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NADPH oxidase: a therapeutic target for hyperoxaluria-induced oxidative stress – an update

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“to reduce the production of ROS and cellular damage associated with hyperoxaluria, a combined treatment which restores mitochondrial structure and function, prevents further mitochondrial damage and inhibits NADPH oxidase activation may prove valuable.”

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NADPH oxidases act as a catalyst in the formation of superoxide anion radical and production of other reactive oxygen species (ROS) such as hydrogen peroxide, which leads to oxidative stress (OS) and causes damage to macromolecules. NADPH oxidase has seven known isoforms: NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1 and DUOX2. These isoforms are comprised of various core catalytic subunits: p22phox, p47phox, p67phox, p40phox, DUOX activator 1, DUOX activator, NOXA1 and NOXO1.

OS & antioxidant defense

There is a subtle equilibrium between the ROS and the antioxidants present in the biological system. Adequate levels of ROS play an essential part as secondary messengers in numerous intracellular and intercellular signaling pathways maintaining an equilibrium. When this balance is disturbed, excess ROS leads to OS which in turn causes damage to numerous macromolecules of the cell. ROS include superoxide anion ($O_2^{\cdot-}$), nitric oxide radical ($NO^{\cdot}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2), all produced by different signaling pathways which has been discussed elsewhere in detail [1]. ROS in excess causes OS and the cells react by increasing intracellular levels of antioxidants. However, the oxidants overpower the production of these antioxidants within the biological system-resulting in the redox state in the cell becoming more oxidizing.

OS has an adverse effect on the kidneys and numerous diseases of the kidneys are affected by ROS. Excessive ROS production leads to inflammatory cell and pro-inflammatory cytokine production, further leading to initial inflammation. At this stage, an important role is played by $TNF-\alpha$ and $IL-1\beta$ as a pro-inflammatory mediator as well as $NF-\kappa B$ for transcriptional regulation of the inflammatory process. At the later stages, there is increased production of $TGF-\beta$ which coordinates the extracellular matrix synthesis.

So, in the initial stages, OS on the kidneys will lead to inflammation which in the end stages leads to the formation of abundant fibrotic tissue that will decrease organ function, leading to renal failure [2]. As reviewed by Pizzino *et al.*, some medicines such as gentamycin, bleomycin, tacrolimus and cyclosporine increase the level of super oxides and OS by lipid peroxidation and hence are nephrotoxic. Also, the heavy metals such as Cd, Hg, Pb and As, and transition metals like Fe, Cu, Co and Cr are robust oxidants leading to different kinds of nephropathy as well as some types of cancers [2].

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NADPH oxidase expression

Of interest to us in this commentary is NADPH oxidase as a therapeutic target for OS – especially in the kidneys. There are other sources of ROS such as XO, uncoupled endothelial NO synthase and mitochondrial dysfunction along with NADPH oxidase which plays a significant role in production of OS [3]. Different forms of NADPH oxidases are expressed in the kidneys as Nox1, Nox2 and Nox4 (shown to be expressed in the cortex), and Nox2, p22phox, p47phox and p67phox have been present in the thick ascending loop of Henle. Nox4 has also been shown to be present in mesangial cells and podocytes, whereas macula densa has abundance of p22phox, p47phox and p67phox subunits [1,4].

Studies have shown that AT-II infusion leads to the activation of NADPH oxidase which causes increased expression of p22phox and Nox1 in the renal cortex. When AT-II production increases abnormally, it causes vascular remodeling and endothelial dysfunction. It not only increases Nox activity but also up regulates SOD enzyme, in order to maintain a balance with increased level of ROS. When this balance is disrupted, ROS production becomes out of control, leading to pathophysiological consequences [3]. A previous in-depth review has shown that if angiotensin remains for a long time in the kidneys, it can cause activation of NADPH oxidase, increased expression of p22phox and decrease in scavenging enzyme SOD leading to renal cortical hypoxia, renal vasoconstriction and hypertension [5]. In the study, there was up regulation of Nox1 in rat cultured vascular smooth muscle cells by PDGF, Ang II and serum, whereas there is down regulation of Nox4 in the renal cortex due to excessive AT-II. AT-II activates p67phox expression in rabbit peri-adventitial fibroblasts and the mouse aorta. These research findings discussed in the review provide an insight on the role of angiotensin on the different subunits of NADPH oxidase and the regulation of expression of NADPH oxidase in the kidneys.

Antioxidants & inhibitors of NADPH oxidase

Antioxidants such as vitamin E (α -tocopherol), vitamin C and the combination of both have been used to eliminate OS; however, many clinical studies have shown that it is not easy to remove OS with increased levels of antioxidants as reviewed by Cifuentes-Pagano *et al.* [6]. This would indicate that the more favorable master plan to tackle the harmful consequences of OS is to directly access and stop the source of ROS generation, which in the majority of the cases involves different NADPH isozymes. As a matter of fact, a long search for specific inhibitors of different NADPH isozymes has been going on for more than two decades. Focusing on NADPH oxidase activity has a lot of therapeutic potential for number of diseases including chronic kidney disease, pancreatic cancer, hypertension, cystic fibrosis, Parkinson's disease, pulmonary fibrosis, acute lung injury, heart failure, ischemia/reperfusion injury and stroke to name a few [6]. There are numerous ways to inhibit NADPH oxidase isozymes due to their multiplex structure and distinct regulation of each isozyme.

OS is considered a significant contributor to oxalate and calcium oxalate crystal induced renal injury and inflammation with the involvement of NLRP3 inflammasome [7,8]. Induction of hyperoxaluria and CaOx crystal deposition in rat kidneys was associated with upregulation of genes involved in inflammasome activation, encoding for NADPH oxidase and production of crystallization modulators. Apocynin treatment caused downregulation of genes and reversed many of the changes. Hyperoxaluria in mice deficient in NLRP3 and ASC adapter protein did not produce CaOx crystal deposition in the kidneys [9]. Previously, we did a comprehensive review on NADPH oxidase as a therapeutic target for oxalate induced injury in kidneys and listed more than 30 inhibitors, along with their mode of action and their pharmacological effects. This review showed that chemicals involved in inhibition of ROS were more efficient than general antioxidants such as vitamin E, which is less efficient due to various factors, including decreased bioavailability [1].

Different kinds of inhibitors

Various peptide and nonpeptide inhibitors are available which inhibit the association of NADPH oxidase complex assembly. Special attention should be aimed at p47phox or the NOXO1 subunits, other molecular subunits for therapy may be the activator such as p67phox and NOXA1 along with Rac. The goal is to develop an inhibitor with increased efficiency and specificity of binding with the protein subunits. Li *et al.*, in their review, separated the NADPH oxidase inhibitors into two classes; peptidic inhibitors and small molecule inhibitors [10]. The peptidic inhibitors include Nox2ds-tat and NoxA1ds. Nox2ds-tat has high specificity for Nox2 isozyme with no effect on Nox1, Nox4 or XO. It is the most extensively used isoform-specific inhibitor of Nox2 isozyme. It inhibits any interaction between Nox2 and p47phox by forming a complex with Nox2 and preventing p47phox translocation to the plasma membrane. The only downside is expected poor oral bioavailability which is undergoing improvement

with emerging technologies for better stabilization, such as peptide stapling and nanoparticle formulations. NoxA1ds inhibits the activation domain of Nox1 activator subunit NoxA1 which is homologous to p67phox domain, thus inhibiting isozyme activation. The small molecule inhibitors include diphenylene iodonium (DPI), apocynin, VAS2870, VAS3947, GKT13690, GKT137831, ML171, ebselen and tetrahydroquinolines to name a few. DPI and apocynin have been widely utilized as traditional and broad spectrum Nox inhibitors; however, they do generate off-target effects. In many studies they have shown protective effects against numerous diseases. The triazolopyrimidine derivatives VAS2870 and VAS3947 are optimistic Nox inhibitors as they have been shown to reduce ROS in cell lines, including primary vascular endothelial and smooth muscle cells without inhibiting XO or eNOS activity. VAS2870 is known as pan-Nox inhibitor as it inhibits Nox1, Nox2, Nox4 and Nox5 [10].

Current reviews & emerging approaches

A recent study has shown antioxidant effects of inorganic nitrite in lipopolysaccharide-induced OS. Inorganic nitrite, formerly considered harmful, was shown to be identified as an important biological NO reservoir with organ protective effects on OS and inflammation. This provides a new insight to regulate the OS and inflammatory response [11]. Another study showed the combined effect of N-acetyl cysteine and apocynin to improve renal functions in hyperoxaluric rats by reducing the activity of NADPH oxidase and mitochondrial dysfunction. This combinatorial approach showed promising therapeutic benefits in hyperoxaluria-induced nephrolithiasis [12]. A current review by Yousefian *et al.* has shown that polyphenolic compounds act as modulators of NADPH oxidase [13]. The active ingredients of different plants such as berberine, thymoquinone, catechin, celastrol, resveratrol and curcumin have shown anti-inflammatory, antibacterial and antioxidants effects. Flavonoids with a hydroxyl group, a methoxy group in ortho position in ring b and saturated 2,3-bond in ring c are the most dynamic NADPH oxidase inhibitors. They showed that the most powerful natural phenolic compound recently studied as NADPH oxidase inhibitors are hesperidin and G-hesperidin [13]. Another study has shown the beneficial effect of a peptide inhibitor NF02 which potently inhibits ROS production of Nox1/Noxo1/Noxa1 complex and did not affect Nox2 and XO [14]. Naringin, a natural flavanone glycoside inhibited Nox4 expression at mRNA and protein levels in streptozotocin induced diabetic nephropathy rats and high glucose-induced podocytes demonstrating that naringin improves diabetic nephropathy [15]. Another study showed GSK2795039, a novel small molecule Nox2 inhibitor to demonstrate Nox2 inhibition *in vivo* in a mice model. It also showed selectivity over other NADPH oxidase isoforms, XO and other eNOS enzymes [16]. A recent study has shown the protective role of ER β in renal calcium oxalate crystal formation by diminishing liver oxalate biosynthesis and renal OS mediated cell injury. ER β prevented oxalate induced OS via transcriptional suppression of the Nox2 by direct binding to estrogen response elements on the Nox2 5' promotor region [17]. To improve the efficacy and bioavailability of iodonium class of NADPH oxidase inhibitor DPI, this cohort synthesized 36 analogs to improve selectivity, solubility and functionalization of DPI. Their results showed that DPI and four analogs NSCs 740104, 751140, 734428, 737392 strongly inhibited colon cancer cell line (HT-29) growth with both 737392 and 734428 showing greater than tenfold activity against ROS production inhibiting DUOX2. They exhibited structural characteristics of iodonium-class inhibitors that affect target selectivity, and this may help in future designing of specific Nox inhibitors [18]. In a recent study of NADPH oxidase inhibitors in terms of potency, specificity and mode of action, DPI, apocynin, diapocynin, Ebselen, GKT136901 and VAS 2870 were tested on cellular and molecular assays. The results showed that DPI generated dose-dependent inhibition of all isoforms in all the assays, and all other molecules showed interesting characteristics and specificity toward different isozymes of NADPH oxidase [19]; although, the role of DPI *in vivo* is not very promising. As mentioned above GSK2795039 stands out as a competitive Nox2 inhibitor which has the off-target effects, specificity, mechanism of action and *in vivo* potency advertently addressed.

Conclusion

We need additional NADPH oxidase inhibitors to be developed which can undergo and pass clinical trials. A significant limitation in the development of NADPH oxidase therapeutics is the absence of reliable methods to measure NADPH oxidase activity *in vivo*. Another problem which can emerge from NADPH oxidase inhibition is the interference with neutrophil's antimicrobial defense. Gene mutations affecting the Nox2 complex lead to an immune deficiency called chronic granulomatous disease (CGD). CGD patients have defective neutrophil oxidative burst and are susceptible to recurrent infections and the development of Granuloma. Since mitochondrial protection has been shown to reduce the production of ROS and cellular damage associated with hyperoxaluria [12,20] a

combined treatment which restores mitochondrial structure and function, prevents further mitochondrial damage and inhibits NADPH oxidase activation may prove valuable.

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