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Role of urate, xanthine oxidase and the effects of allopurinol in vascular oxidative stress

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Abstract: Oxidative stress plays an important role in the progression of vascular endothelial dysfunction. The two major systems generating vascular oxidative stress are the NADPH oxidase and the xanthine oxidase pathways. Allopurinol, a xanthine oxidase inhibitor, has been in clinical use for over 40 years in the treatment of chronic gout. Allopurinol has also been shown to improve endothelial dysfunction, reduce oxidative stress burden and improve myocardial efficiency by reducing oxygen consumption in smaller mechanistic studies involving various cohorts at risk of cardiovascular events. This article aims to explain the role of xanthine oxidase in vascular oxidative stress and to explore the mechanisms by which allopurinol is thought to improve vascular and myocardial indices.

Keywords: allopurinol, vascular oxidative stress, vascular endothelial dysfunction

Introduction

The role of oxidative stress in disease has always been a contentious issue because attempts to reduce oxidative stress using antioxidant vitamins such as in the Heart Outcomes Prevention Evaluation (HOPE) study have consistently failed to demonstrate a mortality benefit.¹ There are many possible reasons for this, not least because of cohort selection and inappropriate dosing of antioxidants. Even if adequate doses were used, at many magnitudes higher than currently taken as part of multivitamin supplementation, there would be further issues with tolerability and safety. Therefore, recent research has concentrated on mechanisms to reduce the formation of reactive oxygen species (ROS) rather than a scavenging approach to already-formed ROS. The two major ROS generating systems are the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the xanthine oxidase (XO) systems.

Background

ROS consists of molecular oxygen and all of its aerobic cellular metabolites including superoxide (O_2^-) and hydroxyl radical (OH^\cdot). Other substances such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and hypochlorous acid (HOCL) have oxidative properties although they are not free radicals.² McCord and Fridovich were one of the first to describe the deleterious effects of ROS in inflammation in an article describing the enzyme superoxide dismutase.³ Since then, there has been an abundance of research demonstrating the role oxidative stress plays in the pathophysiology of abnormal vasorelaxation. There is also increasing evidence that oxidative stress plays a direct role in myocardial remodeling.^{4,5} above and beyond its effects on vasomotion.

Enhanced production of reactive oxygen species (ROS) is a major cause of endothelial dysfunction. This has been demonstrated in animal studies using p66^{SHC} knockout mice which demonstrate reduced aortic endothelial cell superoxide production and a 30% prolonged lifespan.⁶ The precise role of p66^{SHC} remains unclear but in studies looking at its effects on the p53 signaling pathway demonstrate that it is involved

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in stress-activated p53-induced elevation of intracellular oxidants, apoptosis and regulation of the intracellular redox state.⁷ This is consistent with the finding that ROS promotes apoptosis and microvascular rarefaction in spontaneously hypertensive rats.⁸ Furthermore, in chronic heart failure (CHF) patients with the Glu298Asp variant (Glu to Asp amino acid substitution for codon 298 of endothelial nitric oxide synthase (eNOS)) have a significantly shorter eNOS half-life. This translates to a significantly lower event-free survival.⁹ There is also evidence that the same polymorphism results in blunting of the endothelial-dependent vasodilation in healthy individuals.¹⁰

NADPH oxidase catalyzes the reduction of oxygen through electron donation from either NADH (predominantly) or NADPH to generate superoxide (O_2^-). This system is thought to be the predominant driver of O_2^- formation. The most potent inducer of the NADPH oxidase system is angiotensin II,^{11,12} and there are angiotensin II antagonists in the form of ACE inhibitors, angiotensin II receptor antagonists and direct renin inhibitors (DRA) in clinical use. The other enzyme system, XO, has been largely ignored with regards to managing oxidative stress despite the availability of XO inhibitors allopurinol, oxypurinol and febuxostat. This is primarily because most of the data for the beneficial effect of these agents have come from smaller mechanistic studies and the lack of large clinical trial evidence to support widespread use. The two larger studies using the XO inhibitor oxypurinol either showed neutral.¹³ (in LA-PLATA) or negative results (OPT-CHF)¹⁴ which may have been due to an insufficient dose-effect or patient selection. A subsequent follow-up study¹⁴ found a significant benefit in patients with high baseline urate, which could imply either high XO activity or that urate itself is detrimental. A recent study in patients with type II diabetes suggests that urate lowering per se does not improve endothelial function.¹⁵

Xanthine oxidase (XO)

Xanthine oxidoreductase (XOR) is part of a group of enzymes known as the molybdenum iron-sulfur flavin hydroxylases. It was first discovered in milk by Schardinger in 1902¹⁶ and is thought to be involved in reactions that produce ROS such as nitrite which enable newborn infants to overcome gut-associated bacterial gastroenteritis.^{17,18} XOR is widely distributed throughout various organs including the liver, gut, lung, kidney, heart, brain and plasma¹⁹ with the highest levels being found in the gut and the liver.²⁰ In the myocardium, it is localized to the capillary endothelial cells.²¹ The gene encoding for XOR is located at the short arm of chromosome 2.²² It exists

in two inter-convertible forms known as XO (EC 1.1.3.22) and xanthine dehydrogenase (XDH) (EC 1.17.1.4).²³ Both enzymes consist of two identical subunits of 145 kDa.

Mammalian XOR is present *in vivo* as the dehydrogenase form but is easily converted to XO by oxidation of the sulfhydryl residues or by proteolysis.¹⁹ Although XDH has a much greater affinity for NAD^+ compared to oxygen (and therefore is practically incapable of directly producing ROS), both XO and XDH can oxidize NADH which results in ROS formation.²⁴ Physiologically, XOR is involved in the hydroxylation of purines, pterins, and aldehydes but its primary role is as the rate-limiting enzyme in the conversion of hypoxanthine to xanthine and xanthine to urate (Figure 1). XOR is the only enzyme capable of catalyzing the formation of urate in man.²⁵ In lower mammals, an enzyme, urate oxidase further metabolizes uric acid to allantoin but this enzyme is inactivated in primates.²⁶ There is also a suggestion from teleological studies that urate may have even evolved as a compensatory mechanism in higher primates that have lost the capacity to generate other antioxidants like ascorbate *in vivo*.²⁷

The mechanism by which XOR catalyzes hypoxanthine and xanthine conversion is extremely complex and has been previously described in detail.^{16,28} A fully reduced XO contains six electrons and its re-oxidation involves electron transfer to oxygen molecules which generates two H_2O_2 and two O_2^- species²⁸ for every fully reduced XO molecule (Figure 1).

There is a large variability in human XOR expression which can be up to three-fold and on average 20% higher in men than in women.²⁹ Although basal expression of XOR is low in humans, hypoxia, IL-1, IL-6, TNF- α , lipo-polysaccharides as well as steroid treatment have been shown to upregulate transcription.¹⁶ XO is significantly elevated in a variety of conditions including limb ischemia,³⁰ major surgery³¹ coronary artery disease (CAD)³² and heart failure.³³ Circulating XO binds to glycosaminoglycans on the surface of endothelial cells where it is thought it acquires modified kinetics (higher K_m and K_i , oxidant producing capacity, and increased stability).³⁴ There are suggestions that this form of induced, circulating and depositing XO appears to be more important in the pathogenesis of endothelial injury compared with XO constitutively produced from endothelial cells.³⁵

Despite this, the activity of *endothelial bound* XO is increased by more than 200% in patients with CHF.³⁶ Furthermore, studies using electron spin resonance have demonstrated that endothelial oxygen tension is thought to regulate XO activity at a post-translational level as demonstrated by a doubling in XOR activity post exposure to hypoxia without

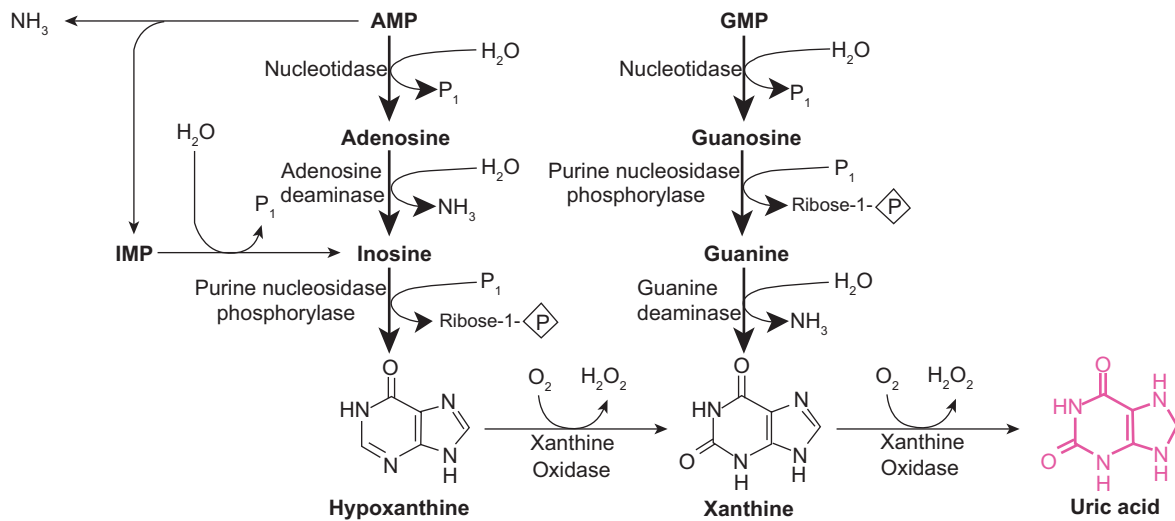


Figure 1 The purine degradation pathway. Reproduced with permission from Berry CE, Hare JM. Xanthine oxidoreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications. *J Physiol.* 2004; 555(Pt 3):589–606.¹⁶ Copyright © Blackwell Publishing.

any increase in mRNA expression for 24 hours in bovine aortic endothelial cells.³⁷ Cells produce a marked elevation in XO levels when exposed to ischemia³⁸ and XDH conversion to XO is also accelerated in hypoxia.³⁹ When infused acutely, XO produces a marked decrease in cardiac contractility, cardiac index and left ventricular systolic pressure.⁴⁰ In atherosclerotic plaques, urate levels are found to be elevated six-fold, reflecting accelerated purine oxidation within these plaques. Therefore XO production may not necessarily be reflected by systemic levels of XO metabolites.⁴¹

The XO inhibitor allopurinol

Recent evidence indicates that allopurinol improves endothelial dysfunction in high risk primary prevention patients such as those with metabolic syndrome.⁴²

Allopurinol has also been shown to normalize endothelial dysfunction in type 2 diabetics with mild hypertension and reduced plasma malondialdehyde (MDA) levels.⁴³ MDA results from acid hydrolysis of lipid peroxides which are formed by free radical attack on plasma lipoproteins. It is therefore used as an indirect measure of oxidized low-density lipoprotein (LDL).

In the experimental murine myocardial infarction model, allopurinol significantly attenuated LV dilatation, hypertrophy, fibrosis and dysfunction. Once again, XO expression (as determined by electron spin resonance spectroscopy) and myocardial ROS generation were markedly increased in the post-myocardial infarction ischemic model.⁴⁴ This suggests a role for allopurinol in LV remodeling, a possibility that we are investigating

at present in our unit. Allopurinol has also been shown to be beneficial in conditions such as post coronary artery bypass surgery where it reduced ischemic events and produced less ST segment depression⁴⁵ as well as in hypercholesterolemic patients.⁴⁶ There are mixed data from ischemic-reperfusion studies. Pacher et al,¹⁹ in an excellent in-depth review on this topic, have summarized the data in the Table 1.

Allopurinol in CHF was assessed by Doehner et al⁴⁷ and by Farquharson et al.⁴⁸ Doehner et al showed that the degree of improvement in forearm blood flow correlated with the degree of urate lowering. Interestingly, they also measured allantoin, a marker of oxygen free radical generation, which was reduced by 20% following 300 mg/day allopurinol. Farquharson et al⁴⁸ from our unit, found a 181% change in forearm blood flow with 300 mg allopurinol. They also found a 33% reduction in plasma MDA levels in patients treated with 300 mg allopurinol suggesting that the improvement in endothelial function and NO bioavailability seen was due (at least in part) to a reduction of ROS generation or oxidative stress burden. Allopurinol also reduced B-type natriuretic peptide (BNP) in stable CHF patients, although the reduction did not correlate with the fall in urate.⁴⁹

We have shown previously that high dose allopurinol is a very effective antioxidant in the vasculature because it abolishes the vitamin C-sensitive component of oxidative stress on vascular endothelial function, ie, in patients on high dose allopurinol, there is insufficient vascular oxidative stress being formed for vitamin C to neutralize the oxidative stress and further improve endothelial function.⁵⁰ This is

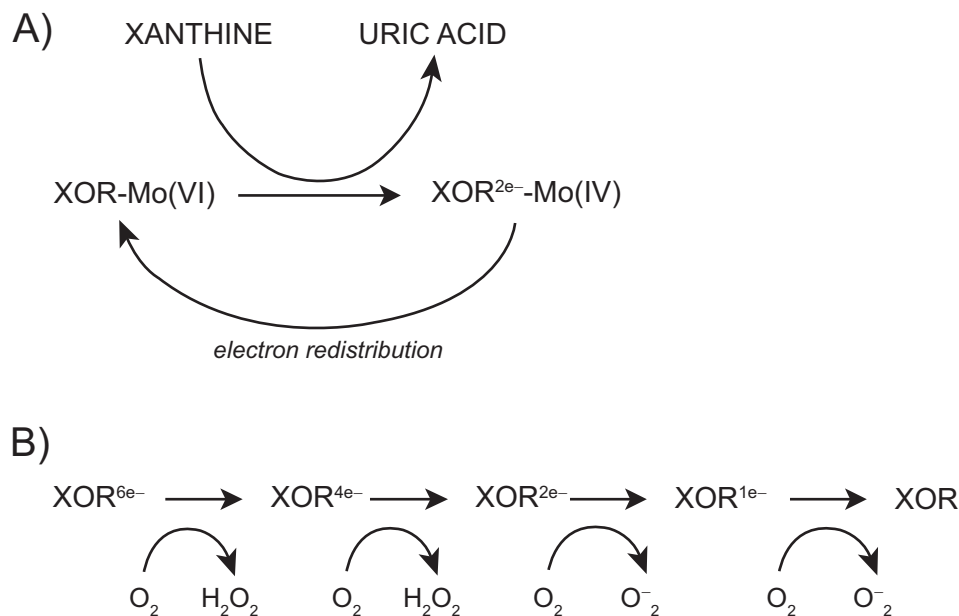


Figure 2 Mechanism of xanthine oxidoreductase XOR reaction with xanthine; **A)** reductive half reaction; **B)** oxidative half reaction. Reproduced with permission from Berry CE, Hare JM. Xanthine oxidoreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications. *J Physiol.* 2004; 555(Pt 3):589–606.¹⁶ Copyright © Blackwell Publishing.

further strengthened by evidence that the beneficial effect of vitamin C co-infusion in patients with CHF was greatest in patients with the highest levels of oxidative stress as measured by extracellular superoxide dismutase (ecSOD)³⁶ and XO activity.

Interestingly, it is the ability of allopurinol to inhibit XO, and therefore ROS, that results in its inclusion as a constituent in the University of Wisconsin solution for organ transport prior to transplantation.⁵¹

The biomarker of lipid peroxidation, F₂-isoprostane, is an indirect but validated marker of oxidative stress. Data from our group (unpublished) also demonstrate the ability of allopurinol to significantly reduce F₂-isoprostane levels in patients with high baseline oxidative stress, further confirming the potent antioxidant effect of allopurinol. This also reflects XO activity within the vascular milieu in these patients because for the same degree of urate lowering as those with low baseline oxidative stress, only patients with high pre-existing oxidative stress were found to have a significant reduction in F₂-isoprostanes. This is consistent with the known cascade effect of multiple superoxide and hydrogen peroxide generation for every urate molecule formation that is catalyzed by XO.^{16,62} In fact, NO and O₂⁻ react at a three-fold greater rate than the rate at which antioxidant defense mechanisms such as SOD can eliminate O₂⁻.⁶³

In chronic diseases such as CHF, sustained high levels of ROS may exceed the capacity of cellular enzymatic and

non-enzymatic antioxidants⁶⁴ to counter its effects. Using electron spin resonance, Spiekermann et al demonstrated that both NADPH oxidase and xanthine oxidase are up-regulated in patients with coronary artery disease.³² Others have demonstrated increased levels in CHF.^{36,65}

Direct antioxidant action of allopurinol

Allopurinol directly scavenges free radicals as demonstrated by Das et al and others^{66–68} in *in vitro* hearts where evidence of free radical scavenging occurred in the absence of XO activity. Animal studies in experimentally induced uveitis show that at very high doses (up to 50 mg/kg), allopurinol behaves as a free radical scavenger with intrinsic antioxidant properties. Crucially, this was only achieved far beyond the XO inhibition dose of 10 mg/kg and not at that dose itself. Further evidence for a possible direct antioxidant effect of allopurinol comes from models of experimental colitis where tungsten (a potent XO inhibitor) failed to improve symptoms whereas allopurinol did.⁶⁹ Augustin et al suggested that this direct effect was only seen at higher doses.⁷⁰ This was also seen in mice paracetamol toxicity models where lower doses (sufficient to block XO activity) of allopurinol failed to show antioxidant protection but higher doses did.⁷¹ There have been other non-XO effects of allopurinol suggested such as copper chelation, preventing LDL oxidation as described above,⁷² inhibition of heat shock protein (hsp) expression⁷³ and

Table 1 Effects of xanthine oxidase (XO) inhibitors in myocardial ischemia-reperfusion injury

Model	Disease or trigger	Mode of XO inhibition	Effects of XO inhibition	Reference
Human (169 patients)	Coronary bypass surgery	Allopurinol	Decreased hospital mortality rate, increased cardiac index	Johnson et al ⁵²
Human (90 patients)	Coronary bypass surgery	Allopurinol	Reduced arrhythmias, need for inotropes and perioperative myocardial infarction in patients	Rashid and William-Olsson ⁵³
Human (140 patients)	Myocardial I	Allopurinol	Increased incidence of infarct extensions in the treatment group	Parmley et al ⁵⁴
Human (80 patients)	Ischemic heart disease	Allopurinol + erinit	Decreases in serum and daily urinary levels of uric acid and lipid peroxidation antioxidative system and an improvement of central hemodynamics	Kaliakin and Mit'kin ⁵⁵
Human (50 patients)	Coronary bypass surgery	Allopurinol	Improves postoperative recovery and reduces lipid peroxidation in patients undergoing coronary artery bypass grafting	Coghlan et al ⁵⁶
Human (20 patients)	Coronary bypass surgery	Allopurinol	Failed to demonstrate a cardioprotective effect of allopurinol in patients with good left ventricular function undergoing elective coronary artery surgery	Taggart et al ⁵⁷
Human (20 patients)	Coronary bypass surgery	Allopurinol	Allopurinol suppressed the reaction rate of XO; therefore, the levels of intermediates, hypoxanthine and xanthine, were high, and the level of the final product, uric acid, was low – however, allopurinol had no efficacy for the level of lactate, pyruvate, CK, and CK-MB	Yamazaki et al ⁵⁸
Human (33 patients)	Coronary bypass surgery	Allopurinol	Better recovery of cardiac output and left ventricular stroke work after bypass surgery and reduction of plasma XO activity and concentrations of uric acid	Castelli et al ⁵⁹
Human (52 patients)	Coronary bypass surgery	Allopurinol	Allopurinol failed to improve left ventricular stroke work after cardiopulmonary bypass surgery	Coetzee et al ⁶⁰
Human (38 patients)	Percutaneous transluminal coronary angioplasty in patients with acute myocardial infarction	Allopurinol	Allopurinol pretreatment was effective in inhibiting generation of oxygen-derived radicals during reperfusion and in the recovery of left ventricular function	Guan et al ⁶¹

Adapted with permission from Pacher P, Nivorozhkin A, Szabo C. Therapeutic effects of xanthine oxidase inhibitors: renaissance half a century after the discovery of allopurinol. *Pharmacol Rev.* 2006; 58(1):87–114.¹⁹ Copyright © 2006 American Society for Pharmacology and Experimental Therapeutics.

⁵Studies concluded with negative results.

Abbreviations: I/R, ischemia-reperfusion; I, ischemia; CK, creatine kinase.

calcium sensitization (below). Allopurinol treatment reduces early changes in inflammation such as leukocyte activation by reducing adherence, rolling and extravasation.⁷⁴

Mechanoenergetic uncoupling

This phenomenon refers to an imbalance between left ventricular performance and myocardial energy consumption.⁷⁵ The role of XO inhibition may either be to maintain cardiac output while reducing myocardial oxygen consumption or even increase cardiac output without increasing myocardial oxygen consumption. In dogs with pacing-induced heart failure, allopurinol improved myocardial contractility and efficiency in oxygen utilization, prevented increases in systemic vasoconstriction and ameliorated reductions in myocardial contractility.^{65,76,77} In murine post-ischemic cardiomyopathy models, allopurinol attenuated the increase in end-systolic and end-diastolic volumes,⁷⁸ increased survival, augmented ventricular function as well as reduced products of lipid peroxidation.⁷⁹ Khan et al found a direct protein-protein interaction between XO and neuronal NOS in the sarcoplasmic reticulum of cardiac myocytes.⁸⁰ Allopurinol improved myofilament calcium sensitivity as contraction force increased without a concomitant rise in systolic Ca²⁺ influx. The effects were not seen in endothelial NOS deficient mice, suggesting a role for neuronal NOS preventing XO inhibition of cardiac excitation-contraction coupling.⁸⁰ The finding that allopurinol is a potent myofilament Ca²⁺ sensitizer, particularly in the setting of ischemia, is thought to be due to the inhibition of basal XO production. As with the previous study by Khan et al, Perez et al found an almost exclusive increase in force generation without a lowering of inward transient Ca²⁺.⁸¹

Despite the small sample size (n = 9), Cappola et al showed using cardiac catheterization that direct intracoronary infusions of allopurinol in these patients resulted in a marked decrease in myocardial oxygen consumption (MVO₂) with no decrease in the rate of left ventricular pressure rise (dP/dT), stroke work or ventricular load.⁸² Patients post coronary artery bypass grafting given allopurinol have also been shown to require less inotropic support.⁴⁵

Conclusion

The evidence for the role of oxidative stress in disease cannot be disputed. However, there are many questions that remain such as (1) exactly what is the contribution of oxidative stress to overall endothelial dysfunction; (2) at what stage should intervention take place; (3) what agents can we employ to effectively deal with this ubiquitous problem, which will be both safe and tolerable to our patients? The emerging evidence

from therapies such as allopurinol are encouraging and should be put to the test in larger randomized studies to determine if the interesting data garnered from smaller mechanistic studies actually translate to a survival advantage with these agents.

Disclosures

The authors disclose no conflicts of interest.

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