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Graphical Review

Xanthine oxidoreductase-catalyzed reactive species generation: A process in critical need of reevaluation ☆

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ABSTRACT

Nearly 30 years have passed since the discovery of xanthine oxidoreductase (XOR) as a critical source of reactive species in ischemia/reperfusion injury. Since then, numerous inflammatory disease processes have been associated with elevated XOR activity and allied reactive species formation solidifying the ideology that enhancement of XOR activity equates to negative clinical outcomes. However, recent evidence may shatter this paradigm by describing a nitrate/nitrite reductase capacity for XOR whereby XOR may be considered a crucial source of beneficial •NO under ischemic/hypoxic/acidic conditions; settings similar to those that limit the functional capacity of nitric oxide synthase. Herein, we review XOR-catalyzed reactive species generation and identify key microenvironmental factors whose interplay impacts the identity of the reactive species (oxidants vs. •NO) produced. In doing so, we redefine existing dogma and shed new light on an enzyme that has weathered the evolutionary process not as gadfly but a crucial component in the maintenance of homeostasis.

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Contents

Introduction

Xanthine oxidoreductase (XOR) is a molybdoflavin enzyme that catalyzes the terminal two reactions in purine degradation in primates; oxidation of hypoxanthine to xanthine and the subsequent oxidation of xanthine to uric acid. XOR is a homodimer of ~300 kD with each subunit consisting of four redox centers: a molybdenum cofactor (Mo-co), one FAD site and two Fe/S clusters, Fig. 1. The Mo-co is the site of purine oxidation while NAD^+ and O_2 reduction occur at the FAD. The two Fe/S clusters provide the conduit for electron flux between the Mo-co and the FAD [1–3]. The enzyme is transcribed as a single gene product, xanthine dehydrogenase (XDH) where substrate-derived electrons reduce NAD^+ to NADH, Fig. 1A. However, during inflammatory conditions, oxidation of key cysteine residues (535 and 992) and/or limited proteolysis converts XDH to xanthine oxidase (XO) [4]. In the oxidase form, affinity for NAD^+ at the FAD is greatly decreased while affinity for oxygen is significantly enhanced resulting in univalent and divalent electron transfer to O_2 generating $O_2^{\bullet-}$ and hydrogen peroxide (H_2O_2), respectively, Fig. 1B [5]. This capacity to reduce O_2 led to XOR being identified as the first source of biological $O_2^{\bullet-}$ formation and subsequently as a significant

Abbreviations: GAGs, glycosaminoglycans; H_2O_2, hydrogen peroxide; I/R, ischemia/reperfusion; •NO, nitric oxide; NOS, nitric oxide synthase; $O_2^{\bullet-}$, superoxide; ROS, reactive oxygen species; XDH, xanthine dehydrogenase; XO, xanthine oxidase; XOR, xanthine oxidoreductase).

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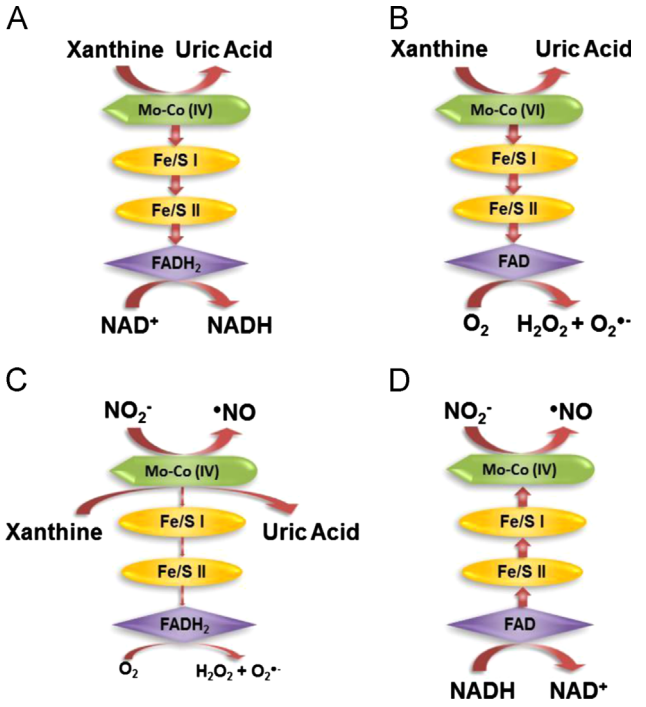

Fig. 1. XOR-Catalyzed Reactions. (A) For XDH, xanthine is oxidized to uric acid and electrons transferred via 2 Fe/S centers to the FAD where NAD^+ is reduced to NADH. (B) For XO, xanthine is oxidized to uric acid and electrons are transferred to the FAD where O_2 is reduced to $O_2^{\bullet-}$ and H_2O_2. Under normal O_2 tension and pH the Mo-co would reside more often in the oxidized +6 (VI) valence as electrons are rapidly transferred to O_2 at the FAD. (C) Nitrite (NO_2^-) undergoes a 1 electron reduction to $^{\bullet}NO$ at the Mo-cofactor of XO (electrons are donated directly to Mo by xanthine). (D) NO_2^- is reduced to $^{\bullet}NO$ at the of XO (electrons are supplied by NADH and transferred retrograde reducing the Mo). Under low O_2 tensions and pH the Mo-co would reside more often in the reduced +4 (IV) valence as electrons are more slowly transferred to O_2. This decrease in electron flux from the Mo-co to the FAD is depicted in (C) as diminished arrows whereas in (D) NADH-mediated electron donation at the FAD is out-competing O_2-mediated electron withdrawal and thus the arrows are reversed indicating flux from the FAD to the Mo-co.

source of reactive species mediating ischemia/reperfusion injury [6,7]. As the redox field progressed, several additional enzymatic and non-enzymatic sources of free radicals and reactive species have been identified yet, to date, XOR remains the most pharmacologically targetable thus incentivizing extensive exploration of inhibition strategies to address disease processes where elevated rates of reactive species formation are contributory.

Oxidant formation

Most reports refer to XO as a source of $O_2^{\bullet-}$ and assume H_2O_2 formation is a result of spontaneous dismutation of $O_2^{\bullet-}$. This premise is completely invalid as attainment of 100% $O_2^{\bullet-}$ generation requires XO turnover at pH 10.0 in an environment of 100% O_2 [8]. However, under room air and pH 7.4, XO transfers over 72% of its substrate-derived electrons to O_2 divalently to generate H_2O_2 and thus 28% to $O_2^{\bullet-}$ formation. This observation is critically important as it clearly demonstrates that, under conditions approaching those encountered *in vivo*, H_2O_2 is the major reactive product of XO-catalyzed O_2 reduction [8,9]. The prime determinate of the relative quantities of $O_2^{\bullet-}$ and H_2O_2 generated by XO is O_2 tension. For example, at pH 7.4 and 10% O_2 XO generates ~26% $O_2^{\bullet-}$ and thus ~74% H_2O_2 whereas at 1% O_2, XO forms ~90% H_2O_2 and only ~10% $O_2^{\bullet-}$, Fig. 2 [9]. In addition to O_2 tension, pH and purine concentration also play a significant role in divalent versus univalent electron transfer to O_2. The reaction of hypoxanthine/xanthine at the Mo-co of XO is based-catalyzed with a pH optimum of 8.9 and a $K_m = \sim 6.5\ \mu M$. Under normal physiologic conditions, hypoxanthine + xanthine levels in humans are ~1–3 μM; however, under hypoxic/inflammatory conditions these levels have been reported as high as 50–100 μM while pH concomitantly drops below 7.0 [10–12]. When this occurs, total purine (hypoxanthine+xanthine) concentration is well above the K_m and thus will not significantly impact either rates of electron deposition at the Mo-co or resultant transfer to the FAD. However, acidic pH will significantly retard purine–Mo-co reaction thereby reducing the electron flux rate which favors divalent transfer to O_2 to generate H_2O_2. Therefore, under ischemic and/or hypoxic conditions, where both O_2 levels and pH are reduced, H_2O_2 formation is favored suggesting that XO activity may be influential in numerous signaling cascades where H_2O_2 has been noted to participate. However, this hypoxia-mediated proclivity for H_2O_2 production cannot overshadow the fact that rates of $O_2^{\bullet-}$ formation by XO under these same conditions are sufficient to mediate alterations in vascular function by reducing $^{\bullet}NO$ bioavailability via direct reaction ($^{\bullet}NO + O_2^{\bullet-} \rightarrow ONOO^-$) [13–15].

While the post-translational conversion of XDH to XO has become synonymous with conversion from a source of reducing equivalents to a source of reactive oxygen species (ROS), it is important to recognize that under certain circumstances XDH effectively reduces O_2 to generate ROS. Although NAD^+ is the preferred electron acceptor for XDH, when levels of this substrate are low XDH will utilize O_2 [16]. These conditions include hypoxia either localized, regional or systemic where O_2-dependent alterations in cellular respiration lead to decreased mitochondrial NADH oxidation and thus significant diminution of NAD^+ levels [17]. This being said, care should be taken not to exclusively associate XDH with the form of XOR that does not produce ROS.

XO-endothelial interaction

In humans, XOR is ubiquitously expressed with the liver and intestines displaying the highest specific activity [18]. Hypoxia as well as inflammatory cytokines (TNF-α, IL-1β, IFN-γ), induce XDH expression in tissues and vascular endothelial cells where it is released to the circulation, Fig. 2 [18,19]. Circulating XDH is rapidly (<1 min) converted to XO where it avidly binds to negatively charged glycosaminoglycans (GAGs) on the apical surface of vascular endothelial cells [20,21]. This XO–endothelium interaction is exemplified in animal models and clinical studies of cardiovascular disease where intravenous administration of heparin results in a substantive increase in plasma XO activity, suggesting heparin-mediated mobilization of XO from vascular endothelial GAGs [21–23]. While XO exhibits a net negative charge at physiological pH, pockets of cationic amino acid motifs on the surface of the protein result in high affinity for GAGs ($K_d = 6$ nM) [21,24,25]. Binding to and sequestration of XO on GAGs: (1) amplifies local XO concentration and subsequent ROS generation; (2) alters XO kinetic properties further shifting oxidant formation from $O_2^{\bullet-}$ to H_2O_2 and (3) confers significant resistance to inhibition from the pyrazalopyrimidine-based inhibitors, allo/oxypurinol [26]. For example, when compared to XO in free in solution, XO–GAG association decreases substrate binding affinity and thus: (1) increases the K_m for xanthine over 3-fold (6.5→21.2 μM); (2) reduces $O_2^{\bullet-}$ production by 34% favoring H_2O_2 formation and (3) induces a 5-fold increase in the K_i for allo/oxypurinol (85→451 nM) [26]. Taken together, inflammation-mediated up-regulation of XDH, export to the circulation, rapid conversion to XO and sequestration by the endothelium coalesce to generate a vascular milieu favoring increased rates of reactive species generation that can participate in mediating the loss of homeostasis, Fig. 2. This deleterious action of XO has been noted in various reports of vascular and cardiopulmonary diseases including

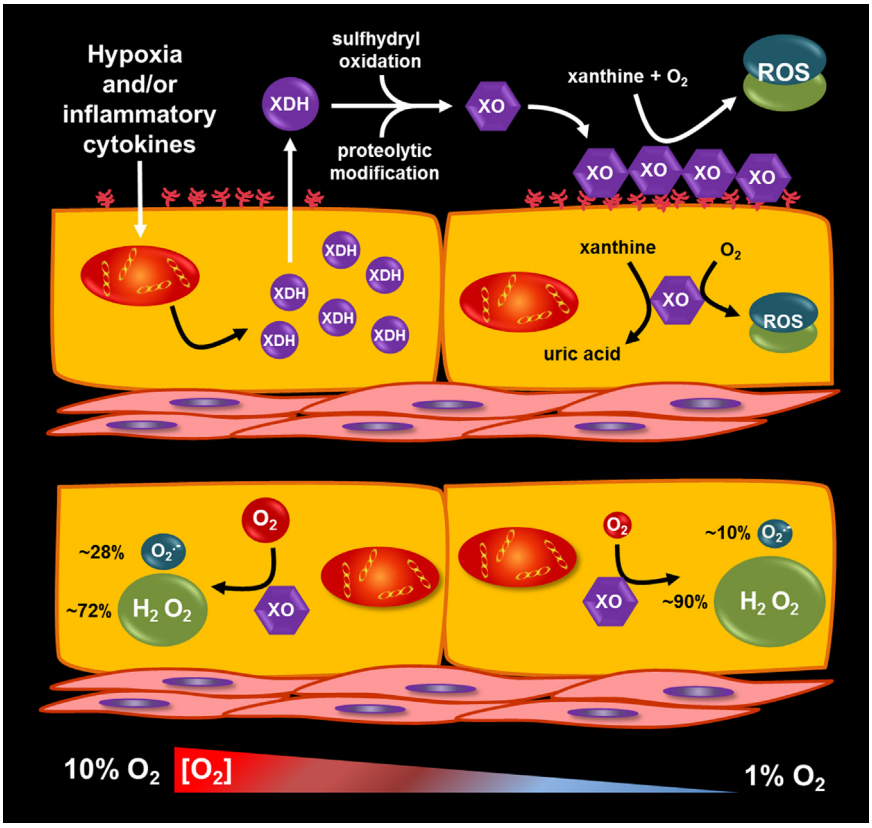

Fig. 2. Hypoxic/inflammatory induction of XOR and vascular consequences. (Top) Inflammatory cytokines and/or hypoxia induce XDH transcription and resultant protein expression. In vascular endothelial cells XDH is exported to the circulation where it is rapidly converted to XO by plasma proteases. However, cellular export is not requisite for XDH conversion to XO as enhanced oxidative stress within the endothelium can induce oxidation of critical cysteine residues that mediate reversible conversion to XO. Once in the circulation, negatively charged glycosaminoglycans (GAGs) on the luminal surface of the endothelium bind and sequester XO by high affinity (K_d=6 nM) interaction with pockets of cationic amino acids on the surface of the enzyme. This sequestration amplifies local XO levels creating a vascular milieu whereby, in the presence of hypoxanthine and/or xanthine, enhanced rates of $O_2^{\bullet-}$ and H_2O_2 formation ensue. (Bottom) A key determinate regulating the relative amounts of $O_2^{\bullet-}$ and H_2O_2 generated by XO is the concentration of molecular O_2. Shown is a cartoon representing the change in relative percentages of $O_2^{\bullet-}$ and H_2O_2 formed by XO at 10% O_2 (~130 μM O_2) compared to 1% O_2 (~13 μM O_2). This range of O_2 tension is critically important as it represents from well above to 50% below the K_m-O_2 at the FAD-cofactor of 27 μM or ~2% O_2. As the O_2 tension drops below this K_m value the FAD-cofactor assumes more time in the fully reduced $FADH_2$ state where, upon reaction with O_2, divalent electron transfer is preferred. This process assumes constant electron from the Mo-co (e.g. [hypoxanthine+xanthine] above the 6.5 μM K_m at the Mo-co) which would be expected under conditions similar to those encountered in the lumen of an ischemic/hypoxic vessel. In addition, it is critical to note that XO–GAG association as well as acidic pH serves to further favor H_2O_2 formation. Taken together, moderate to severe hypoxia induces XDH expression, export and conversion to XO that is subsequently captured by GAGs in an environment the primed for catalyzing the formation of H_2O_2 as well as a little $O_2^{\bullet-}$.

heart failure, chronic obstructive pulmonary disease (COPD), pulmonary hypertension, sickle cell disease and Type I and II diabetes [14,27–30].

XOR knockouts and inhibition strategies

For an enzyme whose activity was described in 1889 followed by it being named xanthine oxidase in 1901 and first purified in 1939, surprisingly little detail is known regarding its regulation and subsequent interplay with biomolecular pathways when compared to other enzymes with much more recent history [31]. Potential reasons for this discrepancy in understanding include: (1) lethality of global XDH knockouts; (2) absence of reports utilizing tissue-specific conditional knockouts; (3) side-effects resulting from pharmacological knockdown with tungsten supplementation; (4) promiscuity of the active site resulting in ambiguity regarding both substrate identity and inhibition by compounds designed to specifically target other molybdopterin enzymes and (5) resistance to inhibition by allo/oxypurinol conferred by binding to vascular endothelial GAGs. Attempts to establish homozygous knockouts of XDH in mice have resulted in the death of pups before 30 days of age due to kidney fibrosis and failure attributed to excessive hypoxanthine deposition [32,33]. Similar effects were obtained with heterozygous XDH knockouts where both nutrient absorption and kidney failure resulted in death in a similar timeframe as $XDH^{-/-}$. These unfortunate side-effects have relegated investigators to utilize allo/oxypurinol-based inhibition or global XOR knockdown with dietary tungsten (W) supplementation for proof-of-principle experimentation. Dietary supplementation with sodium tungstate (NaW) results in replacement of the active site Mo with W producing an enzyme that is inactive with respect to hypo/xanthine oxidation to uric acid. However, it is important to note that W-mediated inactivation of the Mo-co does not affect the capacity of the FAD in XOR to be reduced by NADH and subsequently react with and reduce O_2 to produce $O_2^{\bullet-}$ and H_2O_2. In addition, treatment with NaW also inactivates other members of the molybdopterin family including aldehyde oxidase, sulfite oxidase and mitochondrial amidoxime reducing component 1 (MARC1) which can lead to significant ambiguity regarding interpretation of results. On the other hand, inhibition of XOR with allo/oxypurinol is also not optimal as: (1) allopurinol can mediate effects on other purine catabolic pathways including those resulting in alteration of adenosine levels [34]; (2) reaction of allopurinol with XO induces enzyme turnover resulting in $O_2^{\bullet-}$ and H_2O_2 formation [35] and (3) plasma allo/oxypurinol concentrations (100–400 μM) well above those tolerated clinically (30–90 μM) are incapable of fully inhibiting XO when it is sequestered by vascular

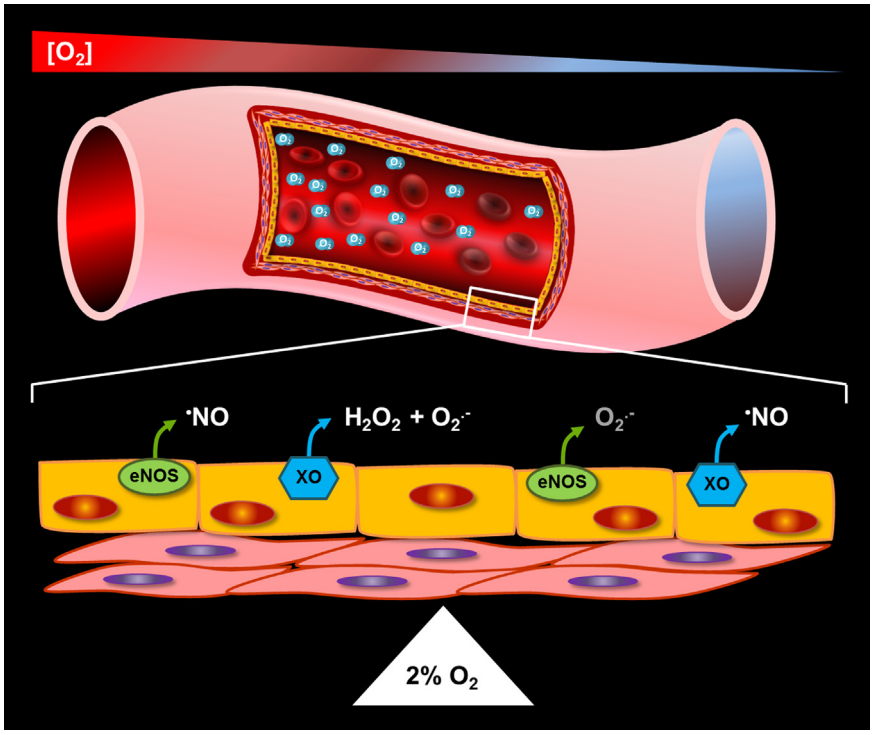

Fig. 3. Hypoxic conversion of XOR from oxidant to •NO production. Hypoxia mediates the alteration of microenvironmental factors that coalesce to both facilitate the conversion of XO from oxidant to •NO production and diminish the capacity of eNOS to catalyze •NO formation as well as enhance its potential to uncouple and produce $O_2^{\bullet-}$. These factors include: (1) acidic pH; (2) elevation of NADH levels; (3) oxidation of biopterin and, of course; (4) diminution of O_2 tension. Nitrite reduction at the Mo-co of XO is acid catalyzed with a pH optimum ~6–6.5 while xanthine oxidation the Mo-co is base catalyzed with a pH optimum=8.9. Therefore, lower pH confers a reduction in affinity for xanthine while increasing affinity for NO_2^-. Thus, lower pH results in an environmental setting more favorable for NO_2^- to compete with xanthine for the Mo-co. This shift in affinity away from xanthine and toward NO_2^- is further augmented by reduction in O_2 tension to values below the K_m-O_2 at the FAD (27 μM or ~2% O_2). Once this occurs, electron withdrawal from the FAD slows resulting in the Mo-co assuming a more reduced state (Mo-co IV, see Fig. 1C and D) which is crucial for two reasons: (1) NO_2^- reduction requires a reduced Mo-co and (2) xanthine oxidation requires an oxidized Mo-co. Therefore, O_2 tensions at or below 2% further assist the ability of NO_2^- to compete with xanthine for reaction at the Mo-co. As seen in Fig. 1D, hypoxia-mediated elevation of NADH levels can also further augment the potential for NO_2^- reduction at the Mo-co by competing with O_2 for reaction at the FAD. In this case, NADH-FAD reaction results in reduction of the FAD to $FADH_2$ inducing Mo-co reduction by retrograde electron flux as well as inhibition of O_2-mediated electron withdrawal. On the other hand, this same inflammatory setting negatively impacts •NO formation by eNOS. For example, as O_2 tensions fall below 2% (27 μM): (1) O_2 becomes limiting as a substrate for eNOS-catalyzed •NO production where the K_m-O_2 for eNOS=23 μM and (2) acid pH coupled with elevated levels of oxidants drive eNOS uncoupling and the propensity for eNOS-mediated $O_2^{\bullet-}$ generation (depicted above the cell on the right in small font). Taken together, diminution of O_2 tension, acidic pH, elevation of NADH levels, and oxidation of biopterin converge to generate an environment whereby the burden for •NO production shifts from eNOS to XOR. Furthermore, the critical O_2 concentration where this shift or *"switch"* is triggered is assumed to be near 2% where the K_m-O_2 values for both XO and eNOS collide (depicted by a pivot point in the cartoon). However, it is crucial to note that if this process is to be of biological relevance then: (1) NO_2^- and/or NO_3^- levels must be significantly elevated by dietary or pharmacologic supplementation and (2) the proposed interplay between the components of these concerted reactions must be vigorously pursued and validated.

GAGs [26,35]. As a result of these limitations we have recently identified febuxostat (Uloric) to be more optimal for exploring contributions of XOR both *in vivo* and tissue culture. For example, febuxostat concentrations (25–50 nM) well below the reported plasma C_{max} (15 μM) for the clinic demonstrate over 3 orders of magnitude greater potency than allopurinol (K_i=0.9 nM vs. 1.6 μM), are not affected by XOR-vascular GAG association and do not alter other purine catabolism pathways [34,35]. *In toto*, these findings clearly demonstrate the potential benefit of using febuxostat to interrogate XOR-dependence in various experimental models.

XOR-catalyzed •NO production

For decades, the dogma in the field has been as described above; specifically that inflammation/hypoxia-induced enhancement of XO activity equates to elevated rates of XO-derived ROS generation, propagation/exacerbation of the disease process and ultimately poor clinical outcomes. This correlation has been substantiated in several disease models where XO inhibition leads to a reduction in symptoms and measurable restoration of function. However, recent reports have posed a bold challenge to the standing paradigm by demonstrating a nitrate/nitrite (NO_2^-) reductase function for XOR ($1e^-$ reduction of NO_2^- to •NO) suggesting XOR to be a source of beneficial •NO under these same hypoxic/inflammatory conditions. In essence, these observations directly countervail a substantive body of literature indicating XO inhibition to be beneficial and as such affirm the need to more closely interrogate XOR-catalyzed reactions and potential factors that alter product formation. For example, reduction of NO_2^- to •NO is indeed catalyzed by purified XO under anoxic conditions when electrons are supplied by either xanthine or NADH [36–38]. Nitrite reduction occurs at the reduced Mo-co (Mo-co IV) and electrons driving this reaction can be supplied directly by xanthine (Fig. 1C) or indirectly by NADH via electron donation at the FAD with subsequent retrograde flow to the Mo-co, Fig. 1D [39]. At this point, it is critical to note that work with the purified enzyme has revealed two issues requiring resolution before the biological relevance of XOR-derived •NO can be substantiated. First, the NO_2^- reductase activity of XOR is inhibited by O_2 which results from oxidation of the Mo-co mediated by electron withdrawal from the FAD [40]. Second, the affinity for NO_2^- at the Mo-co of XOR is 3 orders of magnitude less than for xanthine (K_m-NO_2^-=2.5 mM vs. K_m-xanthine=6.5 μM) [38]. Despite these formidable issues, several reports demonstrate significant reduction

in or ablation of salutary outcomes attributable to NO_2^- treatment upon inhibition of XOR activity with allo/oxypurinol affirming the need for more vigorous investigation to fully elucidate this reductive process. For example, systemic inhibition of XOR activity has diminished protective effects of NO_2^- treatment in models of intimal hyperplasia [41], lung injury [42,43], myocardial infarction [44], pulmonary hypertension [45] and ischemia/reperfusion (I/R)-induced damage [46–49]. It is also important to note that plasma levels of NO_2^- are reported to be enhanced in an XOR-dependent manner by treatment with nitrate (NO_3^-) where XOR serves first as a NO_3^- reductase ($NO_3^- + 1e^- \rightarrow NO_2^-$) and ultimately a NO_2^- reductase ($NO_2^- + 1e^- \rightarrow {}^{\bullet}NO$). This XO-catalyzed process was described over 50 years ago [50] and recently expanded to *in vivo* models [51]. In these experiments treatment of germ-free mice (void of bacterial NO_3^- reductases) with NO_3^- resulted in elevation of plasma NO_2^- levels that were not observed when mice received co-treatment with allopurinol and thus are consistent with previous biochemical reports demonstrating NO_3^- reductase activity for XOR [52]. In the aggregate, there is a new body of evidence suggesting a protective role for XOR under hypoxic and inflammatory conditions in the presence of elevated levels of NO_2^-, summarized in Fig. 3. However, several key issues remain unclear regarding the microenvironmental conditions necessary for operative and biologically relevant nitrite reductase activity of XOR *in vivo* and were recently extensively reviewed [53].

Although XOR has been studied for 114 years, it is clear from the information provided herein that we have only begun to understand the complexity regarding the interplay between crucial microenvironmental factors and the identity/generation of XOR-derived reactive products as well as their impact on cellular signaling both in normal and pathophysiology. Suffice it to say the long-standing dogma identifying XDH as a housekeeping enzyme and XO as a mediator of negative clinical outcomes is beginning to crumble as we uncover new roles for XOR in the network of adaptive responses that serve to maintain homeostasis.

Acknowledgments

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