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Apocynin: Mother Nature's gift to combat oxidative stress

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Abstract

Oxidative stress is a hallmark of several cardio- and neuro-vascular pathophysiological events. However, the approach of using classical antioxidants has not been fruitful due to the vicious redox cycling. In this context identifying the sources of oxidative stress and discovering/developing their selective inhibitors is envisaged to be of therapeutic potential in several clinical conditions. NADPH oxidases are one of such oxidative stress generators, which have gained increasing momentum in the recent years. Apocynin, a compound isolated from natural sources, is the one specific inhibitor of NADPH oxidases with highest potential for drug development. This review briefly describes the biology of NADPH oxidases and the pharmacognosy of apocynin.

Key words: Apocynin, Oxidative stress, NADPH oxidase, Reactive oxygen species

Reactive oxygen species (ROS) are a family of highly reactive molecules that are formed within eukaryotic cells both enzymatically and non-enzymatically by the one electron reduction of molecular oxygen, yielding superoxide anions ($O2^{-\bullet}$). ROS contribute to numerous cardio- and neuro-vascular pathophysiological processes (1, 2). In the recent times NADPH oxidases have been identified as the predominant source of ROS under several pathophysiological processes as they are ubiquitous to all vascular types and layers (2, 3). The structure and function of NADPH oxidase was first characterized in neutrophils, wherein at least 5 protein subunits were described: (a) a large, membrane-bound flavoprotein gp91phox (according to new nomenclature Nox2, which can be replaced in non-phagocytic cells by its isoforms Nox1, Nox 3, Nox4 or Nox5) that contains binding sites for NADPH, molecular oxygen, FAD and two heme groups; (b) a smaller membrane bound subunit, p22phox, whose primary function is to stabilize expression of the flavoprotein in the membrane (4). Together with the flavoprotein, p22phox constitutes the flavocytochrome b558 reductase domain of Nox; (c) a cytosolic activator protein (p67phox or NoxA1) which binds to the reductase domain to induce a conformational change allowing flow of electrons from NADPH to molecular oxygen; (d) a cytosolic organizer protein (p47phox or NoxO1) that facilitates binding of the activator to cytochrome b558; (e) a small G-protein Rac1 or Rac2 which regulates NADPH oxidase activity via a two-step mechanism involving an initial functional interaction with cytochrome *b*558 to catalyze electron transfer to bound FAD, followed by a subsequent interaction with p67*phox* that results in electron transfer to the cytochrome *b*558-bound heme (5) (Figure 1). Translocation of the cytosolic subunits to cytochrome b558 leads to activation of the oxidase and to the well characterized oxidative burst, which is crucial for host defense (6). An important event for activation is the phosphorylation of p47^{phox}, which allows binding of p47^{phox} to the membrane components and initiates the cascade that leads to the respiratory burst (7). The vascular NADPH oxidases differ from the phagocytic one, as they continuously produce low levels of superoxide ($O_2^{-\cdot}$), while the pagocytic NADPH oxidase, which release large amounts of $O_2^{-\cdot}$ upon activation by suitable stimulus. In addition to Nox2, two novel homologues were shown to be expressed in the vasculature, namely Nox1 and Nox4 (8), which share its binding sites for NADPH, FAD and heme. The composition of these Nox1 and Nox4 based oxidase complexes is poorly investigated. The mechanism/s of activation of the vascular NADPH oxidases are by far not as well established as for the phagocytic oxidase, but p47^{phox} (9-11) or its recently discovered homologue NoxO1 (12), p22 phox (4), and/or protein disulphide isomerase (13) appear to be essential. Vascular NADPH oxidases are activated and regulated by a variety of hormones, cytokines and chemokines such as angiotensin II, thrombin, PDGF and TNFα (3), which are well known mediators of vascular pathophysiology.

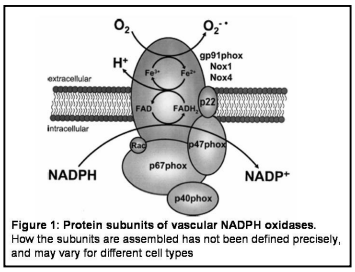

Figure 1: Protein subunits of vascular NADPH oxidases. How the subunits are assembled has not been defined precisely, and may vary for different cell types

NADPH oxidase Inhibitors

Considering the role of NADPH oxidases in vascular pathophysiology, specific inhibitors of these enzymes envisage to be of immense therapeutic potential. Currently available chemical entities proposed to be NADPH oxidase inhibitors (Figure 2,3) are either mechanistically not understood and/or are unspecific. For example, diphenylene iodonium (DPI) is often used to inhibit NADPH oxidases, but little is gained from using

this compound since it is an inhibitor of all flavoenzymes (14), including other potential ROS sources like nitric oxide synthases (NOS), xanthine oxidases (XOD) and the cytochrome P450 enzymes. 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), another putative inhibitor, blocks the subunit assembly of the oxidase complex but is also a protease inhibitor (15). S17834, a benzo(b)pyran-4-one derivative, was shown to block NADPH oxidase in endothelial cells and to reduce atherosclerosis in ApoE knockout mice (16), but since its first publication no further insight in its mechanism of action was provided. Other recently discovered inhibitors are neopterin (17) and plumbagin (18), both compounds are mechanistically poorly understood. PR-39, a proline/arginine-rich antimicrobial peptide, was also shown to prevent the oxidase assembly, but it binds generally to SH-3 binding domains of proteins ((19)). A very elegant way to design a highly specific NADPH oxidase inhibitor was provided by Rey et al (20): they synthesized *gp91ds-tat*, a chimeric oligopeptide consisting of a 9-amino acid peptide (aa) derived from HIV-coat protein (tat) (allowing cell permeation), as well as a 9-aa sequence of gp91phox (known to interact with p47phox). But since peptides cannot be administered orally and possess antigenic potential, the use of *gp91ds-tat* is limited to experimental investigations.

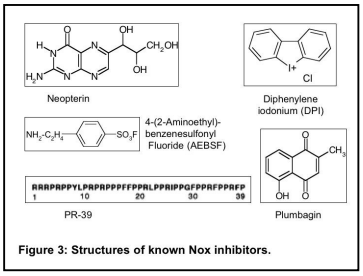

Figure 3: Structures of known Nox inhibitors.

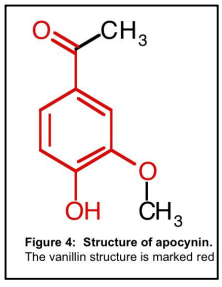
Figure 4: Structure of apocynin. The vanillin structure is marked red

Apocynin (Figure 4), an acetophenone extracted from the roots of *Apocynum cannabinum*, is widely used as specific NADPH oxidase inhibitor and is currently the only specific non-peptide inhibitor freely available. It blocks the oxidase assembly, by preventing the translocation of $p47^{phox}$ and $p67^{phox}$ to the membrane of stimulated leucocytes (21) and endothelial cells (22), however the exact mechanism of this inhibition remains to be elucidated. Apocynin is widely used even *in vivo*, because it is effective when administered orally (23) and shows very low toxicity (24-26) and is observed to show beneficial effects in several models of hypertension and diabetes.

Botanical Source of apocynin

Apocynin (4'-hydroxy-3'-methoxyacetophenone) was first described by Schmiedeberg in 1883 (27) after isolation from the roots of *Apocynum cannabinum*, Linné, (*Apocynum hypericifolium*, Aiton). The plant belongs to *Nat. Ord.*- Apocynaceae and is also referred

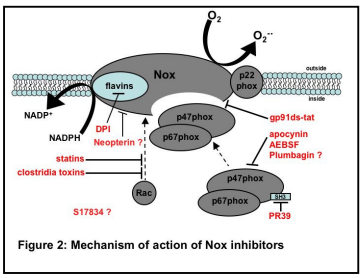

Figure 2: Mechanism of action of Nox inhibitors

by the following local names depending on its geographical location, Black Indian Hemp, Dogsbane, Black Hemp, Canadian Hemp, American Hemp, Amy root, Bowman's root, Bitter root, Hemp Dogbane, Indian Physic, Rheumatism weed, Milkweed, Wild Cotton, Choctaw root. This is an erect, branching plant, from 2 to 4 feet high, the stem is covered with a strong fibrous bark, which is green when the plant grows in the shade, and of a reddish-brown color when in sunny localities. The entire plant exudes a milky juice when wounded. The leaves are opposite, and attached to the stem at an acute angle with flowers which are small, numerous, and in close, peduncled, flat cymes. The calyx is small, with 5 narrow, sharp lobes; the corolla is 5-parted, with erect lobes, white or pale-red color, and but a little longer than the calyx. The stamens are 5, small, distinct, and included. The pistil has 2 ovaries covered by 2 united, sessile, fleshy stigmas. The fruit, which is produced by only a few of the flowers, consists of a pair of slender, diverging pods, containing numerous small seed, which are furnished with a bunch of silky, white hair at the apex. It is replete with a lactescent juice, which becomes hard on exposure to the air. The bark of the stem, when dry, from its fibrous, cohesive nature, is a superior article in the manufacture of rope, giving a white, strong, and durable production. A permanent brown or black dye, according to the mordant used, is obtained from a decoction of the plant (http://www.ibiblio.org/herbmed/eclectic/kings/apocynum-cann.html). Apocynin may also be obtained from other Apocynum species (e.g. A. androsaemifolium) or from the rhizomes of Iris species, provided that the extracts do not contain substantial amounts of cardiac glycosides. Although apocynin was first discovered in *A. cannabinum*, its occurrence is not restricted exclusively to the A*pocynaceae* family (25). In fact, it is a common compound in many plant species although its content may vary from species to species. In 1971, Basu et al. isolated apocynin from the roots of *Picrorhiza kurroa* (28) *(Nat. Ord.*- Scrophulariaceae), which is a plant growing at the high altitudes in the western Himalayas and has been used for ages in the Ayurvedic system of medicine in India and Sri Lanka. Still this drug is in use and fields of application are are as a liver tonic, a cardiotonic, and the treatment of jaundice and asthma (29). In 1990, Simons et al. subjected the roots of *P. kurroa* to an isolation procedure and established the pharmacological potential of apocynin (30). Finally, in the wood and paper industry, apocynin is known as one of the degradation products of lignin (31, 32).

Description of the root of *A. cannabinum*

The root of *A. cannabinum,* gathered in the autumn and dried, is about 1/3 of an inch in diameter, wrinkled longitudinally, and marked by occasional transverse fractures through the bark, which show the white central portion. It consists of a bark, externally ash-gray in color, beneath which is a thin, brown, corky layer, and within this, the inner bark, which is of a pale-pink color. The remainder of the root is composed of white medullary matter, perforated by numerous longitudinal tubes, disposed more thickly in concentric circles about the fortieth of an inch apart, or forming a single circle. Radiating from the center are delicate medullary rays. When gathered in the spring, or in early summer, the center is pierced by light pith, or a small cavity. The root, when dry, is brittle, and snaps readily, giving a clear, smooth fracture. As found in market, there are few, if any, fibers attached although, when fresh, the root is plentifully supplied with secondary roots; but as they are very brittle when dry, they do not remain attached. The woody portion of the root is slightly bitter; the bark is extremely bitter and disagreeable. It is described in the Pharmacopoeia as follows: "Long, cylindrical, somewhat branched, 5 to 10 mm (1/5 to 2/5 inch) thick, gray or brownish-gray, longitudinally wrinkled and transversely fissured, brittle, fracture short, white, the bark rather thick, the wood porous, spongy, with delicate medullary rays, inodorous, taste bitter, disagreeable"-(*U.S.P.*). Water readily takes up the active properties of the root, which are also partially soluble in alcohol, its virtues are impaired by age(http://www.ibiblio.org/herbmed/eclectic/kings/apocynum-cann.html) (25).

Chemical composition of the root

The root consists of tannic and gallic acids, gum, resin, wax, fecula, apocynin, coloring matter, woody fiber, and, probably, caoutchouc. From the precipitate of the fluid extract, a white, waxy component with no taste, cane-sugar crystals were obtained and two component having properties resembling those of digitalin were obtained. Additionally apocynin, an amorphous principle, resinous, nearly insoluble in water, but readily dissolved by ether and alcohol was also isolated (http://www.ibiblio.org/herbmed/eclectic/kings/apocynum-cann.html). Apocynin is an acetophenone with a molecular weight of 166.17 and forms fine needles upon crystallization from water. It possesses a faint vanilla odor and has a melting point of 115°C. The substance is slightly soluble in cold water, but freely soluble in hot water, alcohol, benzene, chloroform, and ether.

Pharmacology and safety of apocynin

Apocynin was initially used for the treatment of osteoarthritis and related inflammatory conditions. In particular, apocynin was found to be effective in retarding progression of osteoarthritis. Further, the inflammatory processes in arthritis and the associated symptoms of stiffness and pain could be alleviated by apocynin administration. The pant extract was used as official remedies for dropsy and heart troubles. Although apocynin is a specific NADPH oxidase inhibitor, it possess other effects such as it markedly decreases the intracellular reduced/oxidized glutathione ratio (GSH/GSSG) and prevents nuclear factor-kappaB (NF-kappaB) activation in stimulated monocytes in addition to inhibiting cyclooxygenase (Cox-2) synthesis and activity (33, 34). While some studies suggest that apocynin displays antioxidant properties, in part, by increasing glutathione synthesis through activation of AP-1 (35). We have observed that apocynin could directly relax blood vessels (Figure 5), an effect that is more pronounced in hypertensive than normotensive rats. This mechanisms of vasodilation is under investigation in our lab, in similar lines apocynin was reported to increase blood flow of iris, ciliary body-iris root, and choroid by 355%, 152%, and 414%, at 120 minutes after drug instillation and was indicated for treatment of ischemic eye diseases (36). Apocynin also inhibits formation of thromboxane A_2 and stimulates release of prostaglandins E_2 and F_2 alpha. Further it inhibits arachidonic acid-induced aggregation of bovine platelets, possibly through inhibition of thromboxane formation suggesting its anti-inflammatory or anti-thrombic properties (24). However it is not clear if these effects are NADPH oxidase dependent. Recently it was reported that although apocynin is an inhibitor of phagocyte NADPH oxidase it also stimulates ROS production in non-phagocyte cells (37) adding further controversies to the field.

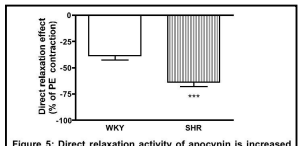

Figure 5: Direct relaxation activity of apocynin is increased in SHR. Aortic rings were precontracted with 500nM Phenylephrine (PE) and 100μM apocynin was added in the plateau of the contraction. SHR showed a significantly higher relaxation response (64.0 ±3.9% of PE induced contraction, n=12) than WKY rings (38.4 ±4.5%, n=9) (unpaired t-test, P<0.001)

Side effects of apocynin are not known. Apocynin has very low toxicity (LD50: 9 g/kg, mice p.o.) (25) and is generally regarded as safe (38). Further, apocynin didn't show genotoxic effects at concentrations up to 600 μM *in Salmonella typhimurium* mutagenicity assay (Ames test) and the sister chromatid exchange (SCE) test (39).

Summary

Considering the extensive role of NADPH oxidases in various pathophysiological processes, the potential of discovering/developing its specific inhibitor is enormous. Although Apocynin is proposed to be the most specific NADPH oxidase inhibitor available to date, weather it is selective for one isoform of NADPH oxidase as well is yet unknown. However, it at least provides a structural basis for going further in the discovery of specific and selective inhibitors. Once such compounds are available, they should be of great benefit in basic research as well as treatment of oxidative stress dependent diseases.

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