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Original Contribution

Febuxostat inhibition of endothelial-bound XO: Implications for targeting vascular ROS production

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ABSTRACT

Xanthine oxidase (XO) is a critical source of reactive oxygen species (ROS) that contribute to vascular inflammation. Binding of XO to vascular endothelial cell glycosaminoglycans (GAGs) results in significant resistance to inhibition by traditional pyrazolopyrimidine-based inhibitors such as allopurinol. Therefore, we compared the extent of XO inhibition (free and GAG-bound) by allopurinol to that by febuxostat, a newly approved nonpurine XO-specific inhibitor. In solution, febuxostat was 1000-fold more potent than allopurinol at inhibiting XO-dependent uric acid formation ($IC_{50} = 1.8$ nM vs 2.9 μM). Association of XO with heparin–Sepharose 6B (HS6B-XO) had minimal effect on the inhibition of uric acid formation by febuxostat ($IC_{50} = 4.4$ nM) while further limiting the effect of allopurinol ($IC_{50} = 64$ μM). Kinetic analysis of febuxostat inhibition revealed K_i values of 0.96 (free) and 0.92 nM (HS6B–XO), confirming equivalent inhibition for both free and GAG-immobilized enzyme. When XO was bound to endothelial cell GAGs, complete enzyme inhibition was observed with 25 nM febuxostat, whereas no more than 80% inhibition was seen with either allopurinol or oxypurinol, even at concentrations above those tolerated clinically. The superior potency for inhibition of endothelium-associated XO is predictive of a significant role for febuxostat in investigating pathological states in which XO-derived ROS are contributive and traditional XO inhibitors are only slightly effective.

The molybdoflavin enzyme xanthine oxidoreductase (XOR) catalyzes the terminal two steps of purine degradation (hypoxanthine → xanthine → uric acid) in humans. XOR is transcribed as a single gene product, xanthine dehydrogenase (XDH), in which substrate-derived electrons at the Mo cofactor of XDH are transferred via two Fe/S centers to a FAD cofactor by which NAD^+ is reduced to NADH. During inflammatory conditions, posttranslational modification by oxidation of critical cysteine residues or limited proteolysis converts XDH to xanthine oxidase (XO) [1,2]. The key difference distinguishing XO from XDH is the structural conformation and electrostatic microenvironment surrounding the FAD cofactor resulting in XO's lower affinity for NAD^+ and enhanced affinity for O_2[3]. Substrate-derived electrons at the Mo cofactor of XO reduce O_2 at the FAD cofactor both divalently, forming hydrogen peroxide (H_2O_2) and univalently, generating superoxide ($O_2^{\bullet-}$). However, conversion to XO is not requisite for ROS production, as XDH displays partial oxidase activity under conditions under which NAD^+ levels are diminished, such as the ischemic/hypoxic microenvironment encountered in vascular inflammation [4]. This same inflammatory milieu leads to enhanced XO levels and thus increased XO-derived ROS formation resulting in activation of redox-dependent cell signaling reactions and alterations in vascular function. Evidence of this role for XO is exemplified by numerous studies in which XO inhibition attenuates vascular dysfunction, including congestive heart failure, sickle cell anemia, and diabetes [5–8].

The splanchnic system, the site of highest XDH-specific activity, readily releases XDH into the circulation in response to ischemic/hypoxic or inflammatory insults [9,10]. Once released, XDH is rapidly converted to XO by plasma proteases. Pockets of cationic amino acid motifs present on XO confer a high affinity ($K_d = 6$ nM) for negatively charged glycosaminoglycans (GAGs) on the luminal face of endothelial cells [11]. This XO–GAG association induces substantial

Abbreviations: GAG, glycosaminoglycans; ROS, reactive oxygen species; XDH, xanthine dehydrogenase; XO, xanthine oxidase; XOR, xanthine oxidoreductase.

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sequestration and thus amplification of local endothelial XO concentration, producing a microenvironment primed for enhanced ROS production. Of crucial importance, GAG association also results in resistance to XOR inhibition by oxypurinol, the active metabolite of allopurinol, increasing the K_i from 230 nM for soluble XO to 405 nM for GAG-bound XOR [12,13]. Combined, amplification of endothelial XO-derived ROS formation and GAG immobilization-induced resistance to inhibition results in a setting that exacerbates inflammation with inextinguishable vascular ROS formation.

Whereas inhibition of XO-derived uric acid formation and resultant symptoms of gout has been accomplished successfully for over 50 years by clinical administration of allopurinol, only partial reduction of vascular inflammatory-related symptoms and restoration of function have been observed by allopurinol-based inhibition approaches. This phenomenon may be explained, in part, by examination of the 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 oxypurinol (the active inhibitory metabolite). Reduction of the Mo cofactor by allopurinol ultimately leads to electron transfer to the FAD, resulting in reduction of O_2[14]. It is equally important to note that oxypurinol binding and resultant inhibition require the Mo cofactor to be reduced [15]. This is accomplished by initial reaction of allopurinol or, in the case of treatment with pure oxypurinol, XO substrates, such as xanthine must provide the electrons. In either case, both allopurinol and 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 may lead to significant misinterpretation of ROS-driven vascular pathology to which XO is contributory. These limitations emphasize the need for alternative inhibitors with mechanisms independent of enzyme redox status and unaffected by GAG immobilization.

Febuxostat, an XO-specific inhibitor recently approved by the FDA for clinical use, is reported to be significantly more potent than allopurinol [16]. Kinetic analysis conducted at pH 8.5, near the pH optimum for xanthine/Mo-cofactor reaction, produced a K_i for febuxostat nearly 6000 times lower than that of allopurinol, 0.12 nM vs 700 nM, respectively [16]. Detailed crystallography studies revealed that febuxostat reaction with XO is confined to critical amino acid residues in the tunnel leading to the Mo cofactor, where it effectively blocks substrate access to the active site [17]. Thus, febuxostat should not be affected by enzyme redox state and interaction with XO should not induce ROS formation. These characteristics of enhanced potency and independence from enzyme turnover suggest that febuxostat may provide new insights into XO-dependent contributions to inflammatory disease. Therefore, we examined the inhibition properties of febuxostat for soluble XO, GAG-bound XO, and endothelial cell-bound XO at physiological pH.

Materials and methods

Materials

Heparin–Sepharose CL-6B and PD10 G25 Sephadex columns were from GE Healthcare (USA). Xanthine, allopurinol, catalase, and native cytochrome *c* were from Sigma (St. Louis, MO, USA), and febuxostat was purchased from Axon Medchem BV (The Netherlands). Xanthine oxidase was purchased from Calbiochem (USA). Medium 199 (M199) and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA, USA). Superoxide dismutase (CuZnSOD) was from Oxis International (Portland, OR, USA). The EPR spin probe 1-hydroxy-4-phosphonooxy-2,2,6,6-tetramethylpiperidine (PPH) was from Enzo Life Sciences (Plymouth Meeting, PA, USA).

Enzyme analysis

Enzymatic activity was determined either spectrophotometrically, by the rate of uric acid formation monitored at 292 nm in 50 mM potassium phosphate (KP_i), pH 7.4 ($\varepsilon = 11\ mM^{-1}\ cm^{-1}$), or electrochemically via reverse-phase HPLC analysis of uric acid production (ESA CoulArray System; Chelmsford, MA, USA) as previously described [12]. Activity is expressed as 1 U = 1 μmol urate/min. Formation of $O_2^{\bullet-}$ was assessed by the SOD-inhibitable reduction of cytochrome *c* (550 nm) [18] and EPR (see below).

Xanthine oxidase binding to heparin–Sepharose 6B

Xanthine oxidase was bound to heparin–Sepharose-6B (HS6B) as we previously reported [12]. Briefly, HS6B was washed with 5 mM KP_i, incubated with XO for 45 min at 25 °C, and washed free of nonassociated enzyme. Activity of the suspension was determined by following the formation of uric acid (λ 292). Reactions were initiated by addition of xanthine (100 μM) and maintained under continuously stirred conditions at 25 °C. Final activity of HS6B–XO used for inhibition studies was identical to that used in studies with XO free in solution.

Cell culture and cell-bound XO inhibition studies

Bovine aortic endothelial cells (BAECs) were isolated as we previously described [19]. Primary cell culture, routine passage, and experimental manipulations were all conducted in the absence of proteases. Cells were propagated by subculturing in a 1:4 ratio in M199 containing 10% FBS and 10 μM thymidine. For inhibition studies, when cells reached confluence, the medium was replaced with fresh M199(−) (formulated without xanthine and hypoxanthine) containing XO (5 mU/ml) for 20 min at 25 °C. We have reported that this process minimizes enzyme internalization [20]. The XO-containing medium was then removed, cells were washed, fresh M199(−) containing designated concentrations of inhibitor was added, and reactions were initiated by the addition of 100 μM xanthine. After 1 h, aliquots of the medium were removed and tested for uric acid formation by reverse-phase HPLC electrochemical analysis. Uricase activity of the medium was undetectable as determined by the addition of known concentrations of uric acid with or without cells (37 °C) and then monitoring the loss of uric acid over time.

EPR studies

Confluent BAECs were exposed to XO (5 mU/ml) for 20 min at 25 °C, harvested by mechanical dissociation, washed thoroughly (three times with ice-cold PBS, pH 7.4), resuspended as a single-cell suspension (1×10^6 cells/ml), and placed on ice (for less than 30 min) until warmed to 37 °C immediately before evaluation by EPR. Aliquots (50 μl) of the cell suspension were exposed to indicated concentrations of inhibitor and 50 μM membrane-impermeative EPR spin probe PPH [21] followed by the addition of xanthine (100 μM). The samples were immediately transferred to a 50-μl glass pipette and placed into a temperature- and gas-controlled Bruker eScan tabletop EPR spectrometer cavity and analyzed for 10 min at 37 °C and 21% O_2. Spectra represent five signal-averaged scans from $t = 9$ to $t = 10$ min. The EPR instrument settings were as follows: field sweep 50 G, microwave frequency 9.78 GHz, microwave power 20 mW, modulation amplitude 2 G, conversion time 327 ms, time constant 655 ms, and receiver gain 1×10^5. To minimize the effects of adventitious metals, all buffers were treated with Chelex resin and contained 25 μM deferoxamine.