Arch Toxicol (2015) 89:1193–1207
DOI 10.1007/s00204-015-1523-8

REVIEW ARTICLE

Dispelling dogma and misconceptions regarding the most pharmacologically targetable source of reactive species in inflammatory disease, xanthine oxidoreductase

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Received: 6 April 2015 / Accepted: 27 April 2015 / Published online: 21 May 2015

Abstract Xanthine oxidoreductase (XOR), the molybdoflavin enzyme responsible for the terminal steps of purine degradation in humans, is also recognized as a significant source of reactive species contributory to inflammatory disease. In animal models and clinical studies, inhibition of XOR has resulted in diminution of symptoms and enhancement of function in a number of pathologies including heart failure, diabetes, sickle cell anemia, hypertension and ischemia–reperfusion injury. For decades, XOR involvement in pathologic processes has been established by salutary outcomes attained from treatment with the XOR inhibitor allopurinol. This has served to frame a working dogma that elevation of XOR-specific activity is associated with enhanced rates of reactive species generation that mediate negative outcomes. While adherence to this narrowly focused practice of designating elevated XOR activity to be "bad" has produced some benefit, it has also led to significant underdevelopment of the processes mediating XOR regulation, identification of alternative reactants and products as well as micro-environmental factors that alter enzymatic activity. This is exemplified by recent reports: (1) identifying XOR as a nitrite reductase and thus a source of beneficial nitric oxide ($^{\bullet}$NO) under in vivo conditions similar to those where XOR inhibition has been assumed an optimal treatment choice, (2) describing XOR-derived uric acid (UA) as a critical pro-inflammatory mediator in vascular and metabolic disease and (3) ascribing an antioxidant/protective role for XOR-derived UA. When taken together, these proposed and countervailing functions of XOR affirm the need for a more comprehensive evaluation of product formation as well as the factors that govern product identity. As such, this review will critically evaluate XOR-catalyzed oxidant, $^{\bullet}$NO and UA formation as well as identify factors that mediate their production, inhibition and the resultant impact on inflammatory disease.

Keywords Nitrite · Xanthine oxidoreductase · Nitric oxide · Uric acid · Inflammation · Hypoxia

Abbreviations

GAGs	Glycosaminoglycans
H_2O_2	Hydrogen peroxide
$^{\bullet}$NO	Nitric oxide
NOS	Nitric oxide synthase
$O_2^{\bullet-}$	Superoxide
ROS	Reactive oxygen species
SOD	Superoxide dismutase
UA	Uric acid
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
XOR	Xanthine oxidoreductase

Introduction

Spanning over 115 years, the history of xanthine oxidoreductase (XOR) is extensive (Massey and Harris 1997). For example, the oxidation of hypoxanthine and xanthine to uric acid (UA) in the presence of cheese and oxygen was first described by Spitzer (1899) as an enzymatic process. The enzyme responsible for catalyzing these reactions was first named xanthine oxidase by Burian (1905), while the reduction of methylene blue by an aldehyde in the presence

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of fresh cow's milk had been reported by Shardinger and named Shardinger's enzyme (Shardinger 1902). For over 20 years, it remained unknown that these two enzymes were one in the same until Morgan and colleagues reported oxidation of xanthine to UA under normoxic conditions and anoxic conditions in the presence of methylene blue. They reported this enzymatic activity in the liver, spleen, kidney and lungs from rats as well as ox milk raising questions regarding the true identity of Shardinger's enzyme (Morgan et al. 1922). This issue was resolved when Dixon and Thurlow (1924) partially purified xanthine oxidase and published a kinetic analysis of both xanthine and methylene blue reactions, demonstrating that Shardinger had also discovered xanthine oxidase. It was not until 1939 in Otto Warburg's laboratory that Eric Ball reported a pure preparation of xanthine oxidase (Ball 1939). These reports and subsequent investigation led to the establishment of XOR as the enzyme responsible for the terminal two steps in purine degradation in primates (hypoxanthine → xanthine → UA) and resulted in the development of the XOR inhibitor allopurinol as the first-line clinical treatment for hyperuricemia associated with gout. However, in the first demonstration of biological superoxide ($O_2^{\bullet-}$) formation, it was the identification of XOR as the source that sparked enthusiasm in the free radical field (McCord and Fridovich 1969). When XOR-derived oxidants were coupled to a demonstrable pathophysiologic impact in ischemia–reperfusion injury by Parks et al. (1982), it vaulted XOR to the forefront of redox biology. This is exemplified by a plethora of reports over the past 30 years demonstrating involvement of XOR-derived oxidants in the pathology of an array of inflammatory disease processes including heart failure, chronic obstructive pulmonary disease (COPD), pulmonary hypertension, sickle cell disease and diabetes (Butler et al. 2000; Desco et al. 2002; Farquharson et al. 2002; Aslan et al. 2001; Heunks et al. 1999; Ahmed et al. 2010; Rajesh et al. 2009). While much has been established regarding XOR, its reactive products and their role in normal and pathophysiology, the field finds itself somewhat deficient regarding its regulation and subsequent interplay with biomolecular pathways when compared to other enzymes with much more recent history. Potential reasons for this deficit in our understanding include: (1) promiscuity of the active site resulting in ambiguity regarding substrate identity and inhibitor specificity (2) lethality of global *xdh* knockouts and the absence of conditional knockouts, (3) side effects resulting from pharmacological knockdown by tungsten administration, (4) resistance to inhibition by allo/oxypurinol conferred by immobilization on the glycocalyx of endothelial and epithelial cells and (5) identification of XOR as a source of salutary $^{\bullet}$NO. These gaps in knowledge affirm a pressing need for further evaluation of XOR and its interplay with pathways associated with the initiation and progression of a wide range of disease processes. This imminent need is further validated by the fact that, to date, XOR is the most pharmacologically targetable source of reactive species as other sources including the mitochondrial electron transport chain, NADPH oxidase, uncoupled eNOS, myeloperoxidase and aldehyde oxidase are not or cannot be specifically inhibited in vivo by FDA-approved compounds. To address these issues, the following sections will describe our current understanding of XOR by focusing on tissue distribution, regulation, oxidant formation, inhibition strategies, alteration of kinetic properties by immobilization on the endothelium, UA production, $^{\bullet}$NO formation and the impact these factors have on treatment approaches.

XOR structure and function

Xanthine oxidoreductase is a complex molybdoflavin protein that catalyzes the terminal two steps in purine degradation in primates; oxidation of hypoxanthine to xanthine and oxidation of xanthine to UA. The human XOR gene (*xdh*) resides on chromosome band 2p23.1 and is transcribed as xanthine dehydrogenase (XDH). The enzyme is a homodimer of ~295 kD with each subunit consisting of four redox centers: a molybdenum cofactor (Mo-co), one FAD and two Fe/S clusters. The Mo-co is comprised of a pterin derivative with a cyclized dithiolene side chain and one Mo atom pentacoordinated with the dithiolene, two oxygen atoms and a sulfur atom. The Mo-co is the site of purine oxidation, whereas NAD^+ and O_2 reduction occur at the FAD, Fig. 1. The two Fe/S clusters serve as a conduit for electron flow between the Mo-co and the FAD. These Fe/S clusters are both of the ferredoxin type, but are not identical and thus are independently distinguishable by their electron paramagnetic resonance (EPR) spectra (Enroth et al. 2000; Nishino and Okamoto 2000; Iwasaki et al. 2000; Hunt et al. 1993). Two major inactive forms of XOR are identified as proteins lacking the Mo atom (demolybdo), commonly found in human breast milk, and proteins in which the Mo=S is replaced with O_2 forming Mo=O (desulfo) (Godber et al. 2005; Gutteridge et al. 1978). In both cases, the enzyme is incapable of oxidizing purines at the Mo–co. It has been proposed that a sulfurase enzyme is responsible for the enzymatic insertion and/or reinsertion of the sulfur atom in desulfo XOR to restore purine oxidizing activity (Bittner et al. 2001; Ichida et al. 2001). XOR lacking a FAD co-factor (deflavo) has not been reported to occur in nature and brings forth a crucial point when considering the contribution of XOR-derived ROS in specific micro-environments (see *Oxidant Production*).

Translation of *xdh* mRNA results in the production of xanthine dehydrogenase (XDH) which utilizes purine-derived electrons to reduce NAD^+ to NADH, Fig. 1. However, during hypoxia/inflammation, reversible oxidation of

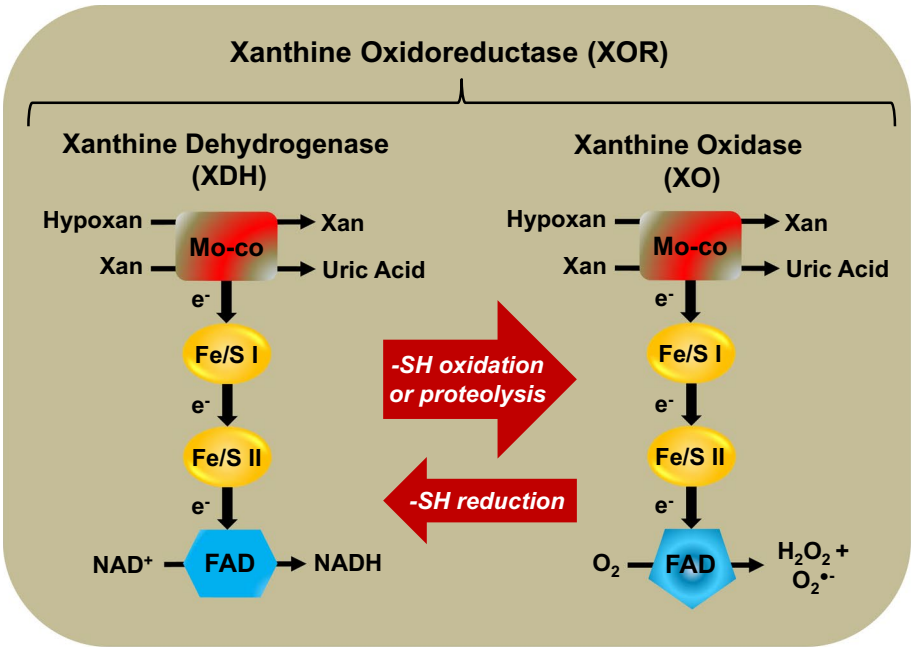

Fig. 1 Xanthine oxidoreductase. For xanthine dehydrogenase (XDH), hypoxanthine is oxidized to xanthine and xanthine is oxidized to UA at the Mo-co. Electrons derived from the purine oxidation at the Mo-co are transferred via 2 Fe/S centers to the FAD where NAD^+ is reduced to NADH. Upon oxidation of critical cysteine residues (reversible) and/or limited proteolysis (irreversible), XDH is converted to xanthine oxidase (XO). For XO, hypoxanthine and xanthine are oxidized to UA at the Mo-co and electrons are transferred to the FAD where O_2 is reduced univalently to generate $O_2^{\bullet-}$ and divalently to generate H_2O_2

critical cysteine residues (535 and 992) and/or limited proteolysis that confers structural alterations in the vicinity of the FAD converts XDH to xanthine oxidase (XO) (Enroth et al. 2000). In the oxidase form, affinity for NAD^+ at the FAD is greatly diminished while affinity for O_2 is enhanced resulting in univalent and divalent reduction of O_2 to generate $O_2^{\bullet-}$ and hydrogen peroxide (H_2O_2), respectively, Fig. 1 (Enroth et al. 2000; Hunt et al. 1993). Therefore, when present in critical settings in the tissue and vasculature, XO is an abundant source of reactive oxygen species (ROS) that subsequently alter vascular tone by reducing $^{\bullet}NO$ bioavailability ($^{\bullet}NO + O_2^{\bullet-} \rightarrow ONOO^-$), inducing cellular damage/dysfunction and altering homeostasis (Aslan et al. 2001; Aslan and Freeman 2004; White et al. 1996).

XOR regulation

For a protein that has been the focus of study for over a century, it is rather disappointing how little detail is actually understood regarding its regulation. Notwithstanding, several factors have been proposed to regulate XOR expression. For instance, cytokines including tumor necrosis factor-α (TNF- α), interferon-γ (INF-γ) and interleukin-1β (IL-1β) are reported to induce XOR expression and protein abundance (Page et al. 1998). In addition, the p38 MAP kinase pathway (Abdulnour et al. 2006) and nuclear factor Y (NF-Y) (Martelin et al. 2000) have been reported to modulate XOR expression. Furthermore, XOR levels are responsive to hormonal control as estrogen has been reported to post-transcriptionally modulate XOR activity independent of receptor activity (Budhiraja et al. 2003). However, one of the most intriguing regulatory effectors of XOR activity is molecular oxygen.

The inverse relationship between O_2 tension and XOR activity is well established yet remains mechanistically unclear. Many studies have shown that hypoxia increases XOR activity and conversely hyperoxia inhibits enzymatic activity (Terada et al. 1988, 1992). The proposed mechanisms underlying hypoxia's effect on XOR activity are variable with studies suggesting transcriptional regulation (Martelin et al. 2000; Hassoun et al. 1994), post-translational modification (Poss et al. 1996; Linder et al. 2003; Kayyali et al. 2001) or both (Terada et al. 1997; Kelley et al. 2006). Of particular interest is the proposed putative hypoxia response element (HRE) in the promoter of *xdh* and thus the potential for responsiveness to HIF-mediated induction (Hoidal et al. 1997). Yet, there has been no supportive reports indicating this pathway to be operative while one study has demonstrated hypoxic induction of XOR independent of the HIF pathway (Kelley et al. 2006). This was validated by elevating HIF-1α in endothelial cells with exposure to CoCl, NiCl or DMOG while failing to observe alteration of XOR expression or enzymatic activity. However, in glioma cells, CoCl does induce a significant elevation in XOR activity that, in turn, serves to further stabilize HIF-1α (Griguer et al. 2006). Other investigators have also shown HIF activation to be, in part, induced by XOR-derived reactive species suggesting XOR-mediated control of the HIF pathway (Nanduri et al. 2013, 2015). More recently, a hypoxia-associated and HIF-independent mechanism for XOR induction was proposed, suggesting that binding of nonmuscle myosin regulatory light chain ($nmMLC_{20}$) to a consensus sequence (GTCGCC) in the

xdh promoter activates transcription in a rat model of myocardial ischemia/reperfusion (Zhang et al. 2015). When assessed in toto, it is abundantly clear that *xdh* expression is modulated by O_2 tension; yet, mechanisms underpinning this process are not only cell/tissue-specific but poorly understood.

Potential reasons for the lack of mechanistic clarity regarding the influence of hypoxia on XOR include variability in exposure times (2–72 h) and the extremely low (0–3 %) levels of O_2 utilized. While these exposure times and O_2 tensions may be compatible with the cellular milieu in a tumor, they are not reflective of the environment encountered by endothelial cells lining conduit vessels or the survival of supported organs, especially when O_2 tensions are below 3 % for extend periods of time such as 24–72 h. Addressing this particular issue, with detail given to clinically relevant O_2 tensions such as those experienced in cardiovascular disease resulting in chronic hypoxemia, it was reported that 10 % O_2 increases endothelial cell XOR protein, activity and ROS production capacity approximately threefold (Kelley et al. 2006). In addition, it is important to note that these studies demonstrated significant export of XOR to the culture medium indicating the endothelium as a potent source of XOR as well as the potential for XOR to travel to distal sites in the vasculature (see *Tissue Distribution of XOR*). Furthermore, this study proposes a mechanistic role for adenosine and adenosine receptors in the hypoxia-mediated regulation of endothelial XOR. These findings, coupled to the reemerging clinical significance of XOR in cardiovascular pathobiology, affirm the need to further examine O_2-mediated regulation of endothelial XOR under clinically relevant O_2 tensions and may help to define the role of XOR-derived ROS in vascular diseases allied to chronic and/or intermittent hypoxemic conditions such as congestive heart failure or sleep apnea.

Tissue distribution of XOR

XOR protein and activity have been reported in numerous tissues including myocardium, vasculature and epithelium; however, the splanchnic system (liver and intestines) displays the greatest specific activity (Sarnesto et al. 1996). An important point to consider when assessing tissue distribution of XOR is the fact that the enzyme is mobile. For example, hepatocellular damage including ischemia/hypoxia, inflammation and viral insult results in release of XOR into the circulation where it is rapidly and irreversibly converted to XO by proteolysis (Fig. 2) (Parks et al. 1988; Houston et al. 1999). This is exemplified in studies demonstrating: (1) ~twofold increase in plasma XO activity in patients undergoing thoracic aorta aneurysm repair, a procedure that renders liver, intestine and all distal tissues ischemic and (2) elevated plasma XO activity in patients with chronic liver disease including steatosis allied to obesity (Tan et al. 1995; Yokoyama et al. 1990; Chiney et al. 2011; Tam et al. 2014). Elevation of plasma XO levels results in binding and sequestration of the enzyme on vascular endothelial glycosaminoglycans (GAGs) (see *Consequences of XOR Binding and Sequestration on Endothelial Cells*) (Houston et al. 1999; Kelley et al. 2004). This affinity for endothelial GAGs provides a mechanism to capture and significantly amplify XO and associated ROS production in distal vascular beds where it may then initiate and/or exacerbate disease.

While insult to the liver and intestines can result in exportation of XO to the circulation resulting in distribution of the enzyme to alternative tissue sites, nonsplanchnic sources of XOR may be equally important. As discussed previously, exposure of cultured endothelial cells to hypoxia results in increased expression, elevated protein abundance, enhanced enzymatic activity and extracellular release of XO (Kelley et al. 2006; Partridge et al. 1992). This phenomenon is realized in vivo where interruption of the blood supply to an upper limb of patients undergoing an orthopedic procedure increases in situ levels of XOR suggesting the endothelium or perivascular tissue as the source (Friedl et al. 1990). These data, combined with a report showing XOR protein and activity in cardiomyocytes and macrophages, clearly demonstrate that splanchnic-derived, plasma-delivered XO is not the sole source of XOR capable of participating in vascular disease (Zhang et al. 2015; Gladden et al. 2011; Kim et al. 2014; Wright et al. 2004). However, it is equally crucial to note that the importance of endogenous XOR expression in nonsplanchnic tissues has been much debated. For example, studies demonstrating immuno-reactive XOR protein in the vasculature including coronary arteries countervail reports indicating the absence of detectable XOR in these same regions. These and numerous published inconsistences regarding XOR distribution have been critically reviewed (Kooij 1994). A plausible explanation for the uncertainty regarding anatomic sites of XOR expression is that immunoglobulins (class A, G and M) co-purify with XO resulting in nonspecific antibody production in antisera raised to purified XOR (confirmed in our laboratory) (Clare and Lecce 1991). As such, immunohistochemical analysis may result in misinterpretation, especially in the absence of parallel evaluation of enzymatic activity. A similar issue surfaces when considering the extent of sequence homology (84 %) between aldehyde oxidase (AO) and XOR which raises the issue of cross-reaction between AO- and XOR-specific antibodies. Another factor contributing confusion regarding XOR expression is the vast range in sensitivity of the various activity assays and/or numerous reports that utilize assessment of UA levels in the plasma or tissue as a surrogate

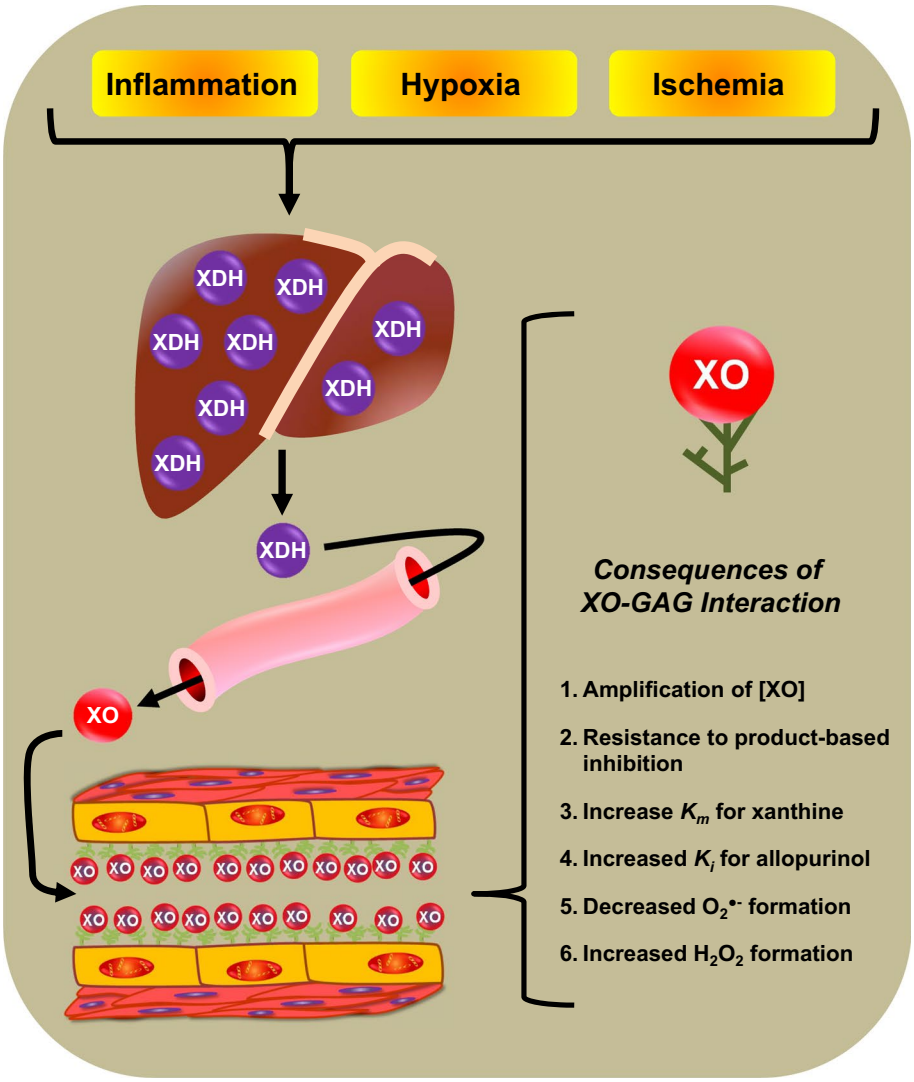

Fig. 2 XOR is mobile. Inflammatory insult or ischemia/reperfusion results in up-regulation of XDH in hepatocytes. Intracellular accumulation of XDH ultimately results in exportation to the circulation where it is: (1) rapidly converted to XO by plasma proteases and (2) can travel as XO and subsequently be sequestered by endothelial cells in distant vascular beds. Accumulation of XO at locations far from the origin results in unwanted amplification of reactive species generation in otherwise healthy tissue

indicator of XOR activity without recognizing UA clearance via the kidney. Inconsistencies are also exemplified by cases where treatment with the XOR inhibitor allopurinol produces salutary actions in organ systems that are reported to be low in or devoid of detectable XOR activity. For example, allopurinol attenuated myocardial ischemia–reperfusion injury in both rabbit models and clinical studies in spite of reports revealing that rabbit and human coronary tissue contain low to undetectable XOR (Grum et al. 1986; Emerit et al. 1988; Eddy et al. 1987). Combined, these data indicate that XOR tissue expression is dynamic due to the effects of chronic and/or intermittent hypoxia coupled to the proclivity of XOR to travel to sites distal from the origin of up-regulation. Thus, it is recommended that: (1) salutary outcomes resulting from XOR inhibition should be buttressed with identification of XOR protein while (2) positive identification of immuno-detectable XOR should be validated with analysis of enzymatic activity not only to confirm XOR activity but to discount the presence of AO.

Oxidant production

As stated previously, post-translation modification of XDH via limited proteolysis or oxidation of critical cysteine residues results in conformational changes that confer alteration of the electrostatic and stereochemical environment in the vicinity of the FAD cofactor ultimately establishing

Table 1 XO-derived $O_2^{\bullet-}$ versus H_2O_2

$[O_2]$ %	(μM)	(mm Hg)	$O_2^{\bullet-re}$	H_2O_2
1	12	7	10.4 ± 0.6	90
2.5	29.5	18.5	14.3 ± 0.9	86
5	59	37	24.6 ± 2.3	76
10	118	75	28.5 ± 0.7	71
15	177	113	27.8 ± 0.5	72
21	235	150	28.1 ± 1.4	72

oxidase capacity (Enroth et al. 2000; Kuwabara et al. 2003). When induced by cysteine oxidation, conversion from XDH to XO is reversible upon endogenous reduction of these residues or by in vitro application of reducing agents such as dithiothreitol (DTT). However, conversion to XO by partial proteolysis of XDH is irreversible (Enroth et al. 2000). While the post-translational modification resulting in XO activity has become synonymous with the conversion from a housekeeping enzyme to a deleterious source of ROS, it is important to recognize that XDH can also reduce O_2 and thus generate ROS (Harris and Massey 1997). Although NAD^+ is the preferred electron acceptor for XDH, in vivo conditions where the concentration of NAD^+ is decreased can result in utilization of O_2 as an electron acceptor. Such conditions include ischemia/hypoxia where O_2-dependent alterations in cellular respiration lead to decreased mitochondrial NADH oxidation and concomitant diminution of NAD^+ concentration (Zhou et al. 2005). Therefore, exclusively associating XO with oxidant generation while referring to XDH as a housekeeping enzyme is ill-advised, especially under conditions as described above where up-regulation of intracellular XDH, in the absence of post-translational modification to XO, may lead to unwanted amplification in intracellular oxidative stress. It is equally important to note that demolybdo and desulfo XOR, while inactive with respect to purine oxidation, retain NADH oxidase activity at the FAD and thus can reduce O_2 and produce ROS (Gardlik et al. 1987). This activity would be unresponsive to inhibition by pyrazalo-based inhibitors such as allo/oxypurinol while remaining sensitive to flavin poisons such as diphenylene iodonium (DPI).

It is unfortunate that XO is mainly referred to as a source of $O_2^{\bullet-}$ with rare mention of XO-catalyzed H_2O_2 production. It is often assumed that H_2O_2 formation results either from reaction of XO-derived $O_2^{\bullet-}$ with superoxide dismutase (SOD) ($2O_2^{\bullet-} \rightarrow H_2O_2$) or spontaneous dismutation of $O_2^{\bullet-}$. This assumption is not only invalid but leads to a significant misrepresentation of XO-derived oxidant identity. For instance, studies conducted over 38 years ago revealed that 100 % O_2 and pH 10 are requisite for 100 % $O_2^{\bullet-}$ production from XO; a setting incompatible with cell biology (Fridovich 1970). This same study demonstrated that under more physiologically relevant conditions (21 % O_2 and pH 7.0) XO generates ~25 % $O_2^{\bullet-}$ and ~75 % H_2O_2, a finding that should have designated XO as a "H_2O_2-producing enzyme that also produces $O_2^{\bullet-}$" (Fridovich 1970). This is further exemplified by work in our laboratory that revealed an inverse relationship between O_2 tension and XO-dependent H_2O_2, Table 1. Evaluation of the relative proportion of H_2O_2 versus $O_2^{\bullet-}$ produced by XO under various O_2 tensions (1–21 %) at normal pH revealed XO-catalyzed H_2O_2 approaches 90–95 % of total electron flux though the enzyme under clinically relevant hypoxic conditions (1–2 % O_2; Kelley et al. 2010). It was noted that the focal point for transition toward enhanced H_2O_2 formation was ~2 % O_2 or ~26 μM saturated O_2; a value similar to the K_m O_2 of ~27 μM at the FAD of XO (Fridovich 1970; Harris and Massey 1997; Olson et al. 1974). This hypoxia-induced proclivity for XO-catalyzed H_2O_2 formation is further amplified by acidic pH (*not shown*). Thus, under ischemia/hypoxia, where both O_2 tension and pH are reduced, H_2O_2 formation is favored suggesting that XO activity may significantly affect numerous signaling events where H_2O_2 is reported to be contributory (Stone and Yang 2006; Veal et al. 2007).

To summarize: (1) under physiologically relevant conditions, the dominant XO-derived reactive product is H_2O_2, (2) under conditions of elevated NADH, XDH is capable of ROS production and (3) under conditions of elevated NADH, demolybdo and desulfo forms of the enzyme are capable of producing oxidants.

Consequences of XOR binding and sequestration on endothelial cells

Up-regulation of cellular XDH can result in release to the circulation where it is rapidly (<1 min) converted to XO. While having a net negative charge at physiological pH, XO contains pockets of cationic amino acid motifs on the surface of the protein resulting in its high affinity ($K_d = 6$ nM) for negatively charged GAGs comprising the glycocalyx of the vascular endothelium (Houston et al. 1999; Kelley et al. 2004; Adachi et al. 1993; Fukushima et al. 1995; Radi et al. 1997). Evidence of the presence of GAG-associated XO is provided by both animal models and clinical studies of cardiovascular disease whereby intravenous administration of heparin results in elevation of plasma XO activity (Houston et al. 1999; Granell et al. 2003; Landmesser et al. 2002). This sequestration of proteins by GAGs (1) substantially amplifies local concentration, (2) diminishes rotational and translational mobility and (3) alters kinetic properties. For example, when compared to XO in free in solution, GAG-immobilized XO demonstrates an increased K_m for xanthine (6.5 vs. 21.2 μM) and a fivefold increase in K_i for allo/oxypurinol; a consequence with significant implications for pharmacological intervention (Kelley et al. 2004). Similar alterations in XO kinetics were observed when XO was bound to the milk fat globule membrane where immobilization of the enzyme in this setting induced a twofold increase in the K_m for xanthine while enhancing affinity for NADH by increasing the K_m threefold at the FAD (Briley and Eisenthal 1974). In addition to affecting kinetic properties at the Mo-co, binding of XO to GAGs confers alterations to the FAD resulting in reduction of $O_2^{\bullet-}$ production

and thus elevation of H_2O_2 formation (Kelley et al. 2004). Combined, XO–GAG interaction results in: (1) diminished affinity for hypoxanthine/xanthine, (2) resistance to inhibition by the pyrazolopyrimidine-based inhibitors allo/oxypurinol and (3) diminished $O_2^{\bullet-}$ production and thus enhanced H_2O_2 generation. This vascular milieu where XO is sequestered on the surface of the endothelium is primed for prolonged enhancement of oxidant formation that is partially resistant to inhibition by the most commonly prescribed clinical agents.

Genetic manipulation and pharmacologic inhibition

Attempts to generate genetic models for XOR proof-of-principle experimentation have met with limited success. For example, global knockout of XOR ($xdh^{-/-}$) in a murine model resulted in pups that died within 10–20 days of birth due to kidney failure resulting from deleterious elevation in hypoxanthine levels (Cheung et al. 2007; Ohtsubo et al. 2004). Global heterozygous knockout of XOR ($xdh^{+/-}$) was reported to result in complications with lactation and abnormal nutrient absorption that questioned the feasibility of the model (Vorbach et al. 2002). However, recent work with the $xdh^{+/-}$ mice suggest that this strain is viable and void of any observable negative phenotype leaving the field a bit uncertain (Murakami et al. 2014). In addition, to our knowledge, there have been no reports demonstrating the generation of *xdh* tissue-specific conditional knockouts. Thus, the absence of transgenic animal models has relegated investigators to utilize either siRNA or pharmacology for validating XOR involvement in their various experimental models. For instance, in vivo and in vitro global XOR inactivation has been accomplished by supplementing the diet/drinking water or culture medium with sodium tungstate (NaW) (Schroder et al. 2006; Hille 2002). The presence of W results in its incorporation into the active site of XOR in place of molybdenum resulting in inactivation of purine oxidation activity and subsequent electron transfer to the FAD. While this approach results in significant XOR inactivation, it: (1) only inactivates the Mo-co leaving an operative FAD which can reduce molecular O_2 in the presence of NADH (*see Oxidant Production*) and (2) is not specific for XOR as alternative molybdopterin enzymes such as AO and sulfite oxidase (SO) are also inactivated. This is a crucial issue when attempting to establish XOR-mediated effects by inhibition of XOR-derived oxidant formation as AO is also a significant source of ROS (Kundu et al. 2012). Although some progress has been made regarding molecular and genetic approaches to alter XOR expression and/or activity, the overwhelming majority of studies addressing XOR-related enzymology and pathobiology have utilized the pyrazolopyrimidine-based inhibitor allopurinol.

Gertrude B. Elion, George Hitchings and James W. Black were awarded the Nobel in 1988, in part, for the discovery of the XOR inhibitor allopurinol. Allopurinol (1*H*-pyrazolo[3,4-d]pyrimidin-4(2H)-one) was approved by the FDA for treatment of gout in 1966 and remains the anchor therapy for hyperuricemia (Pacher et al. 2006). While inhibition of XO-derived UA formation to address symptoms of gout has been accomplished successfully for over 50 years by clinical administration of allopurinol, only partial reduction in symptoms and restoration of function has been observed when applied to address vascular inflammatory disease. This phenomenon may be explained by the examination of allopurinol reaction with the Mo-cofactor of XO. Allopurinol is a classic "suicide inhibitor" as its binding to and reduction of the Mo-cofactor induces self-oxidation to form the active, tight-binding competitive inhibitor, oxypurinol (1,2-dihydropyrazolo[4,3-e]pyrimidine-4,6-dione; Massey et al. 1970). Reduction of the Mo-cofactor by allopurinol ultimately leads to electron transfer to the FAD resulting in reduction of O_2 (Galbusera et al. 2006; Malik et al. 2011). It is equally important to note that oxypurinol binding and resultant inhibition require the Mo-cofactor to be reduced (Galbusera et al. 2006; Malik et al. 2011). This is accomplished by initial reaction of allopurinol or, in the case of treatment with pure oxypurinol, XO substrates such as xanthine must provide these initial electrons. In either case, both allopurinol and/or oxypurinol require enzyme turnover resulting in ROS formation before inhibition is attained. This undesirable action of allo/oxypurinol combined with the reduced capacity to inhibit endothelial GAG-associated XO and propensity to affect alternative purine catabolic pathways (Takano et al. 2005) may lead to significant misinterpretation of ROS-driven vascular pathology where XO is contributory. These limitations emphasize the need for alternative inhibitors with superior specificity, reactivity in the absence of enzyme turnover and resistance to GAG-immobilization alterations in kinetics.

Attempting to address the limitations of pyrazalopyrimidine-based XOR inhibition strategies, the XO-specific inhibitor febuxostat (Uloric®) was identified and approved by the FDA for clinical use in treating gout. Febuxostat is significantly (5833-fold) more potent than allopurinol demonstrating a K_i of 0.12 nM versus 700 nM for allopurinol at pH 8.2 (Okamoto et al. 2003) and confirmed at a more physiologically relevant pH of 7.2 (Malik et al. 2011). Detailed crystallography studies revealed that febuxostat reaction with XO is confined to electrostatic interactions with amino acid residues in the pocket leading to the Mo-cofactor where it effectively blocks substrate access to the active site (Okamoto and Nishino 2008). As such,

Table 2 Modulation of uric acid levels also alters ROS formation

Desired Outcome	Agent	Target	Effects on Uric acid and ROS
decrease uric acid	febuxostat or allopurinol	XOR	Xanthine ⊘→ ↓Uric Acid + ↓$O_2^{\bullet-}$ + ↓H_2O_2
increase uric acid	oxonic acid	uricase	↑Uric Acid ⊘→ ↓allantoin + ↓H_2O_2
decrease uric acid	rasburicase	augment uricase	↓Uric Acid → ↑allantoin + ↑H_2O_2

febuxostat–XO interaction is not affected by the redox state of the Mo-co and does not induce ROS formation (Malik et al. 2011). In addition, inhibition properties of febuxostat remain constant for XO in solution and XO bound to artificial GAGs (heparin-Sepharose) or endothelial cells indicating no apparent alteration in capacity to inhibit endothelial-associated XO (Malik et al. 2011). Furthermore, febuxostat is noted to be more selective than allopurinol by: (1) demonstrating no observable effect on alternative purine catabolic pathways and (2) producing only mild cross-over inhibition of AO at levels well above clinical applicable concentrations (K_i = 613 μM for AO whereas C_{max} in human plasma = 15 μM) (Malik et al. 2011; Takano et al. 2005; Weidert et al. 2014). These characteristics of enhanced potency, specificity and independence from enzyme turnover suggest that febuxostat may provide new insights into XO-dependent contributions to inflammatory disease.

Uric acid

UA, the product of XOR-catalyzed xanthine oxidation, has been proposed to assume an antioxidant role in primates as a surrogate for the loss of capacity to synthesize ascorbate (Ames et al. 1981). While it is established that UA readily reacts with singlet O_2 ($^1O_2$) and peroxynitrite ($O = NOO^-$), the extent of its utility as a small molecule antioxidant remains unclear (Kooy et al. 1997; Wagner et al. 1993; Frei et al. 1989). This may be, in part, due to different actions assumed by UA based on local concentration where levels of UA approaching and exceeding saturation result in pro-inflammatory crystal formation while lower levels serve an antioxidant role. However, this too is a gray area. For example, obese/diabetic animals and patients demonstrate elevated levels of circulating UA. This obesity-related hyperuricemia correlates positively with loss of metabolic homeostasis, allied cardiovascular disease and is reported to be predictive of metabolic syndrome/diabetes (Chaudhary et al. 2013; Aroor et al. 2013; Hare and Johnson 2003; Baldwin et al. 2011; Nakagawa et al. 2006; Masuo et al. 2003). This has led to the suggestion that UA may play a mechanistic role in the pathogenesis of the metabolic abnormalities of obesity, possibly through activation of inflammatory pathways (Baldwin et al. 2011) and/or the renin–angiotensin system (Zhang et al. 2014). This same rationale has been applied to cardiovascular disease where elevation in circulating UA is correlative and potentially predictive (Hare and Johnson 2003; Jia et al. 2014; Tamariz et al. 2014; Zhao et al. 2013; Soltani et al. 2013). However, in vivo studies attempting to address this issue directly have been relegated to pharmacology for proof-of-principle experimentation as global uricase deletion ($uox^{-/-}$) is lethal and heterozygote deletion ($uox^{+/-}$) does not affect circulating UA levels when compared to wild-type littermates (Wu et al. 1994). Thus, the vast majority of reports have manipulated UA levels via: (1) inhibition of XOR activity or (2) inhibition of or augmentation with the UA-catabolizing enzyme urate oxidase (uricase) (Baldwin et al. 2011; Nakagawa et al. 2006; Fabbrini et al. 2014). An alternative pharmacologic approach is modulation of UA clearance with uricosuric compounds such as probenecid; yet, off-target effects are a considerable disincentive leaving XOR inhibition and uricase inhibition (oxonic acid) or augmentation (Rasburicase®) as the working options. While both of these approaches are effective in modulating UA levels, they are both inextricably linked to modulation of ROS levels. This is evidenced by the fact that both XOR and uricase utilize molecular O_2 as an electron acceptor for xanthine and UA oxidation, respectively, Table 2. Thus, for every molecule of UA produced by XO, O_2 is reduced to $O_2^{\bullet-}$ or H_2O_2 and likewise every molecule of UA oxidized to allantoin by uricase (UA + O_2 → allantoin + H_2O_2) produces one molecule of H_2O_2 (Bentley and Neuberger 1952). Therefore, altering UA levels by targeting production and/or catabolism also alters ROS production. This is no dismissible issue when considering the relative impact on total ROS output in experiments conducted in rodent models which, unlike primates, express uricase. For example, lowering UA in a mouse by inhibiting XO would confer a fourfold decrease in electron flux to O_2; two electrons for xanthine oxidation to UA and two electrons for UA

oxidation to H_2O_2. It is therefore crucial that this unappreciated concept be considered when formulating conclusions from studies designed to define contributory roles for UA and ROS as well as the relative impact of one versus the other.

XOR as a source of nitric oxide

The canonical pathway for $^{\bullet}NO$ production is via O_2-dependent enzymatic conversion of arginine to citrulline by nitric oxide synthases (NOS). However, it is now established that alternative pathways exist and serve to augment this system, especially under hypoxic and acidic conditions related to micro-environments associated with inflammatory disease. In particular, the nitrite anion (NO_2^-) has been shown to represent a substantive reservoir of $^{\bullet}NO$ when subjected to a one-electron reduction reaction (Crawford et al. 2006; Gladwin 2008; Gladwin et al. 2006; Lundberg and Weitzberg 2005). There are several candidate mechanisms for NO_2^--dependent $^{\bullet}NO$ generation including reduction by hemoglobin and/or myoglobin (Crawford et al. 2006; Cosby et al. 2003; Huang et al. 2005). However, a recently accumulating body of evidence has demonstrated that mammalian molybdopterin enzymes can effectively catalyze reduction of NO_2^- to $^{\bullet}NO$. Nitrite reductase activity has thus far been assigned to AO, sulfite oxidase and XOR with the majority of reports focused on the later (Li et al. 2009; Millar et al. 1998; Millar 2004; Wang et al. 2014; Li et al. 2001, 2005, 2008). Evidence for XO-catalyzed $^{\bullet}NO$ formation has originated from basic biochemical studies with purified enzyme preparations as well as in vivo models of ischemia/reperfusion where salutary outcomes attributable to NO_2^- administration were abrogated by concomitant XOR inhibition (Alef et al. 2011; Samal et al. 2012; Pickerodt et al. 2012; Sugimoto et al. 2012; Zuckerbraun et al. 2010; Baker et al. 2007; Tripatara et al. 2007; Webb et al. 2004; Lu et al. 2005). In the aggregate, these studies reveal a novel function for XOR as a source of salutary $^{\bullet}NO$; yet, they identify a significant dilemma as they were conducted using models similar to or the same as those that have previously revealed XOR inhibition, in the absence of NO_2^- treatment, to be of benefit. Therefore, the field is now presented with the question: In disease processes resulting in elevation of XOR activity, will inhibition strategies or approaches to supplement NO_2^- be the most efficacious treatment? The answer to this question remains unknown; however, it is clear that there is a pressing need for a better understanding of the factors that mediate oxidant formation versus $^{\bullet}NO$ generation.

Under anoxic conditions, XO catalyzes the 1-electron reduction of NO_2^- to $^{\bullet}NO$, Fig. 3 (Millar et al. 1998; Li et al. 2001, 2005). The active site for NO_2^- reduction has been assigned to the Mo-co, and reducing equivalents can be supplied directly by hypoxanthine or aldehydes or indirectly via NADH-mediated reduction of the Mo-co via retrograde electron flow from the FAD, Fig. 3c. With regard to kinetics, the K_m values are 6.0 μM (xanthine-Mo-co), 0.9 μM (NADH–FAD) and 2.5 mM (NO_2^--Mo-co) (Li et al. 2001). Therefore, a catalytic activity for purified preparations of XO has been defined that requires anoxia and reveals over a 1000-fold difference in affinity between NO_2^- and xanthine for the active site. Considering normal tissue NO_2^- levels are greater than an order of magnitude (~300 nM; Kleinbongard et al. 2003) lower than 2.5 mM and anoxic environments in vivo rarely approach 0 %, especially in the vasculature, then, it would seem very unlikely that XOR-mediated NO_2^- reduction would be biologically relevant. However, this assessment countervails in vivo reports demonstrating XOR-dependence of NO_2^--mediated protective effects upon co-treatment with allopurinol, febuxostat or by dietary supplementation with NaW and affirms the need for a more detailed analysis of the factors that affect this catalytic process. These micro-environmental factors have been reviewed in detail and are summarized below (Cantu-Medellin and Kelley 2013).

A key effector dictating the biological relevance of XO-mediated $^{\bullet}NO$ formation is O_2 tension. Oxygen inhibits the nitrite reductase activity of XO by withdrawing electrons from the FAD to indirectly oxidize the Mo-co, Fig. 3a (Li et al. 2004; Godber et al. 2000). Therefore, the presence of O_2 results in a more electron-deficient Mo-co with diminished capacity to reduce NO_2^- (Cantu-Medellin and Kelley 2013). The O_2 tension where the NO_2^- reductase activity of XOR may become effective is ultimately governed by its affinity ($K_m = 27$ μM or ~2 % O_2) for the FAD cofactor. For example, O_2 tensions near or below 2 % will serve to diminish O_2-mediated inhibition and thus facilitate NO_2^- reaction at the Mo-co (Cantu-Medellin and Kelley 2013). In this context, local NADH levels must be also considered as NADH can not only compete with O_2 at the FAD, but reduce the Mo-co, Fig. 3c. In addition, pH is also crucial as hypoxanthine and xanthine oxidation at the Mo-co are base-catalyzed (pH optimum = 8.9) while reduction of NO_2^- at the Mo-co is acid-catalyzed (pH optimum = 6–6.5) (Fridovich 1970; Godber et al. 2000). During inflammatory conditions, in vivo microenvironments may encounter pHs below 7.0 and thus would facilitate a shift in affinity away from xanthine and toward NO_2^-. This concept was demonstrated by sequentially decreasing pH from 8.0 → 5.0 using either NADH or xanthine as reducing substrates (Li et al. 2004). Therefore, a local milieu where O_2 tension is low, NADH is elevated and pH is acidic is conducive for XO-catalyzed NO_2^- formation. However, it is important to consider that XO may not be the primary isoform of XOR

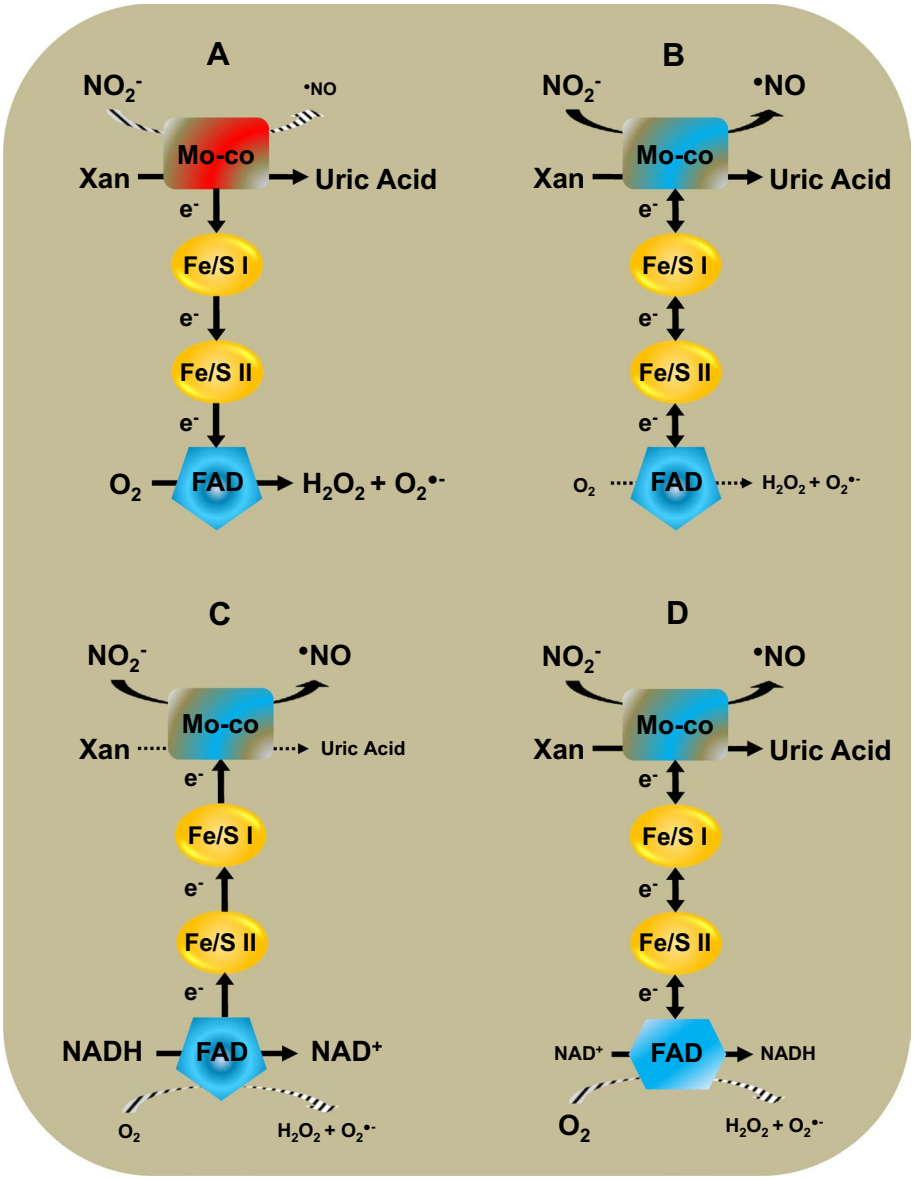

Fig. 3 Nitrite reductase activity of XOR. **a** Under normal O_2 tensions, 1-electron reduction of NO_2^- to $^{\bullet}NO$ at the Mo-cofactor is inhibited due to O_2-mediated electron withdrawal at the FAD (K_m = 27 μM or ~2 % O_2) in XO (designated by the pentagon structure of the FAD). This process results in the Mo-co assuming a more oxidized state (represented by *red color* of the Mo-co) and thus absence of capacity to donate an electron to NO_2^-. **b** Under hypoxia, O_2-mediated inhibition of NO_2^- reduction is diminished and therefore the Mo-co can assume a more reduced state (represented by *blue color* of the Mo-co) and more likely to supply the electrons necessary for $^{\bullet}NO$ formation. This concept is also represented by the double-arrow designators of electron flow indicating an elevation in electron loading of the four cofactors due to less flux via the FAD. **c** Under chronic hypoxia, NAD^+ levels are diminished while NADH concentration is elevated. The presence of excess NADH provides: (1) competition with residual O_2 at the FAD, (2) reduction of the FAD with resultant retrograde electron flow and Mo-co reduction (indicated by the arrows showing electron flow in the direction of the Mo-co) and (3) reduction in affinity for hypoxanthine/xanthine at the Mo-co, a process that requires an oxidized active site. **d** Under normoxia to mild hypoxia, XDH (designated by the hexagon structure of the FAD) can readily reduce NO_2^- to $^{\bullet}NO$ at the Mo-cofactor due to the low affinity (K_m = 260 μM or >21 % O_2) of the FAD in XDH for O_2. In this scenario, NO_2^- would compete for reaction at the Mo-co with hypoxanthine and xanthine. However, maintenance of the Mo-co in a more reduced state would decrease its affinity for reaction with hypoxanthine and xanthine which requires an oxidized active site. Thus, under hypoxia-mediated reduction in NAD^+ levels and slightly acidic pH, the XDH isoform may be the predominant source of $^{\bullet}NO$ generation from NO_2^-. The *font size* and *arrow size* indicate the relative concentration of the substrates and products

responsible for NO_2^- reduction as XDH may assume this role, Fig. 3d. Although NO_2^- reductase experiments with pure preparations of XDH have not been reported, it would be expected that XDH-catalyzed NO_2^- reduction to resist inhibition by O_2 due to substantially lower affinity than XO (K_m O_2 = 260 vs. 27 μM for XDH vs. XO, respectively) (Enroth et al. 2000; Amaya et al. 1990).

As mentioned previously, the vast difference in affinity for the Mo-co between hypoxanthine/xanthine (K_m = 6.5 μM) and NO_2^- (K_m = 2.5 mM) would preclude further consideration of XOR as a biologically relevant source of •NO (Harris and Massey 1997; Li et al. 2001). This is especially true when considering: (1) normal tissue NO_2^- concentration (~0.3 μM) while hypoxia results in diminution of NO_2^- levels and (2) the K_m for xanthine is 6.5 μM while levels of xanthine above 20 μM, commonly seen under pathologic conditions, are reported to induce substrate-dependent inhibition of NO_2^- reduction (K_i = 55 μM; Li et al. 2001; Kleinbongard et al. 2003; Lartigue-Mattei et al. 1990; Pesonen et al. 1998; Quinlan et al. 1997; Himmel et al. 1991). However, it must be noted that hypoxia enhances NO_3^- levels from 30–40 to 120–150 μM depending upon the tissue (Kleinbongard et al. 2003). This is important as reports have revealed XOR-dependent reduction of organic NO_3^- to NO_2^- indicating that XO may also be a source of NO_2^- (Millar et al. 1998; Li et al. 2005; Doel et al. 2001; Huang et al. 2010). Yet, similar issues with affinity also dampen enthusiasm regarding XO-catalyzed NO_3^- reduction. For example, purified XO exposed to NO_3^- produces K_m values ranging from 330 μM to 32 mM depending on the source of NO_2^- ($NaNO_3^-$ or glycerol trinitrate; Millar et al. 1998; Li et al. 2005; Doel et al. 2001). Thus, when considering xanthine as the primary reducing substrate, the potential to encounter an in vivo setting where reduction in xanthine concentration and elevation in NO_2^- + NO_3^- levels would overcome the difference in K_m for the Mo-co is improbable. It could however, be hypothesized that NADH may represent the primary source of electrons necessary to reduce the Mo-co and drive NO_2^- reduction. Ischemia/hypoxia-driven enhancement of NADH levels may serve to efficiently reduce the Mo-co, Fig. 3c. NADH-dependent reduction of the Mo-co would also decrease affinity for xanthine since an oxidized Mo-co is requisite for xanthine reaction and would thus result in diminished potential for xanthine-mediated inhibition of NO_2^- reductase activity. In addition, immobilization of XO on GAGs confers a diminution in affinity for xanthine (*see Consequences of XOR Binding and Sequestration on Endothelial Cells*) which could further facilitate capacity for NO_2^- or NO_3^- to compete for reaction.

Although basic biochemical experiments reveal significant hurdles for readily assigning biological relevance to XOR-mediated •NO generation from NO_2^-, the in vivo studies strongly suggest this process to be operative and affirm the need for additional analysis. It may be prudent, at this juncture, to focus future studies on developing tissue-specific *xdh* conditional knockouts, defining the potential role for XDH and optimizing NO_2^- supplementation approaches.

Summary

For the last 30 years, XOR has been seen as either a housekeeping enzyme (XDH) or a source of deleterious superoxide allied to pathophysiology. This black and white interpretation of XOR's role has come to pass. It is now abundantly clear that: (1) XOR is primarily a source of H_2O_2 that produces some superoxide, the extent of which is dictated by O_2 tensions and its microenvironment, (2) under inflammatory conditions, conversion to XO is not requisite for oxidant formation as intracellular XDH can utilize oxidize NADH and reduce O_2, (3) previous studies may have underestimated contributory roles for XOR in disease processes due to the absence of genetic models and the reduced capacity for allo/oxypurinol to inhibit endothelial-associated XO, (4) inhibited or inactivated (demolybdo, desulfo or NaW) XOR retains oxidase activity, (5) tissue distribution of XOR must be validated by rigorous assessment of enzymatic activity and (6) XOR may be a source of salutary •NO. The union of these concepts reveal, after 114 years, why much is yet to be understood regarding XOR and its influence on signaling pathways for both normal- and pathophysiology. Ongoing and future investigation is primed to reveal an exciting and productive path for XOR into the next 100 years.

Acknowledgments This work was supported by the National Institute on Aging, NIH 3P01AG043376-02S1 and by the University of Pittsburgh, Department of Anesthesiology.

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