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REVIEW

Xanthine Oxido-Reductase, Free Radicals and Cardiovascular Disease. A Critical Review

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Abstract Free radical mediated pathologies occupy a special place in medical semiology and in mechanistic interpretation of diseases. Free radicals, or better reactive oxygen species (ROS) or reactive nitrogen species (RNS) play also an important role in cell signaling. This is the basis of the ambivalent (Jekyll – Hyde) situation of ROS in biology and pathology. Aging itself is attributed by a popular theory to free radicals. A number of ROS – scavenging substances and procedures were described without however reaching credibility for their therapeutic value. An interesting exception is the xanthine oxido - reductase produced ROS and their role in cardiovascular disease. Allopurinol inhibition of xanthine oxido – reductase was shown to be efficient in some cases of cardiovascular diseases. Another important aspect of xanthine oxido – reductase produced ROS is their antibacterial capacity considered to be of importance with newborns fed on milk rich in this enzyme as well as at the gastrointestinal barrier. This ambivalent role of xanthine oxido – reductase justifies this review on the basic enzymatic mechanisms involved, derived ROS production, their role in the above mentioned biological processes and especially the interest of the inhibition of this enzyme as a preventive or curative measure in some cardiovascular pathologies.

Keywords Xanthine oxidase · Free radicals · ROS · Cardiovascular disease · Allopurinol

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Abbreviations

ROS	Reactive oxygen species
RNS	Reactive nitrogen species
XOR	Xanthine oxido reductase
XO	Xanthine oxidase
XD	Xanthine dehydrogenase
AO	Aldehyde oxidase
MFE	Molybdo flavo enzymes
UA	Uric acid
NOS	Nitric oxide synthetase
CVD	Cardio vascular disease
IR	Ischemia reperfusion
MI	Myocardial infarct
HF	Heart failure
BP	Blood pressure
EPR	Electron paramagnetic resonance
NAD	Nicotine adenine dinucleotide
Kb	1000 base-unit in DNA
SOD	Superoxide dismutase

Introduction

A number of free radical- and more generally reactive oxygen species (ROS) -mediated reactions of biological interest were described over the last century. More recently reactive nitrogen species (RNS) had to be added as for instance nitric oxide ($NO^{\bullet}$), to this list. The list of harmful reactions attributed to these reactive species became quite long to comprise progressively most age-related pathologies, osteoarticular, pulmonary and cardiovascular diseases, among others [1]. It was even proposed that aging in general should be considered the result

of life – long "free radical" damage. This theory of aging, proposed originally by Harman [2] is still cited as a valid explanation of aging, supported and extended by a number of scientists, based on examples of oxidative damage to vital tissues (see for a reasonable review Comfort [3] and for an exhaustive series of reviews in a book edited by Ingrid Emerit and Britton Chance in 1992 [4].

In sharp contrast to this general agreement concerning the role of "free radicals" as causative agents of age-related pathologies, very little success was reported on the treatment of such pathologies by free radical scavenging agents [5, 6]. As nutrition contributes also to mechanisms resulting in free radical damage, anti – free radical treatments were proposed but shown mostly unsuccessful, both by pharmacological means as well as by nutritional measures [5, 6]. The only notable exception is the treatment of free radical mediated damage for the prevention of cardiovascular pathologies by the inhibition of XOR, considered as the essential culprit. In the following we shall examine in some detail the validity of this postulate. This critical examination has to take in account another important aspect of "free radical" generating systems, their potential anti-microbial effect, defending the organism against microbial infections. This type of protective effect of XOR was also claimed to play a role, essentially for newborn infants nourished on milk, rich in XOR [7].

The XOR Enzyme

Purine – pyrimidine derivatives are supposed to be among the most ancient bio-organic molecules suggested by their presence in the "primordial soup" at the origin of life, as shown by the Miller-Urey experiment – electric discharges through a chamber containing methane, water and ammonia gave rise among other molecules to purines and pyrimidines [8]. These findings agree also with the early phylogenetic appearance of XOR from prokaryotes to higher vertebrates [9] Adenosine derivatives and especially ATP are ubiquitous and major players in metabolic reactions. Early studies by Szent-Györgyi and his school indicated their importance in muscle contraction and heart function [10]. Another argument for their early biological importance is the presence of purinergic receptors on many cell-types and their physiological importance as for instance in inflammation [11]. XOR and related enzymes as for instance aldehyde oxidases are also of early phylogenetic appearance. XOR is a crucial enzyme for the catabolism of purines, hypoxanthine to xanthine to uric acid and as such involved also in some severe pathologies, gout is one of them. This explains the intense research activity around these enzymes from the early years of the last century up to recent years. Such studies were facilitated by the presence of XOR in bovine milk, source of purification and enzyme-kinetic and reaction mechanistic studies. Milk XOR was shown to be bound to the fat-globules of milk in an inactive form [12, 13]. Several physicochemical treatments were shown to "activate" and "liberate" fat-globule bound XOR, as ultrasounds, heat, pressure or detergents [14–18]. These treatments facilitated the purification of milk XOR and its kinetic studies [19]. Some years later this enzyme was prepared in crystalline form followed by intensive mechanistic studies in several countries [20–25]. Besides the Biochemistry Department of the Paris Medical Faculty [14–19] a team at the Chester-Beatty Cancer research Institute in London took an active part in these studies [20–25]. Their interest for XOR as a cancer research team was motivated by the successful treatment of spontaneous mammary tumors in mice by XOR [26] In this early study, the authors tried to explain their results, mentioning as one possible mechanism the production of H_2O_2 by this enzyme. This was however not the only mechanism envisaged. After intraperitoneal injections of the bovine milk XOR the authors demonstrated an increase of its activity not only in the peritoneal fluid but also in liver homogenates. This experiment is an early example of the curative effect of an enzyme treatment attributable at least partially to an increase of reactive oxygen products (ROS).

But before going more in depth on this subject let us remind some of the characteristics of XOR. As explained in a remarkable review by Enrico Garattini et al. [9], XOR is part of an expanding family of molybdo- flavo-enzymes (MFE-s). This is a homogenous subgroup of molybdo-proteins, associated with a flavin cofactor. As mentioned earlier, these enzymes are present in bacteria, fungi, plants and animals as a structurally and functionally related group of catalytic proteins. XORs are closely related to another important family of MFEs, aldehyde oxidases, denoted as AOX according to Garattini and others [9, 27]. We shall not insist here on other members of this family of enzymes, the above references can be consulted. These are partly cytosolic proteins, the milk enzyme is bound to the lipid — rich membrane of ductal epithelial cells, secreted as fat globules [12, 13]. Although several substrates are oxidized by these enzymes, the physiological substrates are xanthine and hypoxanthine, oxidized to uric acid. Besides milk, liver is a rich source for enzyme purification. Two distinct forms of XOR are known, xanthine dehydrogenase (XD) which gives over the extracted hydrogens to NaD^+ to yield NaDH and the xanthine oxidase form (XO) which reduces O_2 by the substrate – extracted electrons. This second reaction results in the formation of superoxide, O_2^{-} and secondarily to hydrogen peroxide (H_2O_2), both being directly involved in potential oxidative damage to cells and tissues. The conversion of XD to XO can occur reversibly, and also by proteolytic cleavage as for instance by controlled trypsin cleavage catalyzing an irreversible conversion. This results in a cleavage at two sites of the homodimeric form of XOR, one at two of three subunits. The cleaved fragments remain associated with the enzyme. The cleavage sites are situated between the 2 Fe – 2 S–sites and the flavin containing

site of the enzyme and the link of this site to the molybdene containing site. The convertibility of XD to XO is variable and specific for the XOR – s present in different species and tissues. The genes coding for several of these enzymes were cloned, the amino acid sequence as well as the tertiary structures deduced by X-ray studies on crystalline enzymes from several species and tissues and showed a 80 % overall identity of amino acid sequence [9, 27, 28]. These enzymes are composed of three basic structural domains with relatively conserved sequences, linked together by less conserved amino acid sequences acting as hinge regions. The most conserved part of these enzymes is the N-terminal domain containing the 2Fe-2S site with a sequence of eight cysteine residues coordinated with the four iron atoms, strongly conserved. This tripartite structure of these molybdo-flavo-enzymes is schematically represented on Fig. 1. according to Garattini [9] More details on the structural and functional characteristics of this family of enzymes can be found in the cited references.

Xanthine Oxidase, "Free Radical" Production and its Effects

As mentioned in the introduction, the expression "free radicals" is more popular than the "reactive oxygen or nitrogen species" (ROS, RNS) and used often to facilitate understanding for readers less familiar with this subject. As mentioned above, the XO form of XOR can only use molecular oxygen as electron acceptor during the oxidation of its substrates. Therefore ROS – production will to some extent depend on the ratio of XOR converted to XO, although this view was challenged (see later). A second remark of importance in this respect is the fact that several "free radicals" function in intercellular message communication, such as vasodilation, as synaptic neuromediators and others. For these reasons the exact molecular configuration and subcellular location of interacting substances is of major importance for the outcome, beneficial or harmful, of the molecular interactions resulting in "free radical" release. This is the molecular basis of this specific Jekyll – Hyde characteristic of ROS or RNS mediated reactions. Among the harmful effects of XO – generated ROS, the ischemia – reperfusion injury was the first to attract attention [6, 28]. Such lesions occur frequently in myocardial infarction (MI) or stroke. As proposed by Granger et al. [29] in such situations there is loss of energy source in oxygen starved cells, transmembrane ion – gradients collapse with increase of intracellular Ca, leading to Ca – dependent protease (calpain) activation which further converts irreversibly XDH to XO. Degradation of ATP increases hypoxanthine concentration which serves as substrate for XO resulting in an increase of ROS production as soon as oxygen becomes available during reperfusion. The generated superoxide and hydrogen peroxide interact to generate hydroxyl radicals, a strong oxidant of cell constituents, lipids, proteins and nucleic acids. The originally generated ROS were proposed to induce leucocyte infiltration, another source of NaDPH – oxidase, producing superoxide by reducing molecular oxygen with increased superoxide production. These reactions produce a vicious circle with increased production of aggressive free radicals such as the hydroxyl radical. Neutrophils contain also myeloperoxidase catalyzing the formation of the chlorinating agent, HOCl from ambient chloride and H_2O_2, a strong contributor to tissue damage. This mechanism was however disputed and several variants proposed [30]. Upregulation by hypoxia or inflammation of XOD activity might suffice for increased production of ROS and other aggressive products as mentioned above. Without going more in detail of these reactions, we can sum up the proposed mechanism as suggested by the Harrison group [31] as shown on Fig. 2. We should add however that cardiovascular disease is not the only pathology concerned by the above

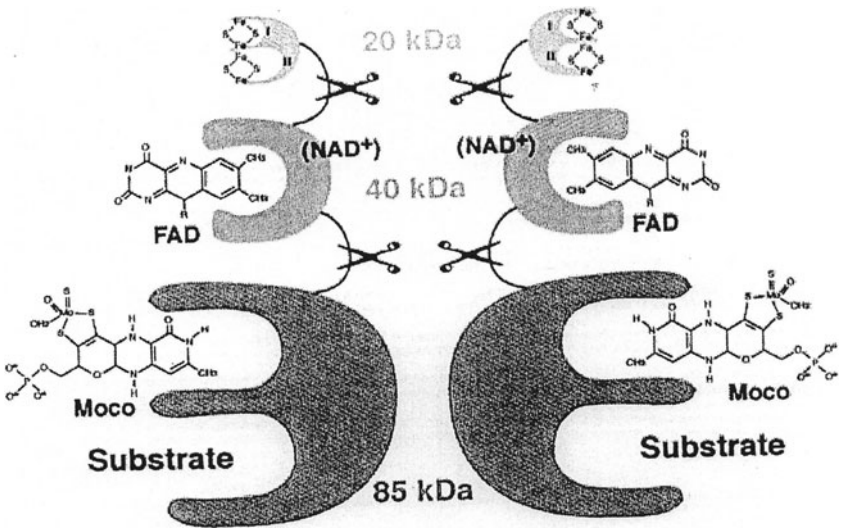

Fig. 1 Subunit structure of XOR, similar or identical to most other molybdo-flavo-enzymes comprising (from top to bottom) a 24 kDa domain, containing the two Fe-centers, linked to a 40 kDa FAD-binding domain, followed by the 85 kDa Mo-cofactor (MoCo site) as well as the substrate-binding site (modified of Fig. 1, from Garattini et al., ref. [9]) (reproduced with permission from author and editor)

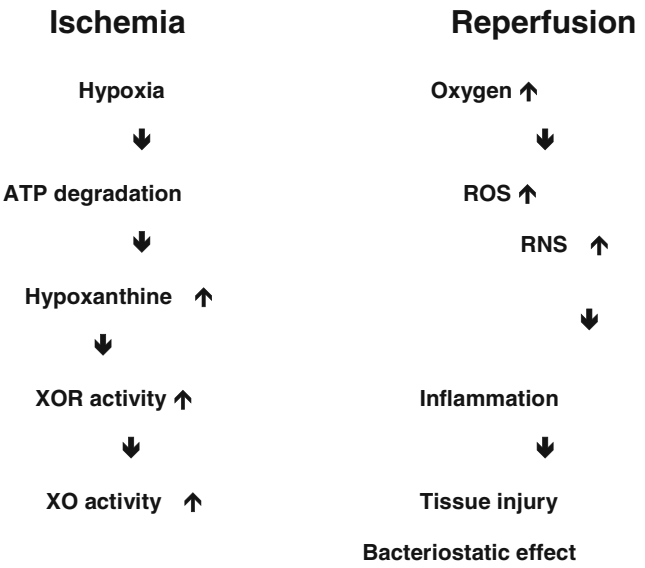

Fig. 2 Mechanisms involved in the generation of ischemia – reperfusion (IR) injury by the generation of reactive oxygen species, due to increased substrate availability by ATP – degradation, upregulation of XOR – availability and its transformation into XO leading to the generation of the inflammatory response

summarized mechanisms. Similar mechanisms were described for the gastro – intestinal tract also [32].

Bactericidal Effect of XOR

Generation of ROS and RNS by XOR and mainly by its XO form were proposed to play a role in antibacterial defense of the organism. Special attention was attracted to this possibility for newborn children nourished on milk as well as the pathology of the gastrointestinal tract, frequent site of microbial attack. Among the before mentioned mechanisms of ROS or RNS production, one further reaction deserves attention, the formation of the highly reactive anion, peroxynitrate, $ONOO^-$. This molecule can produce instantaneous cell death, both of microbs as well as of host cells. Its place in the XOD – generated reactive species is shown on Fig. 3. No doubt, the XOR produced reactive oxygen and nitrogen species might well initiate massive damage in a microbial community. In this context it has to be reminded that human breast milk is by far not as rich in XOR as bovine milk, attributed to a lower Mo coenzyme – content [33, 34]. It has to be added that the XOR protein, independently from its enzyme activity plays an important role in milk secretion by a mechanism not yet elucidated.

XOR (and XO) activity in milk were shown to reach maximal values during the first week post-partum. This is also the period of maximal fragility of the new-born. In this respect, the frequently criticized substitution of bovine milk for human breast milk falls short.

The other organ system where XOR - mediated antimicrobial activity might be of crucial importance is the gastro-intestinal

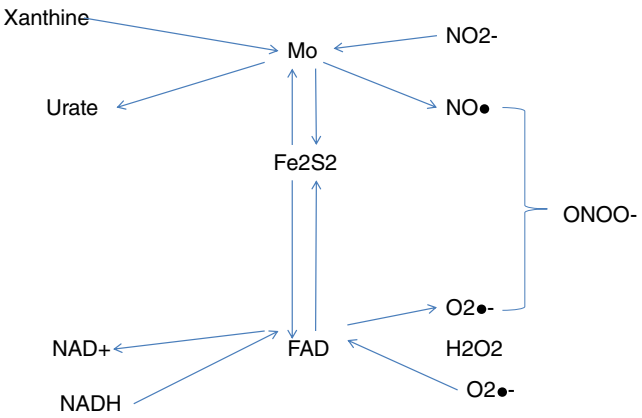

Fig. 3 Xanthine oxidoreductase catalyzed generation of reactive oxygen and nitrogen species (ROS and RNS)

tract. XOR is highly expressed in gastro-intestinal epithelial cells and might well play an important role in the protective barrier of intestinal wall. The first part of the intestine, its goblet cells and the apical layer of enterocytes of the small intestine are rich in XOR, as well as the Paneth cells. This enzyme was demonstrated in duodenal mucus also, as well as in the apical layer of esophagus and tongue epithelial cells. It could be demonstrated that bacteria are surrounded by XO – positive structures in its cornified layer. The demonstration of the reduction of inorganic nitrite to $NO^•$ exhibiting also bactericidal properties followed in presence of oxygen by peroxynitrite production, a potent bactericidal agent. Nitrite excretion by enteric bacteria is a curious example of a suicidal biosynthetic process. By this type of barrier-protective activity, XOR can be considered as part of the innate immune system.

Implications of XOR in CVD and Other Pathologies. From Genes to Diseases

Pathological situations arrive first because of gene – regulation deficiencies and than as a result of ROS – production. The gene encoding human XOR, of about 60 kb, comprises 36 exons, located on the short arm of chromosome 2 [9, 28]. The messenger RNA, with an open reading frame encodes 1,333 amino acids, their sequence is over 90 % homologous with rat and mouse XOR. The Molibdo-protein cofactor binding site is the most conserved with 94 % homology among the above mentioned species. The gene coding for aldehyde oxidase (AO) of much less clearly established function is quite similar to the XOR – gene with about 50 % homologous amino – acid sequence, showing the same redox centers and cofactors as well as sharing several substrates. Their genes are closely located on chromosome 2 suggesting their origin from an ancestral gene by tandem duplication. AO lacks dehydrogenase activity but exhibits oxidase activity and might thus contribute to unwanted side – effects of XOR.

Mutations of the XOR gene result in xanthinuria type I. Xanthinuria type II is the result of mutations of the gene coding human Molybdenum cofactor sulfurase involved in the biosynthesis of both XOR and AO. About half of patients with xanthinuria remain asymptomatic, the other half can suffer of xanthine stones, renal failure and also myopathies [35]. The patients with mutations in the Mo – cofactor sulfurase enzyme suffer from severe neurological disorders, often fatal. As XOR KO mice are runted and do not survive over 6 weeks, suggesting a greater importance of XOR in lower vertebrates than in humans [35]. Some other human diseases are also the result of more or less closely related metabolic disorders as for instance dyspurinic gout – disease resulting from deficit of hypoxanthine – guanine – phosphoribosyl - transferase, the Lesch – Nyhan syndrome (several important publications by the teams of Delbarre et al. [36] and of Ceballos – Picot [37–40].

Important pathologies can be attributed to anomalies in gene expression regulation. We shall insist only on one example, the regulation of expression of the gene coding for XOR involved in its putative role in CVD-s. As shown on Table 1, taken from the Berry – Hare review [28], oxygen tension is an important regulatory factor. Hypoxia upregulates XOR expression and hyperoxia downregulates it. The other factors mentioned on this Table are also of pathophysiological importance, especially the corticoids. Low oxygen tension as in ischemia will upregulate XOR activity. This concerns XOR in vascular cells, especially in the endothelium. This is a crucial argument for the involvement of XOR in damage caused by ischemia – reperfusion (IR) where from its study in CVD started. When the inflammatory process starts, cytokines as TNF – α, IL – 1 and 6, lipopolysaccharides will also participate in the increase of XOR activity and to its conversion to XO. Some of these effects are mediated by hypoxia – inducible factors (HIF) activated by low oxygen tension.

For a long time the conversion of XOR into XO was considered as a crucial step for an increased potential of ROS release, as mentioned above. More recent experiments shed however considerable doubt about this mechanism for XOR – damage to tissues. It appeared that even slight modifications at the FAD – site can modify electron acceptor preference [9, 28, 41]. Slight modification around this site (disruption of the neighboring amino – acids (Phe ^{549}, Arg^{335}, Trp^{336}, Arg^{427}) leading to loss of interaction between Phe^{549} and Trp^{336} alters the flavin site structure and renders it inaccessible to NaD^+, resulting in a preference for oxygen as electron acceptor. The same local conformational change facilitates the access of oxygen to the active site.

Table 1 Factors and mechanisms involved in the regulation of the expression of the gene coding for XOR. (Modified from ref [28])

Factors acting on XOR – gene expression:	Upregulation: + Downregulation: -
Hypoxia	+
Hyperoxia	–
Interleukin – 1	+
Interleukin – 6	+
Interferon – γ	+
TNF - α	+
Dexamethasone	+
Cortisol	+
Prolactin	+

As mentioned earlier, XOR to XO conversion by limited proteolytic activity is irreversible. On the contrary, thiol group oxidation (Cys^{535} and Cys^{992}) results in reversible conversion as well as freeze – storage or anaerobic conditions. During the re-oxidation of fully reduced XO, oxygen is reduced to H_2O_2 and by the transfer of further electrons to O superoxide, $O_2^{\cdot-}$ is produced. The overall reaction results in the formation of two H_2O_2-s and two $O_2^{\cdot-}$ s. It appears however that XOR in its XDH form generates more superoxide per mole O_2 because of the greater thermodynamic stability of the FAD form reacting with oxygen. This reaction is however slower, its maximal rate is about 25 % of that of XO – V_{max}. For further details see the review of Berry and Hare [28]. Furthermore XOR may also produce $NO^{\bullet}$, especially at a low oxygen environment as in IR, as shown by Li et al. [41] in hypoxic myocardium in presence of NOS inhibition. By the intermediary of its Molybden site, XOR can reduce organic nitrates and nitrites. In humans there are great individual variations in XOR activity, from about 1 to 3, this also might represent a source of individual susceptibility to XOR – derived damage. Tissue distribution also introduces another variable in XOR – reactivity.

XOR activity in vascular endothelial cells (bovine) doubles during hypoxia without increase in mRNA. Same was observed with fibroblasts. Increased oxygen tension inactivates XOR by posttranslational modification. Regulation of XOR activity might depend on phosphorylation of the enzyme [42], apparently by a p38 kinase mediated mechanism. Generated $NO^{\bullet}$ is also able to downregulate XOR activity as does also superoxide and hydroxyl radicals. These reactions might well be responsible for XOR inactivation in hyperoxia.

Details of tissue distribution of XOR may also play a role in its biological activity. Its presence in heart muscle is certainly of importance in this respect, as does its presence in small intestine, potential site of oxygen tension variations. Same holds for its presence in capillary endothelial cells. Subcellular localization is also of relevance. XOR was demonstrated in the cytoplasm and in cell – membranes where glycosaminoglycans as heparin were shown to play the role of anchors [43]. Injected heparin can thus liberate membrane – localized XOR, resulting in a significant (2–30 %) increase of plasma XOR. Heparin injection stimulates endothelial XO – release [43]. Peroxysomes in hepatocytes were also shown to contain XOR, as well as rough endoplasmic reticulum (RER)

and lysozymes in the liver. Uric acid (UA) the end – product of XOR action on xanthine and hypoxanthine has also important biological functions, well beyond its accumulation in gout. It is known that UA is a potent free radical scavenger. This capacity may have a role in the regulation of ROS – mediated signal transmission. XOR – mediated ROS was also shown to play a role in atherogenesis by oxydating LDL, thus enhancing its deposition and plaque formation. ROS produced by XOR can disrupt lysosomal membranes and liberate hydrolytic enzymes. The generated superoxide as hydrogen peroxide, via the Haber – Weiss reaction ($Fe^{+++} + O_2^{\cdot -}$ yield Fe^{++} and O_2, Fe^{++} and H_2O generate hydroxyl radicals, $^{\bullet}OH$ with a very short (1 ns) half life and will therefore oxidise any closest target. The production by XOR of $NO^{\bullet}$ will lead, via combination with $O_2^{\cdot -}$ to peroxynitrite ($ONOO^-$) formation, also an exceedingly reactive species. Nitration of proteins produced by peroxynitrite appears to be implicated in severe CVD including heart failure (HF) as well as ischemic bowel injury, involving rapid loss of ATP. Ischemic damage was shown to be attenuated by superoxide dismutase (SOD). This and other experiments tend to confirm the role of XOR in the ischemia – reperfusion (IR) syndrome [44]. Electron paramagnetic resonance (EPR) studies confirmed birsts of ROS – generation from re-perfused hearts, such as $O_2^{\cdot -}$, $OH^{\bullet}$, H_2O_2 and $ONOO^-$ resulting in heart tissue damage. Generated ventricular dysfunction could be alleviated with ROS scavengers and also by allopurinol [44]. Increasing XOR activity was shown to lead to decline of contractile function of the heart muscle, possibly through $ONOO^-$ generation. Reperfusion damage is attenuated by ROS scavengers. Another important finding was the demonstration of $ONOO^-$ - mediated activation of matrix metallo proteases (MMP-s) [45, 46] mediating further tissue damage and contributing to myocardial remodeling.

ROS- generated cell damage disrupts Ca – signaling, which has an important role in ryanodine receptor signaling, inhibited also by $ONOO^-$ as well as in Ca – ATP-ase function, representing a strong contribution to decreased myocardial function [47]

Another argument for XOR involvement in tissue damage is its strong increase (over 10^3-fold) in post-ischemic lavage fluids, with a similar increase in endothelial cells (~8x).

ATP degradation in ischemia strongly increases substrate availability for XOR as shown also by the increase of urinary excretion and serum concentration of oxidation products (xanthine, uric acid) in acute coronary syndrome and myocardial infarction. XOR - generated ROS products were shown to produce neutrophil infiltration. These white blood cells do also contribute to tissue damage in IR, they also generate ROS. Vasoactive pressure regulation is strongly perturbed by superoxide radicals inactivating $NO^{\star}$– production by the endothelium, impairing vasorelaxation. $NO^{\star}$ – reaction with superoxide and peroxynitrite production are much faster (~3×) than SOD – mediated scavenging. This is an important argument for ROS – mediated endothelial damage and increased CVD – risk. Endothelium based XOR also inhibits $NO^{\star}$ – dependent cGMP – generation in SMC-s, an important factor of $NO^{\star}$ – mediated vasodilation.

Harmful effects of XOR were further confirmed in hypercholesterolemia where superoxide production is increased (~3×) [48]. Diseased vessels exhibit a strongly increased XOR activity (›200 %), shown to be colocalized with lipid deposits in atherosclerotic plaques, exhibiting also a strong increase of UA, resulting from the increased XOR activity in plaques. XOR was shown also to play a role in hypertension, inhibited by a Tungsten – rich diet which inactivates XOR and lowers BP. Similar results were obtained in salt induced hypertension. Tungsten and oxopurinol lower BP. Salt intake increases XOR activity in Dahl salt sensitive rats. UA levels appear to correlate with BP, elevated UA level is associated with increased CV – risk in hypertension. A further argument in favor of ROS – mediation in CVD-s is the finding of reduced endothelial SOD activity in HF, whereas endothelial XOR activity is increased.

Role of XOR – Inhibition in Preventing and Treating CVDs. Experimental Results

Most of the above discussed data support the hypothesis that inhibition of XOR as well as of XO might provide a reasonable approach to alleviate or even prevent some CVDs. Such tentatives were undertaken and successful results presented as we shall review in this section. Most of these therapeutic experiments were based on the administration of allopurinol, a potent XOR inhibitor. Some other inhibitors were tested also but most clinical data are based on allopurinol administration. We shall summarize some of the experimental and clinical findings in this section.

Allopurinol (4-hydroxypyrazolo[3,4-d]pyrimidine) has a structure only slightly different from hypoxanthine and functions as an efficient competitive inhibitor of XOR (Fig. 4.). It was

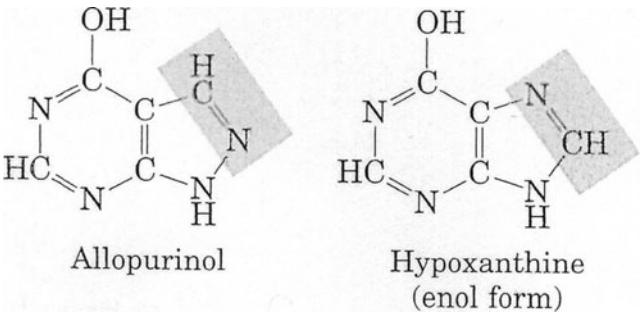

Fig. 4 The structure of allopurinol compared to that of hypoxanthine, a substrate of xanthine-oxido-reductase. A slight difference of structure makes allopurinol an efficient competitive inhibitor of the enzyme

used to treat gout, hyperuricemia [49]. The application for the treatment and prevention of CVD – s and especially of IR came as a result of the detailed knowledge of ROS – mediated tissue injury produced by ischemia and reperfusion accompanied by a decrease and increase of oxygen tension. Allopurinol reacts with the Molybden – cofactor site of XOR to yield alloxanthine (also known as oxypurinol) which binds to XOR by coordination to Molybdenum, inhibiting enzyme – substrate binding [50].

NADH – oxidase activity is resistant to allopurinol inhibition, another substance, diphenylene iodium (DPI) a non – specific FAD – inhibitor does inhibit NADH oxidase activity [51]. For these same reasons allopurinol interfers with the antibacterial activity of XOR. Allopurinol was shown to efficiently alleviate ischemic rat heart injury during reperfusion [52]. Similarly allopurinol pretreated rabbit hearts showed attenuated ventricular dysfunction on reperfusion as compared to untreated control animals. Allopurinol and also oxopurinol were shown to improve force generation by stanned cardiac myofilaments by reducing systolic Ca – transients and enhancing Ca – sensitivity of myofilaments. Allopurinol pretreatment of feline intestine attenuated neutrophil infiltration at reperfusion [53].

Allopurinol as well as Tungsten treatment decreased also neutrophil infiltration after reperfusion [53] as well as adhesion of neutrophils to endothelial cells. It was the successful attenuation of myocardial lesions produced in animal studies by IR that encouraged clinical trials in humans. This was successful in aorta – coronary bypass surgery where allopurinol attenuated myocardial damage [54]. These observations were followed by similar success in acute MI – patients treated by primary coronary angioplasty where allopurinol prevented reperfusion – produced lesions and increased left ventricular function. This improvement was confirmed 6 months later [55]. Allopurinol improved $NO^{\bullet}$ – stimulated blood flow in diabetes [56] reversing endothelial dysfunction. Oxopurinol was shown to lower blood pressure in SHR – hypertensive rats [57]. Hypertension produced in rats by urate oxidase inhibition with oxonic acid could be prevented by allopurinol. Oxonic acid produced blood pressure increase produced renal arteriolar thickening, also prevented by allopurinol. Increased serum uric acid levels are associated by increased CVD risk in hypertensive patients. In heart failure – patients, allopurinol attenuated the mechano – energetic uncoupling, decreased myocardial oxygen consumption and increased myocardial contractility. Curiously high doses of ascorbate imitated these effects of allopurinol, but NOS – inhibition prevented the beneficial effects of allopurinol and also of ascorbic acid, as demonstrated in HF – dogs. According to Kögler et al. [58], rat myofilament twitch tension is enhanced by oxopurinol especially in failing myocardium where XOR activity is elevated. These inotropic effects of oxopurinol appear to be selective for the failing heart with elevated XOR levels. Confirming these findings was the improvement of myocardial efficiency of patients with HF by allopurinol infusion in the coronary circulation, accompanied by decreased oxygen consumption [59].

Clinical — Epidemiological Results

After the rapid overview of experimental data on XOR and its involvement in pathological tissue modifications, let us summarize clinical – epidemiological data on xanthine-oxidase inhibitors as a preventive and/or curative drug for CVD – s. As developed above, xanthine oxidase inhibitors – mainly allopurinol – are used for lowering serum uric acid level in patients with gout. Epidemiological and clinical studies have consistently shown that gout is strongly associated with major CVD risk factors as hypertension, obesity and diabetes. However high uric acid level is also an established risk factor for CVD, independently of classical risk factors [60–62]. An independent association between serum uric acid level and CVD risk was described for coronary heart disease [63], peripheral artery disease [64], as well as for overall mortality from cardiovascular disorders [65, 66]

Most clinical evidence for a possible beneficial effect of xanthine oxidase inhibitors on the cardiovascular system comes from studies in patients with heart failure [67]. In a randomized placebo controlled crossover trial in 65 patients with chronic stable angina, allopurinol increased time to ST segment depression on ECG, median total exercise time and time to chest pain [68]. These results suggested that allopurinol could be a useful anti-ischemic drug for patients with angina. Another controlled study in adolescents with newly diagnosed essential hypertension, treatment with allopurinol resulted in a statistically significant reduction in both systolic and diastolic blood pressure [69]. This study, as many others, support therefore the hypothesis that hyperuricemia could be a predictor of high blood pressure. Although these findings need to be confirmed in larger clinical trials, they suggest that allopurinol could be considered as a useful cardiovascular drug [70].

Discussion

This review was motivated by the worldwide interest in the treatment of CVD – s of all sorts by a rapidly aging world population. Although significant progress was accomplished both in the diagnostics as well as the treatments of the most frequent varieties of CVD – s, it still remains the leading cause of morbidity and mortality. It was realized since decades that this family of diseases is multifactorial, several more or less well identified mechanisms contribute to their, sometimes insidious manifestations. Among the most studied etiological factors “free radical” induced tissue damage was claimed in a large number of pathologies, CVD – s among them [4]. In sharp contrast to this

situation, very few of these pathologies profited from an efficient treatment with free radical scavengers [5, 6]. We mentioned above several reasons for this contradictory situation. One of them is the fact that reactive oxygen and nitrogen species are involved in intercellular signaling. Let us cite as one of the most interesting examples nitric oxide, $NO^{\bullet}$, known to be an endothelial- derived vasodilator as well as a neuromediator. We mentioned above its rapid reaction with superoxide, $O_2^{\cdot -}$, to yield the highly aggressive anion, peroxynitrite, $ONOO^-$, capable of killing cells in the proximity of its formation in nanoseconds. There are other examples of this Jekyll – Hyde behavior of reactive oxygen or nitrogen species . Another, experimental finding as mentioned above is the low efficiency of scavenging mechanisms aimed at the neutralization of such reactive molecules [5, 6]. And thirdly, the organism is provided with molecular devices capable of neutralizing most reactive species. Reduced glutathione, superoxide dismutase, glutathione reductase and peroxidase, ascorbic acid, vitamin E are among the most cited players of the organisms defense system. Nevertheless, in most instances as those discussed above, these defense mechanisms are inefficient or insufficient to protect the organism against "free radical" mediated injuries or pathologies.

A notable exception is the efficiency of XOR – inhibition for the alleviation of at least some of the mechanisms involved in CVD – s. This is perhaps due to the fact that XOR – inhibition is not directly targeted against the reactive oxygen or nitrogen species but targets instead the enzyme which produces them. Such strategies are much more difficult to implement as those targeting directly the reactive molecular species. More than that, the XOR (and to some extent aldehyde oxidase also) are not only an early "invention" of evolution as shown by their presence from prokaryotes to humans, as well as in plants, but also to their wide tissue distribution. Those tissues involved in CVD – s, intima of blood vessels, heart muscle are among other tissues rich in XOR. A further factor in favor of its role in CVD – s is the upregulation of XOR – activity during anoxia and its downregulation at higher oxygen tension. An important factor of continued XOR activity is the relative abundance of its substrates, xanthine and hypoxanthine, resulting from the rapid turnover of ATP and related or derived nucleotides.

A further fact in favor of the rather convincing arguments for the role of XOR in CVD – s is the rapid increase of experimental work on this enzyme family of Molybdo – Flavo enzymes, of crucial importance in several metabolic processes. Since the early 1950-ies when some laboratories, as also ours, got interested in these enzymes, the number of scientists and publications increased considerably. The most cited and consulted references for this review, [4, 9, 27, 28] and some others of the cited references are remarkably extensive with a large number of citations. Nevertheless, as we have seen, open questions remain as well as some unsolved problems which still need further experimental work to be settled. However as often in clinical medicine, the most convincing arguments come from epidemiological studies on a large number of case-controlled observations. As discussed in the last section of this review, this type of argument is decisively in favor of the use of XOR – inhibitors for the treatment of some CVD – s.

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