** and promoting inflammation and neurovascular degeneration.

Differential Roles of NOX4 and NOX2:

- **NOX2** is also implicated but appears to play a more prominent role in acute **glutamate-induced excitotoxicity**, as shown in **Publications 2 and 3**. The NOX2 inhibitor (GLX7013170) reduced damage from excessive glutamate, which is critical in ischemic and diabetic insults.

- While both enzymes contribute to oxidative stress, **NOX4's chronic and constitutive activity** makes it particularly damaging in the sustained hyperglycemic environment of DR.

Role of Müller Cells and Glutamate Toxicity:

- Under diabetic and hypoxic stress, **Müller cells switch from a protective to a harmful phenotype**, failing to buffer glutamate properly and releasing **pro-inflammatory cytokines** (as described in multiple studies).
- The resulting **glutamate accumulation** exacerbates excitotoxic damage to retinal neurons, highlighting a vicious cycle of inflammation, oxidative stress, and neurodegeneration.

Integrated View from GB-Crete Publications (ref. 1, 2 and 3):

Together, these studies propose a **pathophysiological model** where:

1. **NOX4-driven oxidative stress** is central to early and chronic vascular damage in DR.
2. **NOX2 contributes acutely** to neuronal injury via glutamate pathways.
3. Targeting both pathways **pharmacologically may offers an improved therapeutic strategy**, in DR to prevent irreversible damage.

Comment: NOX4 also will be part of glutamate toxicity in the DR pathology based on preventing Müller cells switch from a protective to a harmful phenotype. Thus NOX4 will be preferred pharmaceutical target.

3. Philosophy of Glucox Biotech AB's Pharmaceutical Approach: Targeting NOX4 Inhibition

Glucox Biotech AB (GB) embraces a unique pharmaceutical philosophy built on the inhibition of NOX4 as a versatile platform to treat multiple serious conditions. This document outlines the rationale behind our approach, highlighting the pathophysiological links between diseases such as **Diabetic Retinopathy, ischemia/reperfusion injury, and Acute Kidney Injury**, and demonstrating how strategic timing, local delivery, and liver-metabolized (yet chemically stable) drug design can restore and preserve physiological balance.

Cellular and metabolic factors that contribute to fibrosis and tissue damage, especially in the context of aging and metabolic disorders. Aging leads to mitochondrial dysfunction. NOX4 (NADPH oxidase 4) is a major ROS-producing enzyme that is up-regulated in response to stress, hypoxia, and aging. Localized to mitochondria, it contributes to further mitochondrial oxidative stress. Chronic activation of NOX4 Promotes TGF-β signaling, a central driver of fibrosis. Induces expression of pro-fibrotic and pro-inflammatory cytokines (e.g., IL-6, TNF-α). Inflammatory cytokines reinforce NOX4 activity and further mitochondrial injury—a vicious cycle. Hyperglycemia and lipid accumulation (as in diabetes or obesity): Cause mitochondrial overload, leading to ROS generation and oxidative stress. Drive ER stress and inflammatory signaling (e.g., NF-κB activation). Accelerate NOX4 expression and activity. These factors are key contributors to diabetic complications, such as nephropathy, cardiomyopathy, and retinopathy, all involving fibrosis.

Table Summary

Factor	**Effect on Mitochondria**	**Effect on NOX4**	**Outcome**
Aging	↓ Efficiency, ↑ ROS	↑ Expression	Fibrosis, increased reperfusion injury

High glucose/lipids	↑ Mito stress	↑ NOX4	Inflammation, fibrosis
Inflammation	↑ ROS, mitochondrial injury	↑ NOX4	Feedback loop of damage
Hypoxia/reperfusion	ROS burst	Activates NOX4 and NOX2	Tissue injury, fibrosis

This table summarizes the long-term effects, primarily characterized by increased pathological fibrosis, as well as the short-term cell death induced by hypoxia, followed by additional cell death during the reperfusion phase.

Common Denominators in Glucose-Induced Fibrosis and Reperfusion-Induced Apoptosis: The Role of NOX4 and Mitochondria

This document elaborates in greater detail on the shared mechanisms underlying glucose-induced fibrosis and reperfusion-induced apoptosis, with a particular focus on the critical roles played by NADPH oxidase 4 (NOX4) and mitochondrial dysfunction.

Understanding the Foundation: Balancing Metabolic Complexity

Our bodies are in constant metabolic motion, striving to maintain homeostasis. Cellular energy production is fundamental to life, but excessive or dysregulated metabolic activity can tip the scales toward pathological states.

In the late 20th century, **replacement pharmacology** gained traction with the successful treatment of hormone deficiencies such as insulin and growth hormone. These advances were powered by the ability to express human proteins in single-cell systems and purify them to high homogeneity, minimizing immunogenicity through tight control over glycosylation and oxidation.

Yet, while replacement therapies address missing components, **intervening in the complex web of metabolic signaling without disrupting systemic balance is significantly more challenging**.

A Shift in Therapeutic Strategy: Small Molecule Targeting

With the turn of the century, many pharmaceutical companies shifted focus toward small, non-peptide pharmaceuticals. High-throughput screening identified molecules that could inhibit specific molecular targets, with low IC_{50} values being the gold standard. The goal was to create metabolically stable compounds that offered long-lasting action.

However, blocking a single target too efficiently can disturb systemic equilibrium. In complex diseases, the pathological process is rarely isolated; it's often embedded within broader, tightly regulated signaling networks. This is where **NOX4 inhibition** offers a uniquely balanced approach.

NOX4 – A Central Player in Oxidative Stress and Organ Damage

NOX4, a member of the NADPH oxidase family, is a key source of pathological reactive oxygen species (ROS) in several chronic and acute diseases. Unlike other NOX isoforms, NOX4 is constitutively active and regulated primarily at the expression level. Its activity is closely linked with fibrotic, inflammatory, and degenerative processes.

Diabetic Retinopathy

In diabetic retinopathy, chronic hyperglycemia induces oxidative stress in the retinal vasculature. NOX4 overexpression leads to endothelial dysfunction, breakdown of the blood-retina barrier, and

pathological neovascularization. Local NOX4 inhibition may offer a **non-invasive pharmacological option** to preserve vision and prevent disease progression without systemic side effects.

Ischemia/Reperfusion Injury

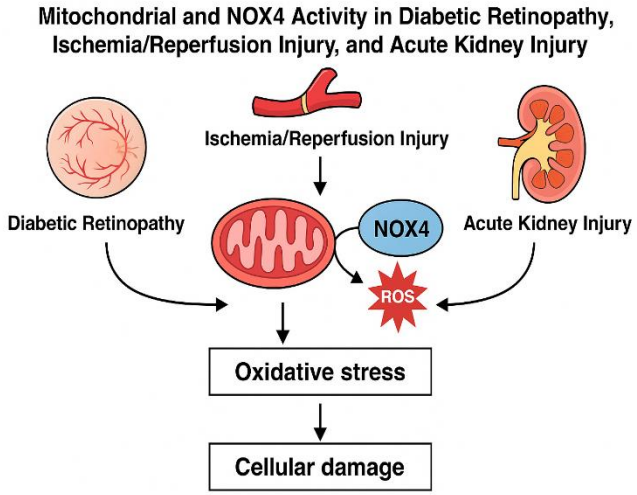
Tissues subjected to ischemia followed by reperfusion (e.g., in stroke, Kidney injury, myocardial infarction, or surgical revascularization) experience a surge in ROS. NOX4 is upregulated in the ischemic zone and contributes to oxidative damage upon reperfusion. Targeted, **local NOX4 inhibition** can blunt the destructive burst of ROS, reducing tissue injury and improving outcomes.

Acute Kidney Injury (AKI)

In AKI, whether from sepsis, toxins, or ischemia, NOX4 is upregulated in tubular epithelial cells and drives mitochondrial dysfunction, inflammation, and fibrosis. Local inhibition of NOX4 in the kidney may **protect renal function** and reduce the risk of long-term chronic kidney disease.

4. Mitochondria and NOX4 important junction in pathology

In **diabetic retinopathy**, **I/R injury**, and **AKI**, the synergy between **mitochondrial dysfunction and NOX4 overactivity** drives a shared mechanism of **oxidative stress-induced tissue injury**, positioning them as key therapeutic targets across these diseases.



Common Role of Mitochondria and NOX4:

1. **Reactive Oxygen Species (ROS) Generation**:
    - Both mitochondria and NOX4 are major sources of ROS in cells.
    - Under pathological conditions such as hyperglycemia (in DR), hypoxia-reoxygenation (in I/R), or nephrotoxicity/inflammation (in AKI), mitochondrial electron transport becomes inefficient, and NOX4 is upregulated, leading to excessive ROS production.
2. **Cellular Dysfunction and Death**:
    - The resulting oxidative stress damages lipids, proteins, and DNA.
    - This contributes to endothelial dysfunction, inflammation, and apoptosis in the retina (DR), brain/heart (I/R injury), and renal tubular cells (AKI).
3. **NOX4 as a Central Amplifier**:

- NOX4 is uniquely localized in mitochondria and endoplasmic reticulum, linking it directly to mitochondrial ROS signaling.
- It acts as a redox sensor and amplifies mitochondrial dysfunction via feed-forward ROS production, exacerbating tissue injury.

4. **Therapeutic Targeting**:
   - Inhibiting NOX4 or restoring mitochondrial function (e.g., via antioxidants, mitochondrial biogenesis promoters, or NOX4 inhibitors) has shown promise in preclinical models for all three conditions.

Mechanistic Summary: Hypoxia/Reperfusion and NOX Isoforms in DR

Glutamate toxicity and the enzymes **NOX2** and **NOX4** are closely linked in the context of **retinal neurodegeneration**, particularly in diabetic retinopathy (DR). Key mechanisms linking oxidative stress, excitotoxicity, and inflammation in diabetic retinopathy (DR), particularly focusing on the roles of NOX2 and NOX4.

Hypoxia/Reperfusion Injury Dynamics

- **Ischemic Phase**:
  - **Prolonged hypoxia** can make NOX4 activation *protective*, possibly via redox-sensitive pro-survival signaling. Hypoxia-Inducible Factor (HIF) plays a central role in protecting neuronal cells under hypoxic conditions. Hypoxia-Inducible Factor (HIF) is a transcription factor that regulates the cellular response to low oxygen (hypoxia). HIF and NOX4 are both crucial players in the cellular response to hypoxia, particularly in the brain and other oxygen-sensitive tissues. Their interaction is complex and context-dependent, involving both protective and damaging roles. HIF-1α Upregulates NOX4 under hypoxia, HIF-1α directly increases NOX4 expression by binding to hypoxia-response elements (HREs) in the NOX4 promoter. This has been shown in various cell types including neurons, vascular cells, and cancer cells. NOX4-Derived ROS Modulate HIF Stability. ROS from NOX4 can inhibit prolyl hydroxylase domain (PHD) enzymes, which normally degrade HIF-1α. Thus, NOX4 activity can enhance HIF-1α stabilization, forming a positive feedback loop.
  - Protective Effects at moderate NOX4 Activity. NOX4-induced ROS act as signaling molecules, activating survival pathways. Promote angiogenesis, metabolic adaptation, and preconditioning effects via HIF targets like VEGF, EPO, and GLUT1. Low levels of ROS can activate Nrf2, leading to antioxidant defenses.
- **Reperfusion Phase**:
  - Damaging Effects of excessive NOX4 Activity. Sudden oxygen influx leads to **excessive ROS production**, particularly **H_2O_2** from NOX4, which becomes **neurotoxic**, damaging neuronal cells. High NOX4 activity leads to oxidative stress, mitochondrial dysfunction, and neuronal death. Contributes to blood-brain barrier breakdown and neuroinflammation in stroke or chronic hypoxia.
  - Linked to neurodegenerative diseases (e.g., Alzheimer's, Parkinson's) and ischemic injury.

HIF and NOX4 mechanism in hypoxia protecting neuronal cells and harmful at reperfusion

Hypoxia → ↑ HIF-1α → ↑ NOX4 → ↑ ROS →

↳ (+) Stabilize HIF-1α (feedback) Hypoxia

↳ (+) Activate pro-survival genes (low ROS) hypoxia

↳ (–) Damage neurons (high ROS) Reperfusion

The Glucox Biotech Strategy: Smart Targeting and Metabolic Harmony

Our approach centers on:

- **Local targeting**: Delivering treatment directly to affected tissues (e.g., eye, kidney), limiting systemic exposure.
- **Timed intervention**: to match drug presence with peak pathological NOX4 activity.
- **Liver-metabolized stability**: Ensuring the compound is efficiently cleared by the liver, minimizing off-target effects and systemic toxicity, while retaining chemical integrity during transit.

We believe **NOX4 inhibition is not just a target — it's a platform** for treating diseases where oxidative stress and metabolic dysregulation converge. Our goal is to restore balance, not just block pathways.

5. Universal NOX4 knockout and Animal survival

Universal NOX4 KO highlight that, although NOX4 is not essential for survival under baseline conditions, it plays significant roles in modulating the body's response to various stressors.

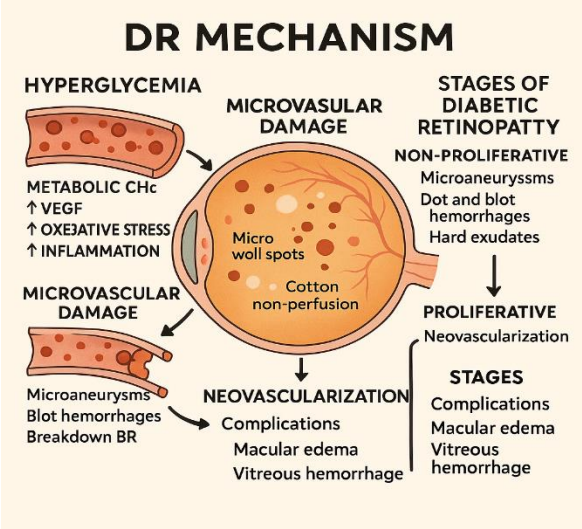
Viability and Development

- Universal (global) NOX4 knockout (NOX4−/−) mice are generally viable and develop normally under standard laboratory conditions. Global knockout (KO) of the NADPH oxidase 4 (NOX4) gene in mice does not adversely affect their viability or lifespan under normal conditions. Studies have shown that NOX4-deficient mice exhibit comparable median and 90% lifespans to wild-type controls, with no statistically significant differences observed. Unlike other NOX enzymes, NOX4 knockout does not cause embryonic lethality, suggesting that it is not essential for early development.

Conclusion: Universal NOX4 knockout is generally **not lethal** and can improve survival in models of **ischemic stroke, cardiac injury, and neurodegeneration** by reducing oxidative stress and inflammation. However, tissue-specific effects suggest that complete inhibition of NOX4 may have **context-dependent outcomes**, requiring caution in therapeutic targeting. This is something GB has been taking in consideration in the various treatable conditions of pathological increased NOX4 activity.

6. Diabetic Retinopathy (DR)

Pathology overview of the induced complexity when untreated effectively. GB strong strategy to block the root cause at several levels of this snowballing cascade.



Stages of Diabetic Retinopathy

Non-Proliferative Diabetic Retinopathy (NPDR):

- Microaneurysms
- Dot and blot hemorrhages
- Hard exudates
- Cotton wool spots (nerve fiber infarcts)

Proliferative Diabetic Retinopathy (PDR):

- Neovascularization
- Vitreous hemorrhage
- Retinal detachment

The pathology of DR is considered as being complex cascade of events. However GB highly specific NOX4 inhibitor demonstrate the importance of NOX4 increased activity in this cascade. NADPH oxidases (NOXs) are implicated in the early pathological events of diabetic retinopathy (DR).

- The NOX4 inhibitor GLX7013114, topically administered, reduced oxidative damage and apoptosis in the rat streptozotocin model of DR.
- GLX7013114 protected retinal neurons and retinal ganglion cell function and reduced the expression of pro-inflammatory cytokines in the diabetic retina.
- GLX7013114 diminished the diabetes-induced increase in vascular endothelial growth factor levels and Evans blue dye leakage in retinal tissue.
- GLX7013114 exhibits neuroprotective, anti-inflammatory, and vasculoprotective properties that suggest it may have a role as a putative therapeutic for the early events of DR.
- GLX7013170 exhibit neuroprotective efficacy connected to induced glutamate toxicity.

Summary of two major parts of the DR pathology

Neuronal part

- Degeneration of retinal neurons
- Reactive macro/microglia

Vascular part

- Pericyte loss
- Leakage of blood vessels
- Neovascularization

Early Management of Diabetic Retinopathy: A Root-Cause and Inflammatory Pathway Approach

In the early management of Diabetic Retinopathy (DR), addressing the underlying mechanisms of disease initiation is key to preventing long-term retinal damage. DR is primarily driven by chronic hyperglycemia, which causes progressive damage to the retinal microvasculature and initiates a cascade of inflammatory and oxidative stress responses [1,2, 12].

One of the central players in this early inflammatory response is Toll-like Receptor 4 (TLR4). Chronic hyperglycemia increases the presence of damage-associated molecular patterns (DAMPs), such as advanced glycation end products (AGEs) and oxidized lipids, which activate TLR4 on retinal cells (e.g., microglia, Müller cells, and endothelial cells) [3,4].

Müller cells are a type of glial cell found in the retina of the eye. Müller cells are essential for maintaining the proper function and structure of the retina, and they play a role in several important physiological processes, such as maintaining the ionic balance of the retina, regulating extracellular glutamate levels, and providing metabolic support to neurons.

NOX4 plays a dual role in Müller cells: contributing to homeostatic signaling under normal conditions and mediating oxidative damage under pathological stress. Understanding the precise mechanisms of NOX4 regulation and its interaction with retinal signaling pathways may offer new therapeutic avenues for diseases such as diabetic retinopathy and AMD.

Activation of TLR4 triggers intracellular signaling cascades—particularly the **NF-κB pathway**—which results in the production of **pro-inflammatory cytokines** (e.g., **TNF-α, IL-1β, IL-6**) [5]. These cytokines contribute to:

- Breakdown of the **blood-retinal barrier (BRB)** [6]
- Capillary occlusion through **leukostasis** [7]
    - Occlusion of capillary causes the local area of retina primarily supplied by that capillary to become hypoxic. Thus induced glutamate toxicity and NOX2 dependency.
- Early **neuronal and vascular injury**, even before visible vascular changes occur [8]

Early intervention, therefore, includes not only tight glycemic control and management of blood pressure and lipids, but also potentially targeting the TLR4-mediated inflammatory axis. Experimental therapies that inhibit TLR4 signaling have shown promise in reducing inflammation and protecting retinal integrity in preclinical models [9,10].

This proactive, root-cause-focused strategy contrasts with therapies used in the advanced stages of DR, such as anti-VEGF (Vascular Endothelial Growth Factor) agents, which are designed to block abnormal blood vessel growth and vascular leakage [11]. While anti-VEGF treatments are effective in managing neovascular complications and preserving vision, they do not address the initial immune and metabolic disturbances that initiate DR.

Thus, the GB approach, emphasizing early intervention targeting metabolic control and inflammatory modulation represents a more holistic and preventative strategy. By intervening at the earliest

stages, this approach seeks to delay or prevent the progression of DR, reducing the need for invasive treatments later on (ref 12).

In detail pathological drivers in DR

NOX4 and TLR4 in Diabetic Retinopathy: Partners in Crime

NOX4 (NADPH Oxidase 4):

- NOX4 is a major source of reactive oxygen species (ROS) in retinal cells.
- In hyperglycemic conditions, NOX4 expression is upregulated, especially in endothelial cells, pericytes, and Müller glia.
- Excessive ROS leads to:
    - Oxidative stress
    - Mitochondrial damage
    - Activation of pro-apoptotic and pro-inflammatory signaling

TLR4 (Toll-like Receptor 4):

- TLR4 detects DAMPs (e.g., AGEs, oxidized LDL) that are increased in diabetes.
- Activation leads to NF-κB and MAPK signaling → production of cytokines like TNF-α, IL-1β, and IL-6.
- Contributes to inflammation, vascular leakage, and leukocyte adhesion in the retina.

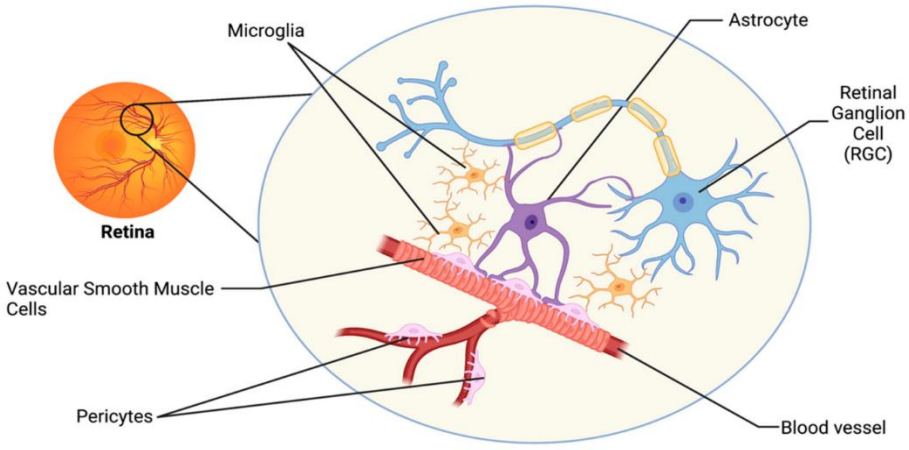
Muller in DR pathology

Müller glia and NOX4 play key roles in the pathology of **diabetic retinopathy (DR)**, particularly in the development of neuroinflammation, oxidative stress, and vascular dysfunction.

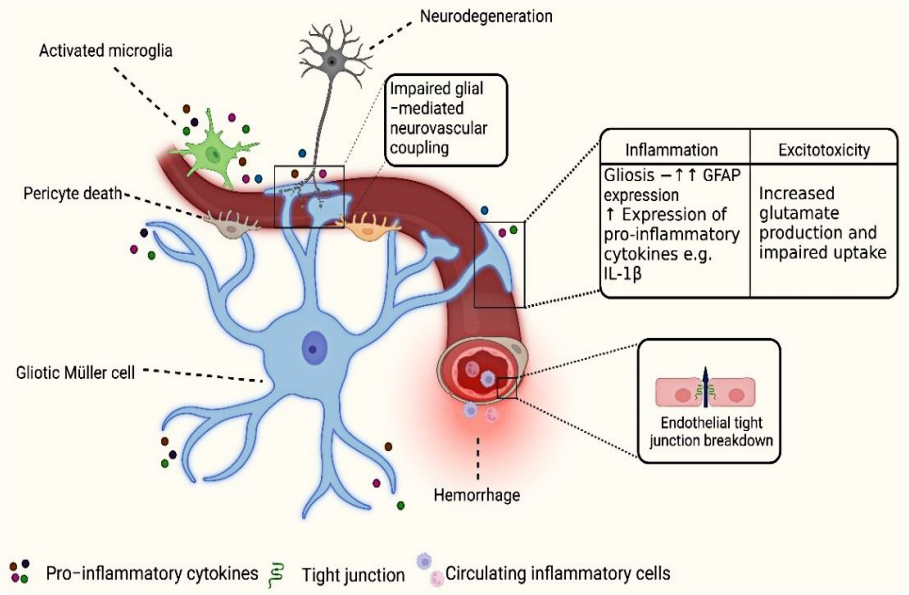
Müller glial cells are the principal macroglial cells in the retina, crucial for maintaining retinal homeostasis. In DR, they become **reactive**, contributing to disease progression through:

- **Gliosis**: Upregulation of GFAP (glial fibrillary acidic protein) and morphological changes.
- **Pro-inflammatory cytokine release**: Including IL-1β, TNF-α, and IL-6.
- **Disruption of blood-retinal barrier**: By releasing VEGF and other mediators.
- **Glutamate toxicity**: Due to impaired uptake leading to excitotoxicity.

Retinal Neurovascular Unit (NVU) in health and disease



Healthy retina



Unhealthy retina

Pictures from Ph.D. Thesis **"INVESTIGATION OF THE ROLE OF NADPH OXIDASE INHIBITORS IN EXPERIMENTAL ANIMAL MODELS OF RETINOPATHIES"** Stavroula Dionysopoulou Biologist, Heraklion, September 2023

Diabetic Retinopathy (DR) Pathophysiology including NOX2 and NOX4 at excessive glutamate

- **Hypoxia** is a key trigger in DR inducing excess glutamate.
- Affected **amacrine cells** are protected by **NOX2 inhibition** (ref 13, 14 e.g., **GLX7013170**).
- **NOX2**: Activated by glutamate in **neurons and microglia**, promoting **ROS generation** and cell death (ref. 12, 13, 14, 15).
- **NOX4**: **Upregulated in Müller glia** by high glucose and hypoxia. Produces **ROS** → **inhibits glutamate transporters** → extracellular glutamate accumulates (ref.12, 15).

Glutamate Toxicity & Crosstalk

- **AMPA injection** mimics **glutamate excitotoxicity** (Ref 13, 14).
- **Extracellular glutamate** → activates **NOX2** → amplifies **ROS** production.
- **ROS + Glutamate** → activate **NF-κB**, leading to:

- **Pro-inflammatory cytokines** (e.g., TNF-α, IL-1β).
- Further **neuroinflammation** and **cell death**.

Therapeutic Insight

- **NOX4 inhibition in early stage DR thus upstream the pathological cascade will also protect amacrine cells in a diabetic condition**. Thus GLX7013114 the specific NOX4 inhibitor (ref 12) major therapeutic target to inhibit the overall harmful DR cascade of evens progressing from NPR to PDR.
- The NOX4 inhibitor, **GLX7013114**, administered topically as eye-drops is able to:
  - reduce oxidative nitrative stress, activation of caspase-3 and micro/macroglia and attenuation of neuronal markers caused by diabetes
  - attenuate the diabetes induced increase in VEGF, vascular leakage and proinflammatory cytokine levels (TNF-α protein, IL-1β/IL-6 mRNA)
  - protect RGCs function (PERG analysis amplitude values)
- NOX2 deletion protects retinal ganglion cells related to glutamate toxicity
- NOX4 inhibition reduces ROS and enhances glutamate clearance.

Conclusion: NOX4 inhibition (GLX7013114) stands alone as the most effective protective therapeutic target preventing DR progression (ref 12). Combined NOX2/NOX4 inhibition however may offer **synergistic neurovascular protection** in DR. GB have NOX4/NOX2 specific inhibitors in the pipeline as potential second generations DR treatment (ref 12, 13, 14, 15).

7. Ischemia/reperfusion injury

Ischemic stroke remains a major cause of mortality and disability worldwide, largely due to limited therapeutic options beyond reperfusion. Oxidative stress, particularly mediated by NOX4, plays a central role in ischemia/reperfusion (I/R) injury. Preclinical studies have demonstrated that NOX4 drives neuronal death, blood-brain barrier breakdown, and inflammation following stroke. Targeting NOX4 offers a novel approach to mitigate ischemic brain injury.

Ischemia/reperfusion AND NOX4

Ischemic stroke occurs when cerebral blood flow is blocked, leading to oxygen and nutrient deprivation. Reperfusion therapies, such as intravenous thrombolysis and mechanical thrombectomy, are the standard of care but do not address the underlying oxidative damage that contributes to neuronal death. A growing body of evidence identifies reactive oxygen species (ROS) as key mediators of ischemic injury, with NOX4 as a primary source. Unlike other NOX isoforms, NOX4 is induced by hypoxia and generates hydrogen peroxide persistently, exacerbating brain damage during and after reperfusion.

Ischemic Stroke Pathophysiology: A Two-Phase Mechanism of Neuronal Death - early and late phase

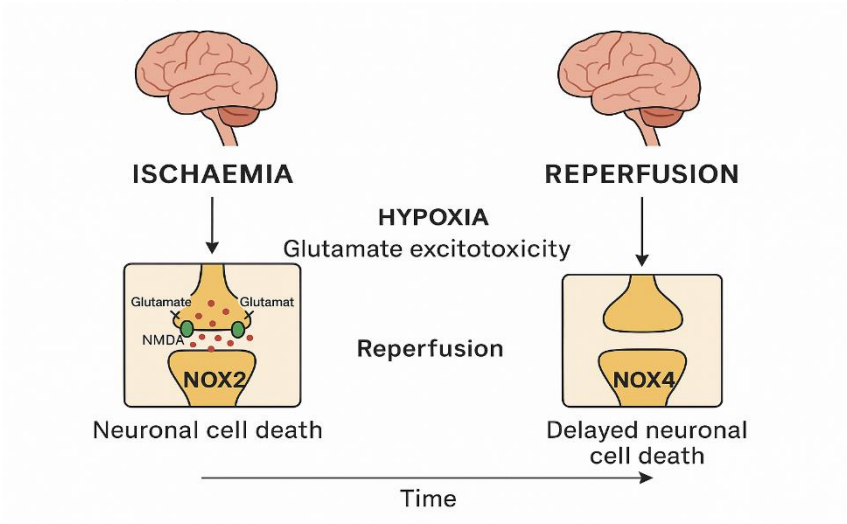
Factor	NOX2	NOX4
Timing	Early (ischemia phase)	Later (reperfusion phase)

Factor	NOX2	NOX4
Primary Cell Types	Microglia, neurons, endothelium	Neurons, endothelium, astrocytes
ROS Generated	Superoxide ($O_2^{-}\bullet$)	Hydrogen peroxide (H_2O_2)
Pathological Role	Amplifies glutamate toxicity, inflammation	Promotes apoptosis, BBB breakdown
Therapeutic Targeting	Acute inhibitors (e.g., NOX2)	Delayed inhibitors (NOX4), longer window

Phase 1: Ischemia (Acute Hypoxia) – Glutamate Excitotoxicity + NOX2-Mediated Oxidative Stress

When a stroke occurs due to an arterial blockage (ischemia), the affected brain tissue is deprived of oxygen and glucose. This leads to several critical, early pathological events:

1. **Energy Failure (ATP Depletion):**
    - The Na^+/K^+ ATPase pump fails, leading to neuronal depolarization and excessive Ca^{2+} influx.
2. **Glutamate Excitotoxicity:**
    - Due to depolarization and impaired glutamate reuptake, extracellular glutamate accumulates.
    - Glutamate overstimulates NMDA and AMPA receptors, causing a massive Ca^{2+} influx into neurons.
    - Elevated intracellular Ca^{2+} activates enzymes (proteases, lipases, nucleases) that damage cell structures, and leads to mitochondrial dysfunction.
3. **NOX2 Activation:**
    - In the hypoxic environment, NOX2 —predominantly found in microglia, neurons, and endothelial cells—is upregulated.
    - NOX2 generates superoxide ($O_2^{-}\bullet$) by transferring electrons from NADPH to oxygen, fueling reactive oxygen species (ROS) accumulation.
    - NOX2-derived ROS amplify glutamate toxicity by:
        - Damaging lipids, proteins, and DNA (oxidative stress).
        - Further impairing mitochondrial function.
        - Promoting inflammation and immune activation.
4. **Neuronal Death:**
    - The combined effects of glutamate excitotoxicity and NOX2-mediated oxidative damage lead to necrosis and apoptosis of neurons in the ischemic core.



Phase 2: Reperfusion – NOX4-Mediated Delayed Neuronal Death

When blood flow is restored (reperfusion therapy or spontaneous recanalization), new challenges arise. Increased expression of NOX4 will be detrimental to the brain (penumbra) and damage neuronal cells outside the ischemic core as well. The penumbra is the name of the part of the injured tissue around the ischemic core that can be targeted with NOX4 inhibition to prevent further brain damage at reperfusion:

1. **Reoxygenation Injury:**
    - The sudden influx of oxygen reactivates metabolic pathways, but the damaged mitochondria cannot handle the load, leading to excessive ROS generation.
2. **NOX4 Activation:**
    - Unlike NOX2, NOX4 is predominantly expressed in endothelial cells, astrocytes, and neurons, and is upregulated during hypoxia/reperfusion.
    - NOX4 produces H_2O_2 as its primary ROS product (rather than superoxide), which can diffuse across membranes and cause widespread damage.
    - **NOX4 activity:**
        - Promotes endothelial dysfunction → blood-brain barrier (BBB) breakdown → edema and hemorrhage.
        - Contributes to delayed neuronal apoptosis, distinct from the acute necrotic death in the ischemic phase.
3. **Secondary Injury:**
    - NOX4-induced ROS also activate matrix metalloproteinases (MMPs), further compromising the BBB.
    - The inflammatory cascade persists, worsening neuronal death and expanding the infarct volume.

Therapeutic Implications regarding the use of NOX inhibitors

NOX2 inhibitors may protect neurons during the acute ischemic phase and NOX4 inhibitors in reducing secondary injury during reperfusion and may extend the therapeutic window for intervention.

Ischemia/Reperfusion (I/R) Injury and NOX4:

- In **ischemia**, blood flow (and thus oxygen) is reduced or cut off to tissues — in the case of stroke, to the brain.
- Hypoxia will induce hypoxic inducible factor (HIF) and Nrf2 (transcriptional factor important in elimination of ROS). HIF closely connected to NOX4 activity upstream as well as downstream.
- **Reperfusion** is the restoration of blood flow after ischemia.
- While reperfusion is essential to rescue tissues, it can cause additional injury (I/R injury) mainly because of a burst of reactive oxygen species (ROS).
- **NADPH oxidases (NOX enzymes)** are major sources of ROS in this setting.
- Induced NOX2 activity is connected to the immediate induced glutamate toxicity and induced NOX4 to short and long term protection of the cellular insult but at reperfusion induced cell death
- **NOX4** (NADPH oxidase 4) is particularly important because:
    - It is highly expressed in endothelial cells, neurons, and astrocytes.
    - Unlike other NOX isoforms, NOX4 produces hydrogen peroxide (H_2O_2) rather than superoxide directly.
    - NOX4 expression is upregulated during ischemia/reperfusion.
    - Its activity contributes to oxidative stress, BBB (blood-brain barrier) breakdown, neuronal death, and inflammation after stroke.
    - Studies (including mouse models) show that genetic deletion or pharmacologic inhibition of NOX4: Reduces infarct size; Preserves BBB integrity; Improves neurological outcomes.
- Thus NOX4 is like a "silent assassin" that wakes up during a stroke. If we can block it safely at the reperfusion phase, we reduce brain damage dramatically without messing up normal brain functions.

Clinical Potential of NOX4 Inhibition

- **Pathological Role**: After ischemic stroke, NOX4 drives oxidative stress, BBB disruption, neuroinflammation, and neuron death.
- **Selective Target**: Unlike other ROS sources (like mitochondria, xanthine oxidase), NOX4 is more disease-specific — it's *induced* during injury but relatively *silent* under normal conditions.
- **Less Risk**: Its selective inhibition might cause fewer side effects than global antioxidant therapy.

- Need highly selective NOX4 inhibitors that must cross the blood-brain barrier (BBB) efficiently. Treatment must be at the reperfusion phase to be efficient (Ref. 6)

Summary

- **NOX4 Upregulation**: NOX4 expression increases significantly after cerebral ischemia, contributing to oxidative stress and neuronal injury.
- **Therapeutic Targeting**: Both genetic deletion and pharmacological inhibition of NOX4 have shown neuroprotective effects in preclinical models.
- **Mechanistic Insights**: NOX4 influences BBB integrity and neuronal survival through ROS production, and its regulation involves complex molecular pathways, including microRNAs.

Targets a source of ROS rather than scavenging ROS afterward; better therapeutic window.

- Need highly selective inhibitors.

- Must cross the blood-brain barrier (BBB) efficiently.

- Timing: treatment must be made following removal of the blockage of the blood supply. This will be especially important for patients suffering of a pronged blockage of blood supply to the brain. The reperfusion damage is driven then by excessive NOX4 activity that at the hypoxic phase is protective and induced for long a longer time management regarding growth stimulating mechanism. Thus timing for NOX4 inhibition is essential for successful treatment to protect the brain from further damage other than the immediate impact of the hypoxic phase. The hypoxic phase will induce excessive glutamate that will mediate cellular damage mediated by increase NOX2 activity.

Conclusion
Targeting NOX4 represents a promising new avenue for ischemic stroke treatment, potentially overcoming the limitations of current therapies. Development of BBB-penetrant inhibitors are essential. GB have highly specific and selective NOX4 inhibitors in the pipeline that correspond to the properties needed for safe and efficient therapy to prevent reperfusion damage.

8. Acute Kidney Injury (AKI):

Ischemia-reperfusion (IR) injury is a major cause of acute kidney injury (AKI) and occurs when blood supply to the kidney is temporarily cut off (ischemia) and then restored (reperfusion). The restoration of blood flow paradoxically leads to further tissue damage due to oxidative stress, inflammation, and cell death. AKI is a sudden loss of kidney function, typically occurring over hours to days. It leads to the accumulation of waste products, fluid imbalance, and electrolyte disturbances.

Causes include:

- **Ischemia** (e.g., shock, hypotension)
- **Nephrotoxins** (e.g., drugs like cisplatin, aminoglycosides)
- **Sepsis**
- **Obstruction** (e.g., stones, tumors)

NOX4 is an isoform of the NADPH oxidase enzyme family, which generates reactive oxygen species (ROS), especially hydrogen peroxide (H_2O_2).

NOX4 is:

- **Highly expressed in the kidney**, especially in renal tubular cells and endothelial cells.
- **Constitutively active**, producing ROS under physiological and pathological conditions.
- **Linked to oxidative stress**, inflammation, and fibrosis.

Connection Between AKI and NOX4

Mechanistic Role of NOX4 in AKI:

1. **ROS Production:** NOX4 contributes to oxidative stress in the kidney by generating ROS, which can damage cells during ischemia-reperfusion injury or toxin exposure.
2. **Cell Death:** Elevated ROS levels from NOX4 can induce **apoptosis** or **necrosis** of renal tubular epithelial cells.
3. **Inflammation and Fibrosis:** NOX4 activation promotes **pro-inflammatory cytokine release** and **fibrotic signaling pathways** (e.g., TGF-β/Smad).
4. **Mitochondrial Dysfunction:** ROS can disrupt mitochondrial function, exacerbating injury.
5. **Endothelial Dysfunction:** NOX4 in renal vasculature affects blood flow and capillary integrity.

Evidence from Studies:

- **NOX4 knockout or inhibition** in animal models reduces AKI severity (e.g., after ischemia-reperfusion or cisplatin exposure).
- **Pharmacologic inhibitors of NOX4** (like GLX7013114) show **protective effects** against AKI in preclinical studies (ref. 4).

Clinical Implications

- **Targeting NOX4** might be a promising **therapeutic strategy** for reducing oxidative stress and injury in AKI.
- Understanding NOX4's role may help **stratify risk** or **monitor progression** in AKI patients.

Conclusion

These studies collectively underscore the pivotal role of NOX4 in various models of AKI and support the potential of NOX4-targeted therapies in mitigating kidney injury.

9. The Common Pathophysiological Thread: Hypoxia/Reperfusion Injury

Hypoxia/reperfusion injury is a major pathological mechanism in ischemic stroke, AKI, and DR (specifically in glutamate toxicity). These similarities are:

Ischemic Stroke:

- **Initial insult: A blood vessel blockage leads to ischemia (lack of oxygen and nutrients).**
- **Reperfusion phase: When blood flow is restored, reperfusion injury occurs due to:**
    - **ROS burst (Reactive Oxygen Species)**
    - **Mitochondrial dysfunction**
    - **Calcium overload**

- **Excitotoxicity (glutamate accumulation) → Overactivation of NMDA receptors → Calcium influx → Neuronal death**

Inflammatory response: Activation of microglia, release of cytokines, and BBB breakdown.

Acute Kidney Injury (Ischemic Type):

- **Initial ischemia: Reduced renal perfusion → Tubular epithelial hypoxia.**
- **Reperfusion injury: Restoration of blood flow causes:**
  - **ROS generation (especially in mitochondria)**
  - **ATP depletion → Ion pump failure → Intracellular Ca^{2+} overload**
  - **Activation of inflammatory pathways (NF-κB, TNF-α, IL-6)**
- **Mitochondrial damage → Cell death (apoptosis/necrosis).**
- **Tubular cell death → Loss of renal filtration function → AKI.**

Glutamate Excitotoxicity in Diabetic Retinopathy:

- **Chronic hyperglycemia → Retinal hypoxia (due to microvascular damage).**
- **Hypoxia triggers:**
  - **Glutamate accumulation in extracellular space (due to impaired clearance by Müller glia).**
  - **Excitotoxicity via overactivation of NMDA/AMPA receptors → Ca^{2+} overload → Mitochondrial dysfunction → ROS generation.**
- **Reperfusion-like state: Fluctuating blood flow in DR, especially in ischemic zones (non-perfused capillaries) followed by revascularization (pathological neovascularization) mimics reperfusion.**

Outcome: Retinal ganglion cell death, microvascular leakage, and progression of DR.

Shared Molecular Pathways:

Pathway	Ischemic Stroke	AKI	DR (Glutamate Toxicity)
Hypoxia	↓ oxygen/glucose	↓ oxygen/nutrients	Retinal hypoxia (ischemia)
Reperfusion injury	Blood flow restoration	Reoxygenation	Fluctuating blood flow (ischemia-reperfusion-like)
ROS generation	Yes	Yes	Yes
Mitochondrial dysfunction	Yes	Yes	Yes
Calcium overload	Glutamate receptors	Ion pump failure	Glutamate receptor overactivation

Pathway	Ischemic Stroke	AKI	DR (Glutamate Toxicity)
Glutamate excitotoxicity	**Yes (NMDA/AMPA)**	**Indirect (less prominent)**	**Major player**
Inflammation	**Microglia activation**	**Leukocyte infiltration**	**Microglial activation**
Cell death	**Neurons (apoptosis/necrosis)**	**Tubular epithelial cells**	**Retinal neurons, ganglion cells**

Conclusion:

- **Hypoxia/Reoxygenation → ROS + Calcium overload → Mitochondrial failure → Cell death is a shared pathological cascade in these conditions.**
- **Glutamate toxicity is a common feature in CNS tissues (brain, retina) but less in the kidney.**
- **Inflammatory and oxidative pathways are universal contributors, suggesting common therapeutic targets**

10. Beta-cells and NOX4 as an important modulator of mitochondrial function

Additionally regarding other indications investigated by GB:
The role of NOX4 in regulating mitochondrial function and beta-cell survival was further elucidated through the use of the pharmacological NOX4 inhibitor GLX7013114. In EndoC-βH1 cells, NOX4 inhibition enhanced insulin secretion under high glucose conditions and conferred protection against glucose- and palmitate-induced cell death. These findings were consistent across heterogeneous human islets, regardless of their size, functional activity, or glucose responsiveness.
Importantly, the study by Elksnis et al. (Biomedicines 2021, 9, 1865) demonstrated that NOX4 inhibition improves mitochondrial function in both EndoC-βH1 cells and human islets, and this improvement was closely correlated with increased cell survival across diverse islet subtypes. Unexpectedly, NOX4 inhibition was associated with an increase, rather than a decrease, in mitochondrial ROS levels, suggesting a nuanced role of ROS in beta-cell resilience.
These findings collectively support the notion that pharmacological inhibition of NOX4 activity may represent a promising therapeutic strategy in type 2 diabetes, targeting a broad spectrum of islet phenotypes through mitochondrial preservation and enhanced insulin secretory function.

11. Aging and NOX4 Activity

As we age, **NOX4 (NADPH oxidase 4) activity increases**, contributing to both **cellular homeostasis** and **oxidative stress-related aging diseases**. While **moderate NOX4 activity is necessary for normal vascular and cellular function**, excessive NOX4-derived **reactive oxygen species (ROS)** can accelerate **vascular dysfunction, neurodegeneration, and tissue damage**.

Thus the increase in NOX4 expression with aging is driven by a complex interplay of oxidative stress, chronic inflammation, mitochondrial dysfunction, and epigenetic modifications. This contributes to age-related diseases such as cardiovascular disease, neurodegeneration, and fibrosis, making NOX4 a potential target for anti-aging therapies.

In further detail regarding the turnover of NOX4 in Aging

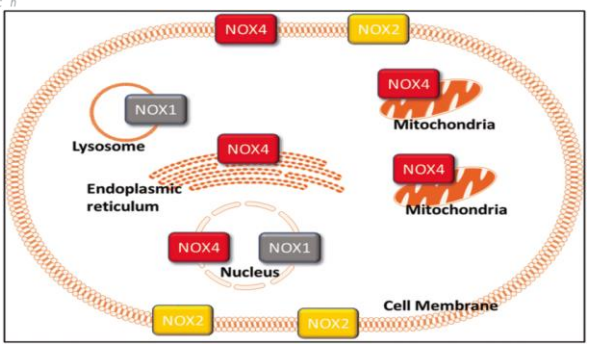
The turnover of NOX4 in older individuals involves both its **synthesis (upregulation)** and **degradation (clearance mechanisms)**. With aging, the balance shifts towards **increased NOX4 expression and reduced degradation**, leading to its accumulation and prolonged activity. Here's a breakdown of NOX4 turnover mechanisms in older individuals:

Increased NOX4 Synthesis and Stability

- **Transcriptional Upregulation**:
    - Aging-related stressors (oxidative stress, inflammation, TGF-β activation) increase **NOX4 mRNA** transcription.
    - Factors like **HIF-1α, NF-κB, and AP-1** enhance NOX4 gene expression.
- **Post-Transcriptional Stability**:
    - **miRNA Dysregulation**: Aging reduces miRNAs like **miR-25**, which normally suppress NOX4 expression.
    - **RNA-Binding Proteins (RBPs)**: Some RBPs involved in NOX4 mRNA degradation become less efficient with aging, leading to prolonged mRNA stability.
- **Reduced Proteasomal Degradation**:
    - NOX4 degradation is regulated by **ubiquitin-proteasome pathways**, but proteasomal activity declines with age.
    - The aging-related decline in **E3 ubiquitin ligases** (such as FBXO proteins) may impair NOX4 clearance.

Reduced NOX4 Degradation and Clearance

- **Limited Proteasomal Turnover**
    - Under normal conditions, NOX4 undergoes **ubiquitination and degradation by the proteasome**.
    - Aging-related **proteasome inefficiency** leads to NOX4 accumulation, particularly in endothelial cells and fibroblasts.
- **Lysosomal Degradation Pathways**
    - **Autophagy Dysfunction**:
        - NOX4 can be degraded through **macroautophagy**, but aging leads to impaired autophagy (reduced expression of LC3, p62, Beclin-1).
        - This prevents NOX4 clearance, promoting oxidative stress and cellular damage.
- **Membrane Anchoring and Persistence**
    - NOX4 is mainly located in **intracellular membranes (ER, mitochondria, perinuclear regions)**, making it less accessible to rapid degradation.



- Unlike other NOX isoforms that require activation, NOX4 is constitutively active, meaning its prolonged presence leads to **continuous ROS production**.

3. Consequences of Prolonged NOX4 Turnover in Aging

- **Vascular Dysfunction**: NOX4 accumulation in endothelial and smooth muscle cells leads to **increased oxidative stress, endothelial senescence, and vascular stiffness**.
- **Fibrosis and Organ Damage**: Excess NOX4-mediated ROS in aged tissues enhances **TGF-β signaling**, promoting fibrosis in the **lungs, heart, and kidneys**.
- **Neurodegeneration**: In the aging brain, prolonged NOX4 activity contributes to **oxidative damage, mitochondrial dysfunction, and neuroinflammation**, linked to Alzheimer's and Parkinson's diseases.

Potential Strategies to Modulate NOX4 Turnover in Aging

- **Enhancing NOX4 Degradation**:
  - **Boosting proteasomal activity** (e.g., proteasome activators, caloric restriction).
  - **Restoring autophagy** using **rapamycin, spermidine, or metformin**.
- **Reducing NOX4 Synthesis**:
  - **Targeting transcriptional regulators (e.g., NF-κB, HIF-1α inhibitors)**.
  - **Restoring miRNA expression (e.g., miR-25 mimetics to suppress NOX4 mRNA stability)**.
- **Pharmacological NOX4 Inhibitors**:
  - **GKT137831 (Setanaxib)**: A NOX4 inhibitor under investigation for fibrosis and vascular aging.
  - **GLX7013114 and GLX7013159 under investigation**

How aging and NOX4 activity affects health

Increased NOX4 Expression with Age

- **Aging cells (senescent cells) show higher NOX4 expression**, particularly in blood vessels, kidneys, heart, and brain.
- The **oxidative stress theory of aging** suggests that accumulated ROS damage from enzymes like NOX4 contributes to cellular aging.

- **Mitochondrial dysfunction** in aging cells can also trigger NOX4 activation, creating a feedback loop of oxidative stress.

Age-Related Oxidative Stress & NOX4-Driven Damage

- NOX4 produces **hydrogen peroxide (H_2O_2)**, which in normal amounts is beneficial (e.g., cell signaling).
- However, in aging tissues, **chronic NOX4 activation leads to excessive ROS, mitochondrial dysfunction, and cellular senescence**.
- **Aged blood vessels** with high NOX4 activity become stiff and less responsive to nitric oxide (NO), leading to **hypertension and cardiovascular disease**.

12. Lifestyle

Lifestyle factors such as diet, exercise, stress, and sleep can influence **NOX4 (NADPH oxidase 4) activity**, which plays a crucial role in oxidative stress, inflammation, and cellular signaling.

Diet

- **Increased NOX4 activity**: Diets high in **saturated fats, refined sugars, and processed foods** can increase oxidative stress, leading to higher NOX4 expression, especially in endothelial cells and cardiovascular tissues.
- **Decreased NOX4 activity**: **Antioxidant-rich foods** (e.g., fruits, vegetables, nuts, and green tea) help regulate NOX4 activity by neutralizing reactive oxygen species (ROS).

Physical Activity

- **Moderate exercise**: Regular aerobic exercise (e.g., walking, cycling) can **balance NOX4 activity**, reducing oxidative stress and inflammation.
- **Intense exercise**: Overtraining may **increase NOX4-mediated oxidative stress**, contributing to muscle fatigue and tissue damage.

Conclusion

A balanced lifestyle—encompassing healthy eating, regular physical activity, effective stress management, sufficient sleep, and moderation in alcohol consumption and smoking—plays a vital role in promoting good health. Central to this process is the NOX4 isoform, which serves as a key regulatory mechanism in oxidative stress balance. While physiological levels of NOX4 support normal cellular functions, excessive or dysregulated NOX4 activity, such as in diabetes or under chronic stress, contributes to pathological outcomes.

By maintaining a healthy lifestyle, individuals can modulate NOX4 activity, reduce oxidative stress, and protect against chronic diseases. **In cases where lifestyle modifications are insufficient or when disease states demand targeted intervention, selective and specific NOX4 inhibition offers a promising and safe therapeutic strategy for a range of conditions.**

13. Abbreviations

Term	Category	Function / Role
HIF-1α	Hypoxia Signaling	Activates genes (e.g., VEGF, GLUT1) under low oxygen.
TGF-β	Cytokine / Fibrosis	Regulates inflammation, fibrosis, EMT, and tissue scarring.
IL-6	Pro-inflammatory Cytokine	Promotes inflammation and immune response.
IL-1β	Pro-inflammatory Cytokine	Key mediator of inflammation and cell death.
TNF-α	Pro-inflammatory Cytokine	Induces fever, apoptosis, and cytokine production.
TLR4	Immune Receptor	Detects pathogens/DAMPs, activates NF-κB.
NF-κB	Transcription Factor	Drives inflammatory gene expression.
EPO	Hormone / Neuroprotective	Stimulates RBC production, protects neurons.
GLUT1	Glucose Transporter	Brings glucose into cells; upregulated by HIF-1α.
MAPK	Signaling Pathway	Regulates cell survival, apoptosis, and stress response.
Glutamate	Neurotransmitter	Excess causes excitotoxicity via NMDA/AMPA receptor overstimulation.
AMPA injections	Excitotoxicity Model	Mimic glutamate-induced neuronal damage.
Proteases	Enzyme	Degrade proteins; involved in apoptosis and inflammation.
Lipases	Enzyme	Break down lipids; excessive activity damages membranes.
Nucleases	Enzyme	Degrade DNA/RNA during cell death.
BBB	Barrier System	Protects brain; regulates molecule entry.
BRB	Barrier System	Protects retina; regulates transport and prevents edema.
Amacrine cells	Retinal Interneurons	Modulate signal in retina between bipolar and ganglion cells.
Gliotic Müller cells	Retinal Glia (Reactive)	Scar-forming, support retina under stress/disease.

Term	Category	Function / Role
Activated microglia	CNS Immune Cells	Respond to injury; secrete cytokines and phagocytose debris.
Astrocyte	CNS Support Cells	Regulate BBB, provide nutrients, and modulate inflammation.
Microglia	CNS Immune Cells	Surveillance and immune response in CNS.
Müller cells	Retinal Glia	Maintain homeostasis; become gliotic in disease.
Pericytes	Vascular Support Cells	Stabilize blood vessels, maintain BBB/BRB.
Endothelial cells	Vascular Cells	Line blood vessels; regulate permeability and flow.
Fibrotic signaling	Pathological Pathway	Mediates tissue scarring (e.g., via TGF-β, CTGF).
Metalloproteinases	ECM-Degrading Enzymes	Break down ECM; contribute to barrier breakdown and remodeling.
EndoC-βH1	Human Cell Line	Model for insulin-secreting pancreatic beta cells.
miRNA	Gene Regulation	Post-transcriptional repression of gene expression.
mRNA	Gene Expression	Translates DNA code into proteins.
DNA	Genetic Material	Blueprint for all cellular functions.
Autophagy	Cell Survival/Death Process	Degrades cellular components; protective or damaging depending on context.
Perinuclear regions	Cellular Region	Area near nucleus; involved in organelle/organelle signaling, stress responses.
ATP	Energy Molecule	Powers all cellular functions; depleted during I/R injury.
IC_{50}	Pharmacological Metric	Concentration of drug that inhibits 50% of its target activity.
I/R injury	Pathological Condition	Reperfusion-induced tissue damage due to ROS, inflammation, and cell death.

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Chapter 2.

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Chapter 7

Key Studies on NOX4 in Ischemic Stroke

1. Post-Stroke Inhibition of Induced NADPH Oxidase Type 4 Prevents Oxidative Stress and Neurodegeneration
   Kleinschnitz et al., PLoS Biology, 2010
   This study identified NOX4 as a major source of oxidative stress in stroke. Mice lacking NOX4 ($Nox4^{-/-}$) exhibited reduced oxidative stress, blood-brain barrier (BBB) leakage, and neuronal apoptosis after cerebral ischemia. Pharmacological inhibition of NOX4 with VAS2870 also provided significant neuroprotection, suggesting NOX4 as a promising therapeutic target.
2. Oxidative Injury in Ischemic Stroke: A Focus on NADPH Oxidase 4
   Li et al., Oxidative Medicine and Cellular Longevity, 2022
   This comprehensive review discusses the upregulation of NOX4 following cerebral ischemia and its role in ROS production, BBB disruption, and neuronal death. It also explores therapeutic strategies targeting NOX4, including natural compounds and novel delivery systems.
3. Neuroprotection After Stroke by Targeting NOX4 as a Source of Oxidative Stress
   Radermacher et al., Antioxidants & Redox Signaling, 2013
   This review emphasizes NOX4 as the predominant source of ROS in ischemic stroke, surpassing other NOX isoforms. The authors advocate for the development of isoform-specific NOX inhibitors as potential neuroprotective agents.
4. Pathophysiology and Therapeutic Potential of NADPH Oxidases in Ischemic Stroke-Induced Oxidative Stress
   Zhang et al., Oxidative Medicine and Cellular Longevity, 2021
   This article highlights NOX4's specific involvement in cerebral ischemia, noting its expression in endothelial cells, neurons, and smooth muscle cells. The study underscores NOX4's role in BBB disruption and neuronal apoptosis, reinforcing its potential as a therapeutic target.
5. Foxo1-Induced miR-92b Down-Regulation Promotes Blood-Brain Barrier Damage After Ischemic Stroke by Targeting NOX4
   Shen et al., Journal of Cellular and Molecular Medicine, 2021
   This research uncovers a molecular mechanism where downregulation of miR-92b leads to increased NOX4 expression, resulting in BBB damage post-stroke. Targeting this pathway could offer new therapeutic avenues.
6. GB internal report together with KI, Huddinge demonstrating that treatment using GLX7013114 protect ischemic/reperfusion injury in ischemic stroke mice models. Improved physical function post stroke.

Chapter 8

Ref. showing NOX4's role in AKI or a summary of key research papers on this topic

1. Jiameng Li et.al, "NOX4 is a potential therapeutic target in septic acute kidney injury by inhibiting mitochondrial dysfunction and inflammation" Theranostics 2023 May 8;13(9):2863-2878.
2. Cristina García-Caballero et. al, "Nox4 is involved in acute kidney injury associated to intravascular hemolysis". Free Radic Biol Med 2024 Nov 20:225:430-444
3. Xiao-Ming Meng et. al, "NADPH oxidase 4 promotes cisplatin-induced acute kidney injury via ROS-mediated programmed cell death and inflammation". Laboratory Investigation (2018) 98, 63–78

4. Tomas A. Schiffer et. al, “Specific NOX4 Inhibition Preserves Mitochondrial Function and Dampens Kidney Dysfunction Following Ischemia–Reperfusion-Induced Kidney Injury” Antioxidants 2024, *13*(4), 489 (Glucox Biotech publication)
5. Wei Wei et. al, “Remote Ischemic Preconditioning Attenuates Mitochondrial Dysfunction and Ferroptosis of Tubular Epithelial Cells by Inhibiting NOX4-ROS Signaling in Acute Kidney Injury” Int. J. Biol. Sci. 2025, Vol. 21

Chapter 11

Ref.

1. Aleksandr E. Vendrov et. al, “NOX4 NADPH Oxidase-Dependent Mitochondrial Oxidative Stress in Aging-Associated Cardiovascular Disease” ANTIOXIDANTS & REDOX SIGNALING Volume 23, Number 18, 2015
2. Tetsuro Ago et. al, “The NADPH Oxidase Nox4 and Aging in the Heart” AGING, December 2010, Vol.2 No.12
3. Elżbieta Cecerska-Heryć et. al, “Activity of erythrocyte antioxidant enzymes in healthy women depends on age, BMI, physical activity, and diet” *Journal of Health, Population and Nutrition (2022) 41:35*
4. Kai Chen et. al, “Regulation of ROS signal transduction by NADPH oxidase 4 localization” J. Cell Biol. Vol. 181 No. 7 1129–1139
5. Chandrika Canugovi et. al, “Increased mitochondrial NADPH oxidase 4 (NOX4) expression in aging is a causative factor in aortic stiffening” *Redox Biology 26 (2019) 101288*
6. Tetsuro Ago et. al, “Upregulation of Nox4 by hypertrophic stimuli promotes apoptosis and mitochondrial dysfunction in cardiac myocytes” Circ Res. 2010 April 16; 106(7): 1253–1264.
7. Andrey Lozhkin et. al, ”NADPH oxidase 4 regulates vascular inflammation in aging and atherosclerosis” J Mol Cell Cardiol. 2017 January ; 102: 10–21.
8. Tamara M. Paravicini et. al, “Increased NADPH-Oxidase Activity and Nox4 Expression During Chronic Hypertension Is Associated With Enhanced Cerebral Vasodilatation to NADPH In Vivo” *Stroke*. 2004; 35:584-589.
9. L E Rikans et. al, “Effect of aging on enzymatic antioxidant defenses in rat liver mitochondria” Gerontology 1992;38(3):133-8.
10. Judge S et. al, “Age-associated increases in oxidative stress and antioxidant enzyme activities in cardiac interfibrillar mitochondria: implications for the mitochondrial theory of aging” FASEB J. 2005 Mar;19(3):419-21
11. Rikans LE et. al, “Sex-dependent differences in the effects of aging on antioxidant defense mechanisms of rat liver” Biochim Biophys Acta. 1991 May 24;1074(1):195-200
12. Öztürk G et. al, “Age-related changes in the rat brain mitochondrial antioxidative enzyme ratios: modulation by melatonin” Exp Gerontol. 2012 Sep;47(9):706-11

Reference of all GB publications

Pancreas and diabetes

1. Pharmacological Inhibition of NOX4 Improves Mitochondrial Function and Survival in Human Beta-Cells. Elksnis A, Cen J, Wikström P, Carlsson PO, Welsh N. Biomedicines. 2021 Dec 8;9(12):1865. doi: 10.3390/biomedicines9121865.PMID: 34944680 Confidential and Proprietary. Revision 2. 21
2. The selective NOX4 inhibitor GLX7013159 decreases blood glucose concentrations and human beta-cell apoptotic rates in diabetic NMRI nu/nu mice transplanted with human islets. Elksnis A, Welsh N, Wikström P, Lau J, Carlsson PO. Free Radic Res. 2023 May-Jun;57(6-12):460-469. doi: 10.1080
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Diabetic Retinopathy

1. Topically Administered NOX4 Inhibitor, GLX7013114, Is Efficacious in Treating the Early Pathological Events of Diabetic Retinopathy Stavroula Dionysopoulou, Per Wikstrom, Claudio Bucolo, Giovanni Luca Romano, Vincenzo Micale, Richard Svensson, Dimitris Spyridakos, Niki Mastrodimou, Spiros Georgakis, Panayotis Verginis, Erik Walum, and Kyriaki Thermos Diabetes 2023;72:638–652 | https://doi.org/10.2337/db22-0515
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Cardiology

A small molecule inhibitor of Nox2 and Nox4 improves contractile function after ischemia–reperfusion in the mouse heart Ferenc L. M. Szekeres, Erik Walum, Per Wikström & Anders Arner Scientific Reports | (2021) 11:11970

Ischemic stroke

Selective NOX4 Inhibitor M114 Reduces Stroke-Induced Brain Damage in Mice
V. Darsalia & C. Patrone Karolinska Institutet, Huddinge, Final Report April 2022

Acute kidney injury (AKI)

Tomas A. Schiffer et.al., “Specific NOX4 Inhibition Preserves Mitochondrial Function and Dampens Kidney Dysfunction Following Ischemia–Reperfusion-Induced Kidney Injury” Antioxidants **2024**, 13, 489.