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Regulation of the nitric oxide oxidase activity of myeloperoxidase by pharmacological agents

Sophie L. Maiocchi [a,b], Jonathan C. Morris [b], Martin D. Rees [a,*], Shane R. Thomas [a,*]

[a] Mechanisms of Disease & Translational Research, Department of Pathology, School of Medical Sciences, Faculty of Medicine, University of New South Wales, Sydney, NSW 2052, Australia
[b] School of Chemistry, University of New South Wales, Sydney, NSW 2052, Australia

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ABSTRACT

The leukocyte-derived heme enzyme myeloperoxidase (MPO) is released extracellularly during inflammation and impairs nitric oxide (NO) bioavailability by directly oxidizing NO or producing NO-consuming substrate radicals. Here, structurally diverse pharmacological agents with activities as MPO substrates/inhibitors or antioxidants were screened for their effects on MPO NO oxidase activity in human plasma and physiological model systems containing endogenous MPO substrates/antioxidants (tyrosine, urate, ascorbate). Hydrazide-based irreversible/reversible MPO inhibitors (4-ABAH, isoniazid) or the sickle cell anaemia drug, hydroxyurea, all promoted MPO NO oxidase activity. This involved the capacity of NO to antagonize MPO inhibition by hydrazide-derived radicals and/or the ability of drug-derived radicals to stimulate MPO turnover thereby increasing NO consumption by MPO redox intermediates or NO-consuming radicals. In contrast, the mechanism-based irreversible MPO inhibitor 2-thioxanthine, potently inhibited MPO turnover and NO consumption. Although the phenolics acetaminophen and resveratrol initially increased MPO turnover and NO consumption, they limited the overall extent of NO loss by rapidly depleting H_2O_2 and promoting the formation of ascorbyl radicals, which inefficiently consume NO. The vitamin E analogue trolox inhibited MPO NO oxidase activity in ascorbate-depleted fluids by scavenging NO-consuming tyrosyl and urate radicals. Tempol and related nitroxides decreased NO consumption in ascorbate-replete fluids by scavenging MPO-derived ascorbyl radicals. Indoles or apocynin yielded marginal effects. Kinetic analyses rationalized differences in drug activities and identified criteria for the improved inhibition of MPO NO oxidase activity. This study reveals that widely used agents have important implications for MPO NO oxidase activity under physiological conditions, highlighting new pharmacological strategies for preserving NO bioavailability during inflammation.

1. Introduction

Nitric oxide (NO) plays a key functional role in maintaining cardiovascular homeostasis by regulating vascular tone and arterial pressure, as well as inhibiting platelet aggregation, vascular smooth muscle cell proliferation and leukocyte–endothelial interactions [1]. NO is also important for pulmonary function where it regulates airway smooth muscle cell relaxation and participates in the innate immune host-defence against microbes [2]. Decreased NO bioavailability is implicated in the pathophysiology of cardiovascular disease and a range of other inflammatory conditions [1], with the extent of impairment of vascular NO bioavailability (indexed as impaired endothelium-dependent vasodilatation) predictive of the risk of clinical cardiovascular events occurring in patients with coronary artery disease [3–7]. Impaired pulmonary NO bioavailability has also been shown to correlate with the presence and severity of cystic fibrosis [8]. There is consequently considerable interest in understanding the processes that impair vascular and pulmonary NO bioavailability during inflammation and how these processes may be regulated by pharmacological agents.

* Corresponding authors at: School of Medical Sciences, Wallace Wurth Building, University of New South Wales, Sydney, NSW 2052, Australia (M.D. Rees). Mechanisms of Disease & Translational Research, Department of Pathology, School of Medical Sciences, Faculty of Medicine, Wallace Wurth Building, University of New South Wales, Sydney, NSW 2052, Australia (S.R. Thomas).

E-mail addresses: martdrees@gmail.com (M.D. Rees), shane.thomas@unsw.edu.au (S.R. Thomas).

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The heme enzyme myeloperoxidase (MPO) is released extracellularly by activated leukocytes and is implicated in playing an important role in impairing vascular and pulmonary NO bioavailability during inflammation [9–13] by acting as an NO oxidase [9,10]. Recent studies in humans and animals highlight that MPO potently impairs endothelium-derived NO bioavailability, indexed as endothelium-dependent vasodilatation [9,14]. Ex vivo studies in isolated rat aortae and in human plasma further implicate MPO NO oxidase activity in these in vivo effects [9,15]. Additionally, intravascular administration of MPO decreases myocardial blood flow and increases pulmonary vascular resistance in pigs involving the enzyme's NO oxidase activity [14]. The clinical importance of intravascular MPO in impairing endothelial NO bioavailability is highlighted by studies showing that levels of circulating and vessel-bound MPO correlate inversely with endothelium-dependent vasodilatation in coronary artery disease patients [15–18]. Also, blood pressure in humans is independently and positively associated with elevated circulating MPO levels [19] and following myocardial infarction, plasma levels of MPO are increased and support enhanced NO oxidase-dependent consumption of NO ex vivo [15]. With regard to pulmonary NO bioavailability, increased MPO activity in sputum from cystic fibrosis patients has been shown to associate negatively with lung expired NO levels and also support enhanced peroxidase-dependent NO consumption ex vivo [20].

The preceding findings implicate MPO NO oxidase activity as an important process that impairs vascular and pulmonary NO bioavailability during inflammation, however the processes by which MPO consumes NO in complex physiological fluids have only recently been detailed [21]. Initial studies with purified enzyme showed that MPO can utilize hydrogen peroxide (H_2O_2) as a co-substrate to consume NO as a direct peroxidase substrate involving the reaction of NO with MPO compounds I and II (Fig. 1, Reactions 1 and 4) [9,10]. Subsequent studies reported that MPO could consume NO by converting certain endogenous peroxidase substrates (e.g. tyrosine, urate) to efficient NO-consuming substrate radicals [9,22] (Fig. 1, Reactions 2, 3 and 5). Recent studies reveal that both of these processes contribute to MPO NO oxidase activity in complex physiological fluids (e.g. human plasma) and that the mechanism and efficiency of NO consumption is critically dependent on the availability of endogenous radical scavengers and MPO substrates [21]. Thus, physiological concentrations of ascorbate were overall protective by scavenging NO-consuming substrate radicals (e.g. urate radicals; Fig. 1, Reaction 6 *vs.* 5) to form ascorbyl radicals, which consume NO less efficiently [21,22]. Thiocyanate (SCN^-), but not chloride (Cl^-), was also protective by acting as a competitive substrate for MPO Compound I, thereby inhibiting the direct oxidation of NO by this highly reactive redox intermediate (Fig. 1, Reaction 9 *vs.* 1) [21]. As well as identifying novel protective functions for physiological MPO substrates and radical scavengers, these data indicate that exogenous drugs with similar activities as MPO substrates and/or radical scavengers also have the potential to influence NO bioavailability during inflammatory conditions by modulating the NO oxidase activity of MPO. However, the effects of pharmacological agents on MPO NO oxidase activity under physiological conditions are currently unknown.

In this study, candidate pharmacological agents with diverse chemical structures (hydrazides, 2-thioxanthine, hydroxyurea, indoles, phenolics and nitroxides) were selected based on their established redox activities as MPO substrates/inhibitors and/or free radical scavengers (Figs. 1 and 2, Table 1). Their effects on MPO-catalyzed NO consumption were screened in dilute human plasma and in protein-free model systems containing physiological concentrations of endogenous MPO substrates and radical scavengers (i.e., tyrosine, urate and ascorbate). Theoretical kinetic analyses were employed to provide further insights into the mechanistic basis of the observed drug activities in order to identify criteria for developing novel pharmacological agents with improved capacity to suppress NO-consuming reactions catalyzed by MPO.

2. Materials and methods

2.1. Materials

Purified human neutrophil myeloperoxidase (MPO) and H_2O_2 (30% solution) were obtained from Merck (Kenilworth, New Jersey, USA). The NO donor NOC-9 was purchased from Santa Cruz Biotechnology Inc. (Dallas, Texas, USA). 2-Thioxanthine (AZD5904, TX4) was kindly provided by AstraZeneca R&D (Mölndal, Sweden). Unless otherwise indicated all other materials were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), were of the highest purity available and used without further purification. The concentration of stock solutions of H_2O_2 were routinely determined by spectrophotometry (H_2O_2 $\varepsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$). All solutions were prepared using water filtered through a four-stage MilliQ system. Phosphate buffer (0.1 M, pH 7.4) was chelex-treated and supplemented with 100 μM DETAPAC to prevent spurious reactions involving transition metals. Working solutions of urate were prepared by first dissolving urate (0.01 M) in aqueous sodium hydroxide solution (0.04 M), then immediately diluting the urate stock into phosphate buffer. Working solutions of resveratrol, trolox and melatonin were prepared from 0.01 M stock solutions in ethanol and then diluted into phosphate buffer. Working solutions of apocynin, 4-ABAH, 2-thioxanthine and isoniazid were prepared from 0.1 M stock solutions in DMSO and then diluted into phosphate buffer. Other reagents used were prepared as 0.001 or 0.01 M stock solutions in water and then diluted into phosphate buffer. Stock solutions of NOC-9 were prepared in ice-cold 0.01 M sodium hydroxide and then diluted directly into buffered reaction mixtures at 22 °C to initiate NO production.

2.2. Preparation of human plasma

Plasma was obtained after centrifugation (5000 rpm, 10 min, at 4 °C) of freshly isolated heparinised blood donated by healthy consenting adult volunteers as per a protocol approved by the UNSW Human Ethics Review Committee. Aliquots of the isolated plasma were immediately frozen and stored at −80 °C. Aliquots of plasma stocks were thawed immediately before experiments and used within 1 h of thawing.

2.3. Electrochemical measurement of NO and H_2O_2

For NO delivery, NOC9 was employed as it: (i) is a member of the well-characterised NONOate class of NO donors that non-enzymatically break-down via a simple mechanism to accurately produce NO in biological systems at consistent rates and amounts [73–75], (ii) exhibits a short half-life ($t_{1/2}$ 2.7 min at 22 °C) and (iii) has been successfully employed in our previous work studying MPO NO oxidase activity [21,22]. For experiments, NOC-9 was added to 0.1 M phosphate buffer (pH 7.4, ~22 °C, air-saturated) supplemented with 100 μM DETAPAC in the presence and absence of other reaction components (i.e., 1–500 μM pharmacological agents, 50 μM tyrosine, 200 μM urate and/or 50 μM ascorbate), with rapid stirring. The amount of NOC-9 added had been optimised such that a steady-state concentration of ~500 nM NO was achieved at the time point at which MPO-catalyzed reactions were initiated (final NOC-9 concentration ~2 μM). In protein-free systems, H_2O_2 (10 μM) and the relevant heme peroxidase (i.e.,

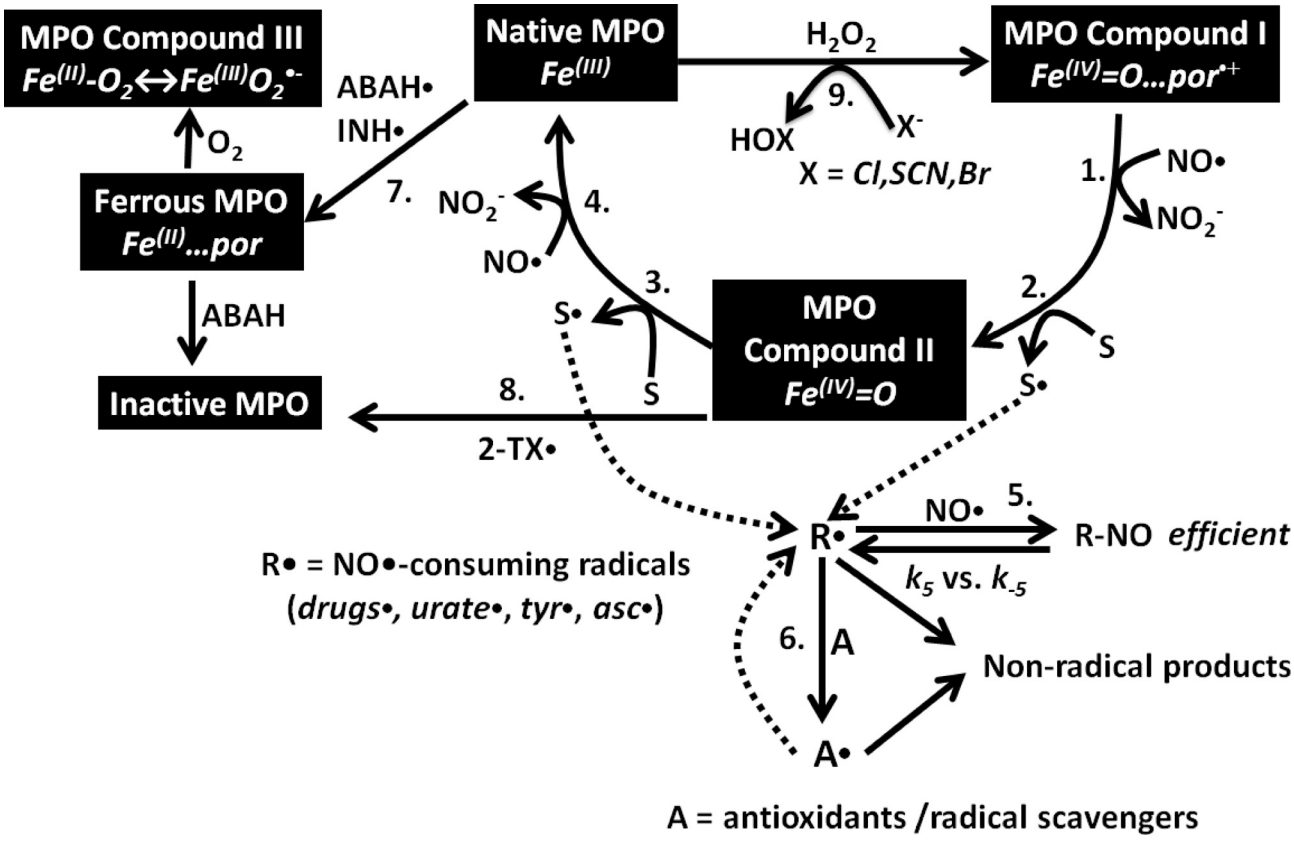

Fig. 1. Regulation of MPO NO oxidase activity by pharmacological agents. H_2O_2 initiates MPO-catalyzed NO consumption by converting native, ferric MPO ($Fe^{(III)}$ heme) to the highly reactive redox intermediate MPO compound I ($Fe^{(IV)}$ = O. . .por^{+}). NO can be directly oxidized by MPO compound I or MPO compound II ($Fe^{(IV)}$=O) to nitrite (NO_2^-) via the enzyme's peroxidase cycle (Reactions 1 and 4), regenerating native ferric MPO. In complex biological fluids, endogenous MPO substrates (S; e.g., urate, tyrosine) are also oxidized via MPO compounds I and II (Reactions 2 and 3), resulting in the production of diffusible radicals (S·). Peroxidase substrates may stimulate MPO-catalyzed NO consumption by (i) stimulating peroxidase turnover via Compound II reduction (Reaction 3, the rate-limiting step in peroxidase turnover) and (ii) by acting as a source of diffusible NO consuming radicals (R·) (Reaction 5), which efficiently react with NO (fast k_5). Scavenging of NO-consuming radicals (R·) by endogenous antioxidants (A; e.g., Ascorbate, Reaction 6) may limit NO consumption. Ascorbate confers incomplete protection through radical scavenging, and the reaction between resultant ascorbyl radicals ($Asc^{\cdot-}$) and NO (Reaction 5) is thermodynamically unfavourable and the reverse reaction spontaneous (reverse of Reaction 5, fast k_{-5}). Whilst oxidation of NO by MPO is preferred in the presence of physiological levels of Cl^-, SCN^- protects by effectively competing with NO for oxidation by MPO compound I (Reaction 9 *vs.* Reaction 1). Hydrazide derivatives ABAH and Isoniazid (INH) may limit NO consumption through enzyme-modifying and -inactivating reactions mediated by their respective hydrazyl radicals (ABAH·, INH·) (Reaction 7). 2-Thioxanthines may limit NO consumption in a similar manner through their enzyme-inactivating reactions with MPO compound II mediated by the 2-thioxanthine radical (2-TX·) (Reaction 8). Pharmacological agents that are peroxidase substrates may limit efficacy of NO consumption through effective competition with NO for the reaction with MPO compound I (i.e., Reaction 2 *vs.* Reaction 1), where the resultant diffusible drug-based radicals (i) have limited reactivity with NO (Reaction 5, where $k_{-5} > k_5$) and/or (ii) are efficiently scavenged by physiological antioxidants leading to a cooperative or co-antioxidant effect (Reaction 2; Reaction 6). Pharmacological agents may also act as efficient scavengers of diffusible NO-consuming substrate radicals (Reaction 6).

15 nM MPO or 200 nM HRP), were added sequentially (2.5 and 3 min after addition of NOC-9).

In some experiments NOC9 was omitted from the reaction mixtures, in which case H_2O_2 (10 μM) and the relevant heme peroxidase (i.e., 15 nM MPO or 200 nM HRP), were added sequentially at timepoints of 3 and 3.5 min. In reactions containing diluted human plasma (20% v/v in 0.1 M phosphate buffer, pH 7.4, 100 μM DETAPAC), H_2O_2 (10 μM) and MPO (15 nM) were added sequentially at 3.5 and 4 min after addition of NOC-9 (~2 μM).

The changes in NO or H_2O_2 concentration during the reactions were measured continuously with an NO-specific electrode (ISO-NOP) or H_2O_2-specific electrode (ISO-HPO-2), respectively interfaced to a one-channel free radical analyzer (TBR1025, World Precision Instruments) and LabScribe software (World Precision Instruments). The initial linear rate of NO consumption was measured by performing a linear regression of the slope over a 5 s time-period beginning at 5 s following the addition of MPO, or from 15 s where explicitly stated in the Figure Legend.

2.4. Measurement of MPO chlorination activity

HOCl production by the MPO-H_2O_2-Cl^- system was determined spectrophotometrically by measuring the conversion of taurine to taurine mono-chloramine using the 2-nitro-5-thiobenzoic acid (TNB) assay [76]. TNB reagent was prepared by dissolving DTNB in aqueous 0.05 M sodium hydroxide solution to make a final concentration of 1 mM DTNB and then diluting 1:40 into 0.1 M phosphate buffer (pH 7.4). Reactions were carried out in phosphate buffer containing MPO (100 nM), taurine (20 mM) and Cl^- (100 mM) in the absence or presence of resveratrol (1–100 μM), and were initiated by the addition of H_2O_2 (50 μM). The final volume of the reaction mixture was 60 μl, and the reaction was allowed to proceed for 5 min, and then stopped by the addition of catalase (50 μg ml^{-1}; 15 μl of 250 μg ml^{-1}). Following this, reaction mixture (60 μl) was added to TNB reagent (250 μl). The extent of TNB oxidation to DTNB arising from HOCl-oxidation of taurine was quantified after 5 min of incubation using a FLUOstar Omega

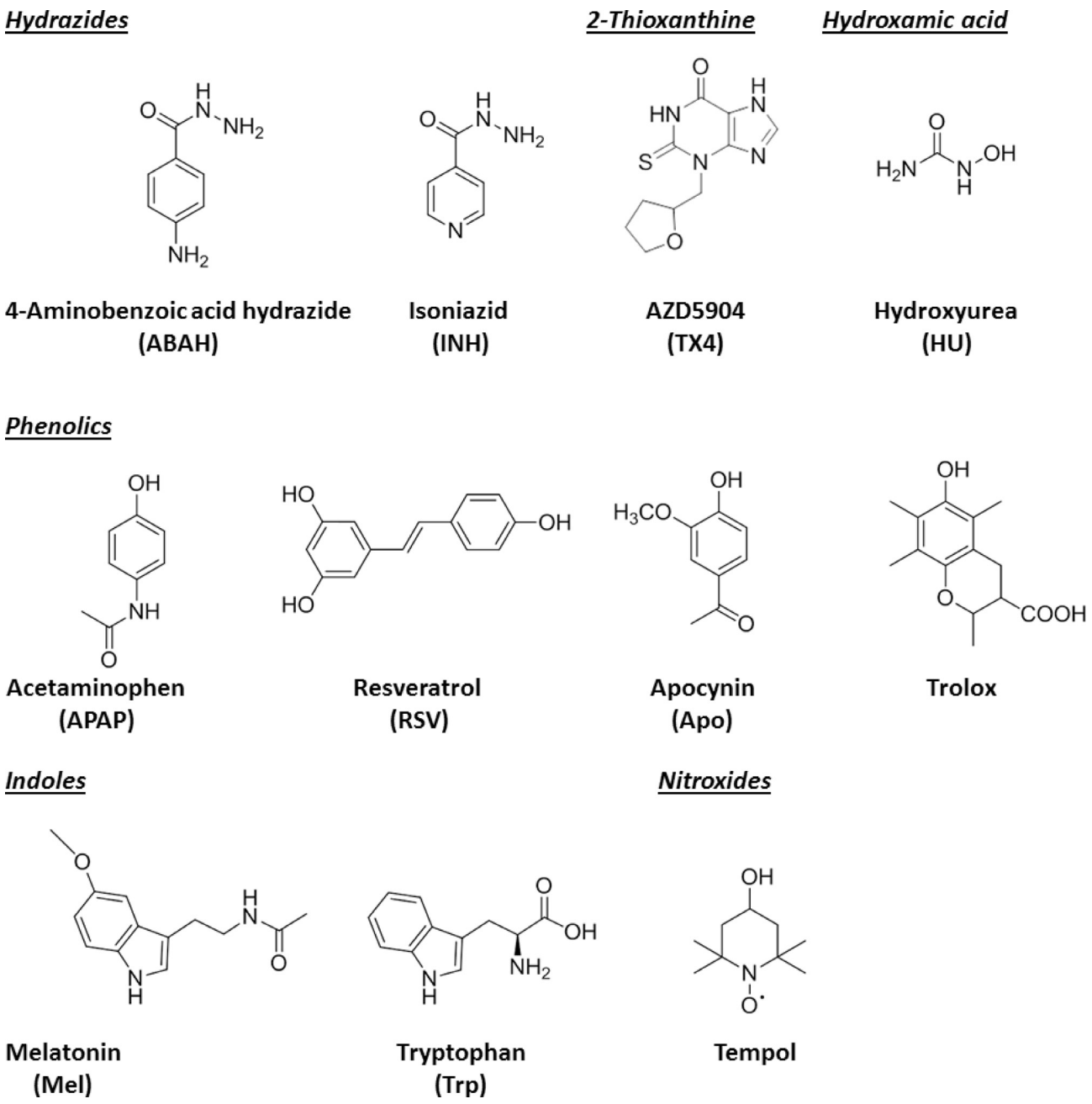

Fig. 2. Pharmacological agents studied in this work.

microplate reader (BMG Labtech) and measuring absorbance at λ = 412 nm.

2.5. Kinetic modeling

Computational kinetic models of MPO-catalyzed NO consumption were constructed using Excel software (Microsoft) using the reactions shown in Fig. 1 and the rate constants compiled in Table 2. Kinetic simulations were performed to predict the initial distribution of oxidized products produced by MPO redox intermediates (i.e., MPO Compounds I and II) during the initial phase of steady state catalysis (when the rates of NO consumption were measured experimentally), essentially as recently described for equivalent experimental systems lacking exogenous pharmacological agents [21]. The contribution of NO consumption by diffusible substrate radicals (Reaction 5, Fig. 1), as well as the effects of reactions between substrate radicals and enzyme-inactivating reactions (Reactions 6–8, Fig. 1; for which many rate constants are also unavailable) were omitted from the kinetic analysis. Calculations for the proportion of substrates oxidized by MPO compound I and compound II as per Reactions 1–4 (Fig. 1) were calculated employing the kinetic analyses as recently described [21]. The rate of NO consumption by peroxidase redox intermediates alone ('$-d[NO_{(per)}]/dt$') can be expressed by the following relationship:

$$-\mathrm{d}[\mathrm{NO}_{(per)}]/\mathrm{dt} = -\mathrm{d}[\text{Compound } II]/\mathrm{dt} \times f\mathrm{NO}_{(per)} \quad (1)$$

where, '$-d[Compound\ II]/dt$' is the rate of Compound II reduction to native MPO and '$fNO_{(per)}$' is the fraction of NO consumed by MPO redox intermediates Compounds I and II in one catalytic cycle. Substituting values for each term in this equation (see above), this becomes:

Table 1
Pharmacological agents studied in this work.

Compound	Pharmacological applications/activities	Relevant redox activities
ABAH (hydrazide)	MPO inhibition demonstrated in vivo in pre-clinical models and ex vivo in activated leukocytes [23–25]	Excellent[1] MPO peroxidase substrate; substrate radicals irreversibly inhibit MPO [26–28]
Isoniazid (hydrazide)	Frontline treatment for tuberculosis [29]	Poor[2] MPO peroxidase substrate; substrate radicals reversibly inhibit MPO [30]
2-Thioxanthine (AZD5904, TX4)	MPO inhibition demonstrated in vivo in pre-clinical models and ex vivo in activated leukocytes [31,32]	Excellent[1] MPO peroxidase substrate; substrate radicals irreversibly inhibit MPO catalytic activity [32–34]
Hydroxyurea (hydroxamic acid)	Treatment of sickle cell anaemia and myelo-proliferative cancers [35,36]; Increases NO bioavailability in vivo [35]	Excellent[1] peroxidase substrate for a related heme peroxidase (HRP[3]); peroxidase-mediated oxidation reported to generate NO [37]
Melatonin (indole)	Treatment of jet lag and sleep disorders [38]; anti-hypertensive and antioxidant actions in humans and animals [39–42]	Poor[2] MPO peroxidase substrate; reversibly inhibits MPO when Compound II substrate availability is limited [43,44]
Acetamino-phen, paracetamol (phenolic)	Analgesic, antipyretic, acutely promotes vasodilation in humans [45]	Excellent[1] MPO peroxidase substrate; competitively inhibits MPO chlorination activity [46,47]
Resveratrol (phenolic)	Preserves vascular NO bioavailability in humans [48–50]	Excellent[1] MPO peroxidase substrate, competitively inhibits MPO chlorination activity (this work) [51–53]; radical scavenger [54,55]
Apocynin (phenolic)	Inhibits NADPH oxidase activity [56], improves NO bioavailability in vivo, anti-hypertensive in pre-clinical models [57]	Excellent[1] MPO peroxidase substrate, competitively inhibits MPO chlorination activity [56,58,59]
Trolox (phenolic, water-soluble Vitamin E analogue)	Protective effects in pre-clinical models of ischemia-reperfusion [60–62]	Peroxidase substrate for related peroxidases (HRP[3] and LPO[4]) [63–65]; inhibits MPO NO oxidase activity in the absence of physiological substrates, and in diluted CF sputum [9,20]; radical scavenger [66,67]
Tempol (nitroxide)	Improves NO bioavailability in vivo, anti-hypertensive in pre-clinical models [68,69]	Poor[2] MPO peroxidase substrate, reversibly inhibits MPO when Compound II substrate availability is limited [70,71]; radical scavenger [68,72]

[1] Excellent peroxidase substrate: Reactive substrate for Compound I and Compound II (cf. rate constant data in Table 2).
[2] Poor peroxidase substrate: Reactive Compound I substrate and poor Compound II substrate (cf. rate constant data in Table 2).
[3] HRP, horseradish peroxidase.
[4] LPO, lactoperoxidase.

$$-\mathrm{d}[NO_{(per)}]/\mathrm{dt} = (k_4[NO] + k_3[S]) \times \{[((k_1[NO])/(k_1[NO] + k_2[S])) + ((k_4[NO])/(k_4[NO] + k_3[S]))]/2\} \quad (2)$$

where:

$k_1 = k_{(NO+\text{Compound I})}[\text{Compound } I] - cf.\ Reaction\ 1,\ Fig.\ 1$

$k_2 = k_{(S+\text{Compound I})}[\text{Compound } I] - cf.\ Reaction\ 2,\ Fig.\ 1$

$k_3 = k_{(S+\text{Compound II})}[\text{Compound } II] - cf.\ Reaction\ 3,\ Fig.\ 1$

$k_4 = k_{(NO+\text{Compound II})}[\text{Compound}II] - cf.\ Reaction\ 4,\ Fig.\ 1$

2.6. Statistical analysis

Statistical analyses were performed with Prism 5 software using one-way ANOVA with the Newman-Keuls post hoc testing or two-way ANOVA with Bonferroni's post hoc testing. IC_{50} values were determined by fitting a rectangular hyperbola to dose-response curves using non-linear regression. All data are means ± SEM of a minimum of three independent determinations. Statistical significance was accepted for P values of <0.05.

3. Results

In the following studies, structurally diverse compounds (Table 1) were screened for their effects on MPO-catalyzed NO consumption in (i) diluted human plasma (20% v/v in 0.1 M phosphate buffer, pH 7.4) or (ii) protein-free model systems in 0.1 M phosphate buffer (pH 7.4) containing various combinations of tyrosine, urate and/or ascorbate at their physiologically-relevant concentrations in human plasma (i.e., 50 μM tyrosine, 200 μM urate, 50 μM ascorbate). Importantly, to model in vivo conditions of inflammation and oxidative stress where ascorbate is preferentially depleted from extracellular fluids and accumulates within activated leukocytes [86,87]; compounds were also screened in the presence of tyrosine and urate alone. NO was employed at the concentration of ~500 nM to model patho-physiological NO levels [88]. All compounds were screened at pharmacologically-relevant concentrations (see relevant subsections below) and at a common drug concentration of 50 μM. Parallel measurements of H_2O_2 consumption were also carried out to allow the effects of test compounds on MPO-catalyzed NO consumption to be correlated with their effects on the rate of MPO peroxidase turnover. Theoretical kinetic analyses were performed to provide further insights into the mechanistic basis of the observed effects of the drugs on MPO peroxidase turnover and NO consumption using available rate constants (Table 2).

3.1. Hydrazides

Hydrazide derivatives ($RCONHNH_2$) typically inhibit MPO by acting as peroxidase substrates and forming reactive substrate radicals (hydrazyl radicals) that reversibly promote the formation of catalytically-inactive MPO redox intermediates (e.g. MPO Compound III) and/or that irreversibly damage the active site heme prosthetic group [26,27,30] (Reaction 7, Fig. 1). Here, we examined the prototypical MPO suicide inhibitor 4-aminobenzoic acid hydrazide (ABAH), which is widely used as a research tool to inhibit MPO's catalytic activity in vitro [26] and in vivo [24,25], and the tuberculosis drug isoniazid (isonicotinic acid hydrazide, INH), whose activity as an MPO substrate and reversible MPO inhibitor is proposed to contribute to its idiosyncratic effects and ability to impair innate immune defences against *Mycobacteria tuberculosis* [30,89]. Whilst pharmacokinetic studies have yet to be performed for ABAH, peak plasma concentrations in mice after a pharmacological dose of 40 mg/kg delivered intraperitoneally [23,24] are likely to be well in excess of IC_{50} values determined for the inhibition of MPO catalytic activities by ABAH in vitro (i.e. ≤2.2 μM). Peak plasma concentrations of isoniazid can reach up to ~50 μM in tuberculosis patients [90].

Table 2
Reaction rates of MPO redox-active heme species.

Key reactions of MPO redox-active heme species		Rate constant with MPO ($M^{-1}\,s^{-1}$)	References
Ferric-MPO + H_2O_2 → Compound (Cmp)-I		2.6×10^7	[77]
MPO Cmp-I + NO → MPO Cmp-II + NO^+ ($NO^+ \rightarrow NO_2^-$)		7.0×10^6 *(estimate)*[a]	[11,78]
MPO Cmp-II + NO → Ferric-MPO + NO_2^-	NO + Fe^{IV} = O → Fe^{III}-ONO Fe^{III}-ONO → Fe^{III}-heme + NO_2^-	8.0×10^3	[11]
MPO Cmp-I + Cl^- → Ferric-MPO + HOCl		2.5×10^4	[77]
MPO Cmp-I + S → MPO-Cmp-II + S·	S = Tyrosine	7.7×10^5	[79]
	S = Urate	4.6×10^5	[22]
	S = Ascorbate	1.1×10^6	[80]
	S = NO_2^-	2.2×10^6	[81]
	S = ABAH	3.34×10^6	[82]
	S = Isoniazid	1.22×10^6	[30]
	S = 2-Thioxanthine	6.8×10^5[f]	[32]
	S = Hydroxyurea	*Peroxidase substrate*[b]	[37]
	S = Melatonin	6.1×10^6, 7.3×10^5 *(estimate)*[a]	[43,83]
	S = L-Tryptophan	4.5×10^5, 2.1×10^6	[84,85]
	S = Acetaminophen	*Very fast*[e]	[46]
	S = Resveratrol	*Occurs*[c]	[51]
	S = Apocynin	*Occurs*[c]	[58]
	S = Trolox	*Peroxidase substrate*[b]	[63,64]
	S = Tempol	3.5×10^5	[71]
MPO Cmp-II + S → Ferric-MPO + S·	S = Tyrosine	1.6×10^4	[79]
	S = Urate	1.7×10^4	[22]
	S = Ascorbate	1.1×10^4	[80]
	S = NO_2^-	5.5×10^2	[81]
	S = ABAH	6.5×10^5	[82]
	S = Isoniazid	9.8×10^2	[30]
	S = 2-Thioxanthine	*Occurs*[c]	[32]
	S = Hydroxyurea	*Occurs*[c]	[37]
	S = Melatonin	9.6×10^2, 1.9×10^2	[43,83]
	S = L-Tryptophan	6.9	[84]
	S = Acetaminophen	1.4×10^5 *(estimate)*[a]	[46]
	S = Resveratrol	*Occurs*[c]	[51]
	S = Apocynin	*Occurs*[c]	[58]
	S = Trolox	*Peroxidase substrate*[b]	[63,64]
	S = Tempol	*Apparent rate constant of* 1.0×10^2 *at low concentrations*[d]	[71]

All reported rate constants were measured at pH ~7.
[a] Estimate indicates that the second order rate constant *k* was calculated from a graph of k_{obs} (pseudo first order rate constant) *vs.* concentration in the cited reference.
[b] Peroxidase substrate' means it is oxidized by other heme peroxidase enzymes; e.g., horseradish peroxidase.
[c] Occurs' means it is a known MPO peroxidase substrate but the rate constant of reaction has not been measured.
[d] There is a binding interaction between tempol and MPO that forms an MPO compound II-tempol complex, which slowly decays to MPO compound II and tempol and the oxoammonium cation of tempol and native MPO.
[e] Very fast' indicates that the reaction occurred faster than stopped flow spectrometry could measure.
[f] The 2-thioxanthine rate constant is that of a related but different 2-thioxanthine derivative than the one used in this study.

3.1.1. ABAH

ABAH (2–50 μM) stimulated the initial rate of NO consumption by MPO in diluted human plasma in a dose-dependent manner with up to a 7-fold increase afforded with 50 μM ABAH (Fig. 3A, B). ABAH also significantly stimulated NO consumption in all protein-free model systems examined (Fig. 3C); i.e., in the presence of (i) no physiological peroxidase substrates (6-fold increase) (Fig. 3D), (ii) tyrosine (1.6-fold increase) (Fig. 3E), (ii) tyrosine and urate (3.5-fold increase) (Fig. 3F) and (iv) tyrosine, urate and ascorbate (7.6-fold increase) (Fig. 3G). In all of these systems the ABAH-mediated increase in NO consumption was sustained over the time-course of the experiment (~9 min after the addition of MPO).

Measurements of H_2O_2 consumption in these protein-free model systems identified that ABAH increased initial MPO peroxidase turnover (0–0.5 min) under all conditions examined when NO was present (Fig. 3H–K, see trace '3' in each panel). However, after 0.5 min, H_2O_2 consumption slowed despite the availability of unconsumed peroxidase substrates, indicating eventual inhibition of MPO activity by ABAH. Importantly, when NO was omitted from the reactions, ABAH immediately inhibited MPO activity under all conditions examined (Fig. 3H–K; compare trace '3' in the presence of NO *vs.* trace '3,-NO' in the absence of NO in each panel). These data show that the inhibition of MPO by ABAH radicals (Fig. 1, Reaction 7) is antagonized by NO.

To further understand the stimulatory effect of ABAH on MPO turnover and NO consumption, kinetic analyses were performed using available rate constants for the reactions of ABAH, tyrosine, urate, ascorbate and NO with MPO Compound I and MPO compound II (Table 2). The reactions of ABAH radicals (Fig. 1, Reaction 7), which are suppressed by NO (see above), were omitted from the simulations. These analyses predict that ABAH is the dominant substrate for both MPO Compound I and Compound II in the presence of physiological levels of tyrosine, urate, ascorbate and NO (accounting for 67% of total substrates oxidized by MPO, Fig. 4).

These kinetic analyses also predict the effect of ABAH on MPO turnover and in turn its effect on the overall rates of MPO-catalyzed NO consumption by MPO redox intermediates (Fig. 1, Reactions 1 & 4). Thus, the analyses predict that at a concentration of 50 μM ABAH stimulates MPO turnover in the presence of urate, tyrosine, ascorbate and NO by 7.8-fold (by increasing the rate of MPO Compound II reduction; Fig. 1, Reaction 3). This theoretical prediction was in accordance with the experimental data, which showed that ABAH initially increases MPO turnover and hence

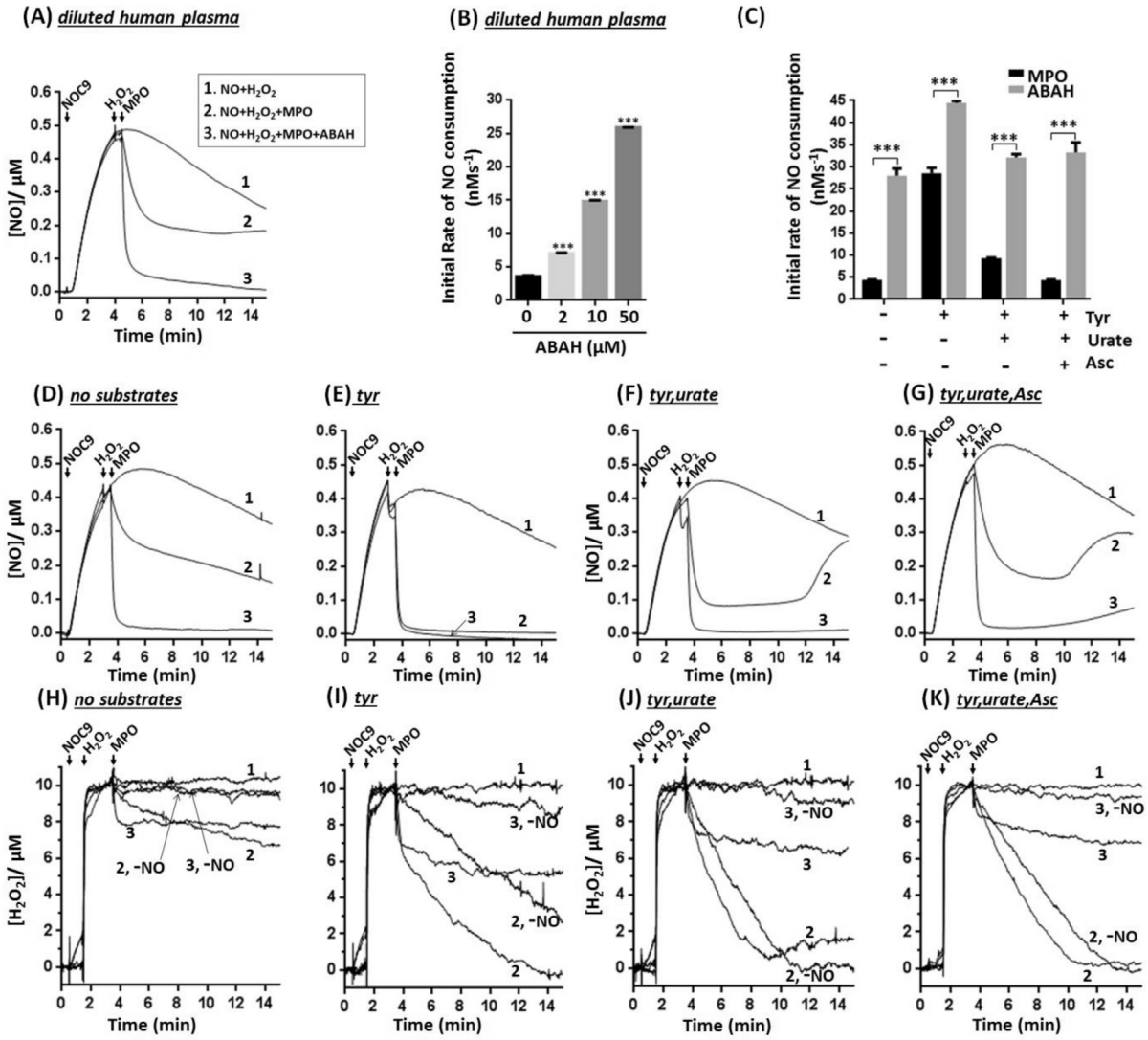

Fig. 3. Effect of ABAH on MPO-catalyzed NO and H_2O_2 consumption. (A, B) For experiments with human plasma, the NO donor NOC-9 was added to air-saturated plasma (diluted 1:5 in 0.1 M phosphate buffer, pH 7.4, 100 µM DETAPAC) followed by the addition of H_2O_2 (10 µM) and MPO (15 nM) at 3.5 min and 4 min after the addition of NOC-9, respectively. (C–K) For experiments in protein-free model systems, NOC-9 was added to air-saturated 0.1 M phosphate buffer (pH 7.4, 100 µM DETAPAC) containing physiological peroxidase substrates and antioxidants; i.e., 50 µM tyrosine, 200 µM urate and/or 50 µM ascorbate. H_2O_2 (10 µM) and MPO (15 nM) were then added at 2.5 min and 3 min, respectively after the addition of NOC-9. Both plasma and protein-free systems' samples were incubated at 22 °C with rapid mixing to achieve a maximal steady-state NO concentration of ~500 nM and NO was quantified continuously using an NO-specific electrode (ISO-NOP, WPI). Data for the initial rates of MPO-catalyzed NO consumption represent the mean ± SEM of a minimum of n = 3 independent experiments for all experimental conditions. (A) Representative trace of the effect of ABAH (50 µM) on NO consumption in diluted human plasma (see panel B for initial rate data). (B) Effect of ABAH (2–50 µM) on the initial rates of NO consumption in diluted human plasma; $^{***}P < 0.001$ relative to 0 µM ABAH (see panel A for representative trace for 50 µM ABAH). (C) Effect of ABAH (50 µM) on the initial rates of NO consumption in protein-free model systems in the absence (no substrates) or presence of 50 µM tyrosine (Tyr), 200 µM urate and/or 50 µM ascorbate (Asc); $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ and ns $P > 0.05$ (n = 3; see panels D–G for representative traces). (D–G) Representative traces of the effect of ABAH (50 µM) on NO consumption in protein-free buffer systems in the absence or presence of 50 µM Tyr, 200 µM urate and/or 50 µM Asc (see Panel C for initial rate data). 'No substrates' refers to the reaction in the absence of Tyr, urate and Asc. (H–K) Representative traces of the effect of ABAH (50 µM) on H_2O_2 consumption in protein-free model systems in the absence or presence of Tyr, urate and/or Asc and in the absence (−NO) or presence of ~500 nM NO (from NOC-9; i.e. under identical conditions as Panels D–G). The H_2O_2 measurements are representative of 3 independent determinations.

H_2O_2 consumption under these conditions (Fig. 3H–K). Furthermore, kinetic analyses predicted that the increase in MPO turnover afforded by ABAH in the presence of tyrosine, urate and ascorbate will translate into a 4-fold increase in the rate of NO consumption by MPO redox intermediates alone. As the experimentally determined increases in NO consumption under these conditions (7-fold increase; Fig. 3C) exceeded this prediction, this indicates that ABAH not only stimulates NO consumption by MPO redox intermediates, but also stimulates NO consumption by promoting the generation of NO-consuming substrate radicals, potentially including ABAH radicals.

Together, these data indicate that NO effectively antagonizes MPO inhibition by ABAH radicals. As a result, where NO is present, ABAH fails to immediately inhibit MPO and instead stimulates MPO turnover through its action as a MPO Compound II substrate. These increases in MPO turnover in turn drive increased NO consumption by MPO redox intermediates and NO-consuming radicals, including ABAH radicals.

3.1.2. Isoniazid

Isoniazid (50 µM) stimulated the initial rate of NO consumption by MPO in diluted human plasma by 1.9-fold (Fig. 5A, B). It also

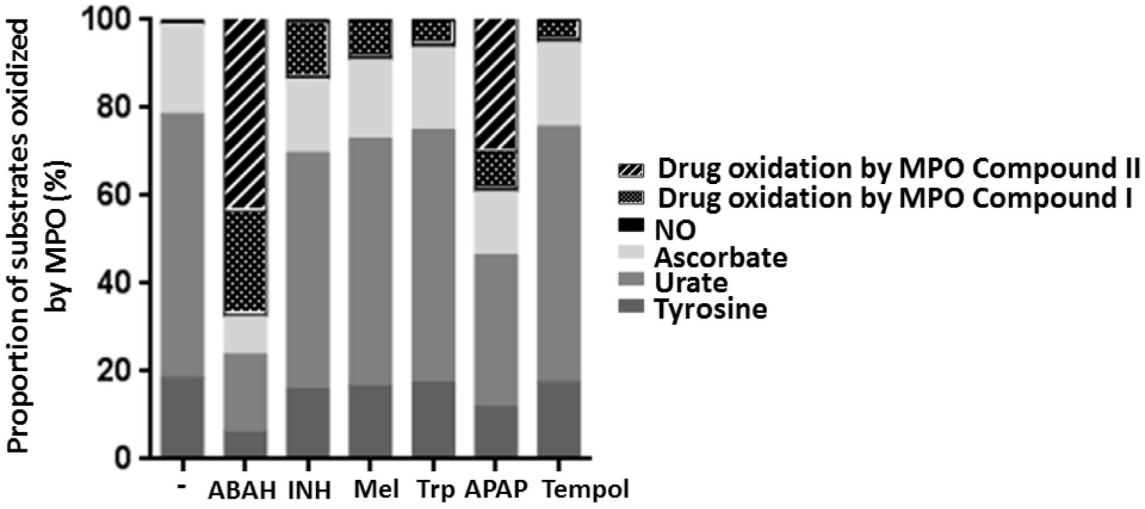

Fig. 4. Kinetic modeling of the distribution of substrates oxidized by the peroxidase cycle of MPO. The predicted individual effect of ABAH, Isoniazid (INH), melatonin (Mel), L-tryptophan (Trp), acetaminophen (APAP) and tempol at a concentration of 50 μM on the distribution of substrates consumed by MPO compound I and MPO compound II in the presence of NO (500 nM), urate (200 μM), tyrosine (50 μM) and ascorbate (50 μM) during the initial phase of steady-state catalysis, when the rates of NO consumption were measured experimentally. The yields of reactive species consumed during steady-state catalysis were predicted by kinetic simulations (using Excel software and employing available rate constants of the reactions with MPO compound I and MPO compound II, Table 2) and were expressed as a percentage of the total reaction yield. The rate constant of reaction for APAP with MPO Compound I has not been determined previously and kinetic analyses were performed with this value set to be equivalent to that for tyrosine (Table 2); however it is expected that it greatly exceeds this value and thus a greater proportion of APAP is predicted to be oxidized.

stimulated the initial rates of NO consumption in most protein-free model systems (Fig. 5C); i.e., (i) in the absence of peroxidase substrates (by 1.3-fold) (Fig. 5D), (ii) the presence of urate and tyrosine (by 1.7-fold) (Fig. 5F) and (iii) the presence of tyrosine, urate, and ascorbate (by 2.8-fold) (Fig. 5G). In contrast, isoniazid did not alter NO consumption in the presence of tyrosine alone (Fig. 5C, E). In all of the systems examined, NO loss promoted by isoniazid was sustained over the time-course of the experiment.

Measurements of H_2O_2 consumption confirmed that in the absence of NO, isoniazid inhibited MPO turnover in all protein-free model systems examined (Fig. 5H–K; see trace '3,-NO' *vs.* trace '2, -NO' in each panel), consistent with its reported action as an efficient reversible MPO inhibitor [30]. In contrast, the presence of NO suppressed the capacity of isoniazid to inhibit MPO turnover in all systems examined (Fig. 5H–K), with the exception of tyrosine alone where inhibition of MPO turnover was apparent after depletion of NO (Fig. 5I). These data reveal that similar to ABAH radicals, MPO inhibition by isoniazid radicals (i.e. Fig. 1, Reaction 7) is antagonized by NO, potentially involving a direct reaction between these two radical species (Fig. 1, Forward Reaction 5).

Notably, whilst a role for ascorbate in antagonizing MPO inhibition by isoniazid has been proposed [30], our current findings do not support this. Thus, our data show that in the absence of NO, the addition of physiological levels of ascorbate (50 μM) did not appreciably alter isoniazid-dependent MPO inhibition (Fig. 5J *vs.* K; Trace 3, -NO). Our data do, however, indicate that NO is a physiological antagonist of isoniazid-mediated MPO inhibition.

Kinetic analyses (performed as described for ABAH, i.e. omitting enzyme-inactivating reactions of hydrazide radicals) predicted that isoniazid (50 μM) should not appreciably increase the rate of MPO enzyme turnover in the presence of tyrosine, urate, ascorbate and NO (Fig. 4), as it is a poor MPO Compound II substrate (Table 2). Additionally, kinetic analyses predicted that at a concentration of 50 μM isoniazid will attenuate the rate of NO consumption by MPO redox intermediates by 20% in favour of the generation of isoniazid radicals. As isoniazid itself does not increase MPO turnover and in fact is predicted to suppress the direct consumption of NO, isoniazid radicals are likely to be responsible for driving the experimentally observed increases in NO consumption (Fig. 5C) and initial MPO turnover (Fig. 5J, K). We therefore propose that isoniazid radicals stimulate MPO peroxidase turnover by reducing MPO Compound II, with this in turn driving an increase in the production of diffusible NO-consuming radicals. The reduction of MPO Compound II by isoniazid radicals is thermodynamically plausible as isoniazid-derived radicals readily reduce native (ferric) MPO [30], which has a significantly lower oxidizing potential than MPO Compound II (reduction potential E^o(Ferric MPO/MPO Cmp I) = 1160 mV; E^o(MPO Cmp II/Cmp I) = 1350 mV [91,92]).

In summary, the experimentally observed increases in NO consumption promoted by isoniazid are likely to relate to the capacity of isoniazid radicals to drive MPO peroxidase turnover resulting in increased NO consumption by MPO redox intermediates and MPO-derived substrate radicals, including isoniazid radicals.

3.2. 2-Thioxanthines

2-Thioxanthines are a recently discovered novel class of mechanism-based, irreversible MPO inhibitors [32–34]. 2-Thioxanthines efficiently react with MPO compound I to form reactive substrate radicals that rapidly react with the active site heme of MPO compound II to form an irreversible covalent thioether bond, resulting in potent MPO inhibition [32–34] (Fig. 1, Reaction 8). Here, we examined the 2-thioxanthine, AZD5904 (TX4), which has been shown to efficiently inhibit HOCl production by MPO with an IC_{50} of 0.2 ± 0.02 μM [32]. 2-thioxanthines have also been employed in vivo with peak plasma concentrations of 12–178 μM reported, which inhibited MPO-mediated inflammation and protein chlorination in a murine model of zymosan-induced peritonitis [32] and halted the progression of chronic obstructive pulmonary disease in guinea pigs exposed to cigarette smoke [31].

3.2.1. 2-Thioxanthine (AZD5904, TX4)

2-Thioxanthine (50 μM) attenuated the initial rate of MPO-catalyzed NO consumption in diluted human plasma by ≈70% (Fig. 6A, B). It continued to be effective at lower concentrations (e.g. 10 μM attenuated by ≈30%; Fig. 6B). 2-Thioxanthine also provided virtually complete protection against MPO-catalyzed NO consumption in the presence of urate, tyrosine and ascorbate

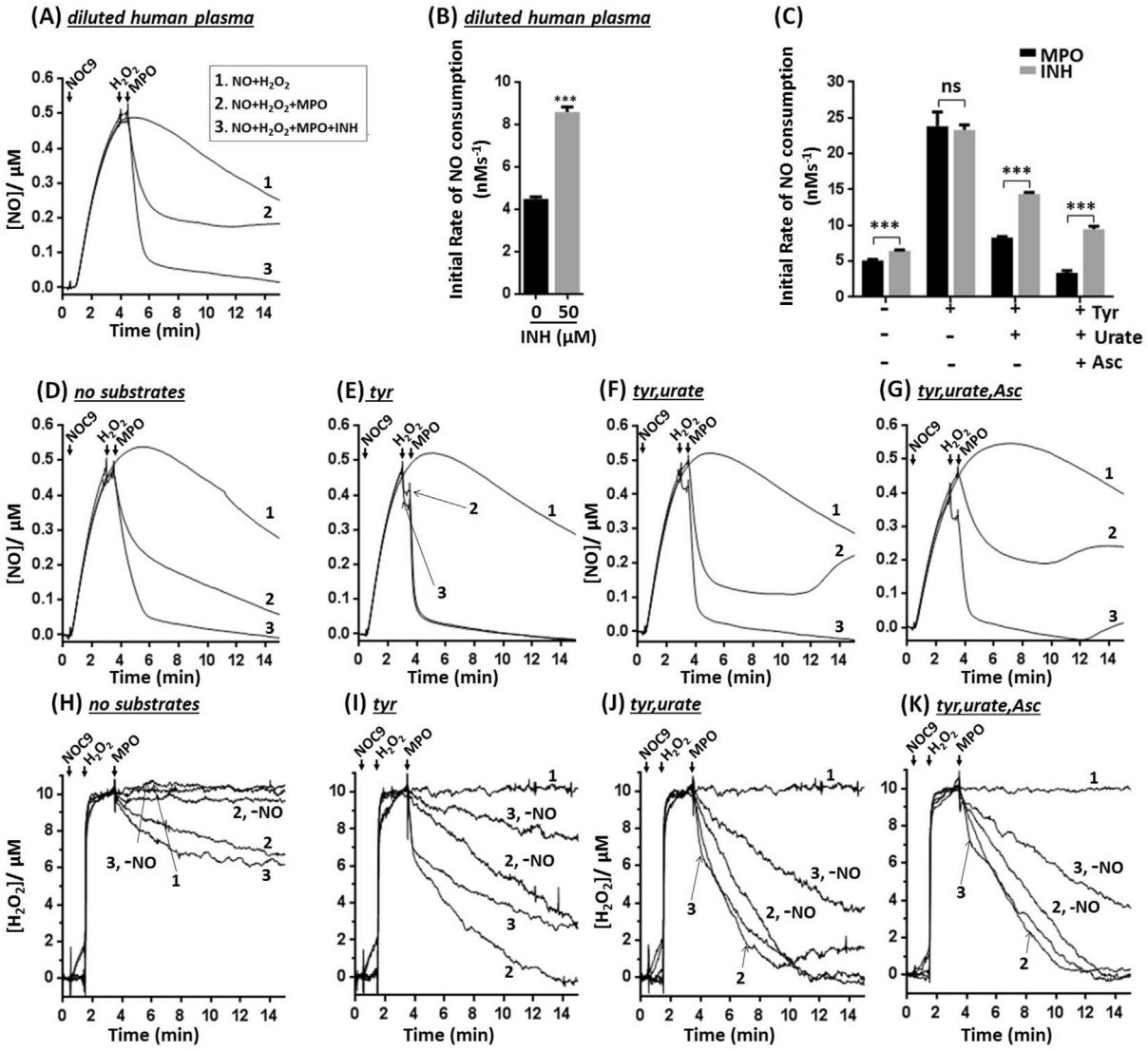

Fig. 5. Effect of Isoniazid (INH) on MPO-catalyzed NO and H_2O_2 consumption. (A) Representative trace of the effect of INH (50 µM) on NO consumption in diluted human plasma (see panel B for initial rate data). (B) Effect of INH (50 µM) on the initial rates of NO consumption in diluted human plasma; $^{***}P < 0.001$ (Data represent the mean ± SEM, n = 3; see panel A for a representative trace). (C) Effect of INH (50 µM) on the initial rates of NO consumption in protein-free model systems in the absence (no substrates) or presence of 50 µM tyrosine (Tyr), 200 µM urate and/or 50 µM ascorbate (Asc); $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ and ns $P > 0.05$ (Data represent the mean ± SEM, n = 3; see panels D–G for representative traces). (D–G) Representative traces of the effect of INH (50 µM) on NO consumption in protein-free model systems in the absence or presence of 50 µM Tyr, 200 µM urate and/or 50 µM Asc (see Panel C for initial rate data). (H–K) Representative traces of the effect of INH (50 µM) on H_2O_2 consumption in protein-free model systems in the absence or presence of Tyr, urate and/or Asc in the absence (−NO) and presence of ~500 nM NO (from NOC-9; i.e. under identical conditions as Panels D–G). The H_2O_2 measurements are representative of 3 independent determinations.

(Fig. 6C, G) and attenuated NO consumption in the presence of (i) urate and tyrosine (by >75%), (ii) tyrosine (by ≈90%) and (iii) in the absence of peroxidase substrates (by >75%) (Fig. 6C–F). In all of the systems examined, inhibition of NO consumption was preserved throughout the time-course of the experiment.

Measurements of H_2O_2 consumption confirmed that 2-thioxanthine effectively inhibited MPO turnover in all protein-free model systems examined (Fig. 6H–K), consistent with its reported action as a potent mechanism-based MPO inhibitor [32]. Notably, in contrast to ABAH and isoniazid, which similarly inhibit MPO via substrate radical-mediated reactions (Fig. 1, Reaction 7), NO did not markedly antagonize the inhibitory capacity of 2-thioxanthine towards MPO catalytic activity (Fig. 6H–K), with only minor differences in MPO-catalyzed H_2O_2 consumption observed for 2-thioxanthine in the absence and presence of NO.

Kinetic analyses provide further support that 2-thioxanthines are efficient mechanism-based inhibitors of MPO NO oxidase activity. Thus, based on the observed rate constant of reaction of 2-thioxanthine with MPO compound I ($6.8 \times 10^5\ M^{-1}\ s^{-1}$, Table 2), a "simple" competition with NO for binding to MPO compound I (Fig. 1, Reaction 1 vs. 2) does not account for 2-thioxanthine's inhibition of MPO NO oxidase activity (Table 2); i.e., kinetic analyses where competitive inhibition is used as the model, predict that 2-thioxanthine is unable to inhibit MPO-mediated NO consumption, which is clearly inconsistent with our experimental observations.

In summary, 2-thioxanthine potently inhibited MPO NO oxidase activity in physiologically relevant model systems in a manner consistent with its reported mechanism-based inhibition of MPO catalytic activity [32].

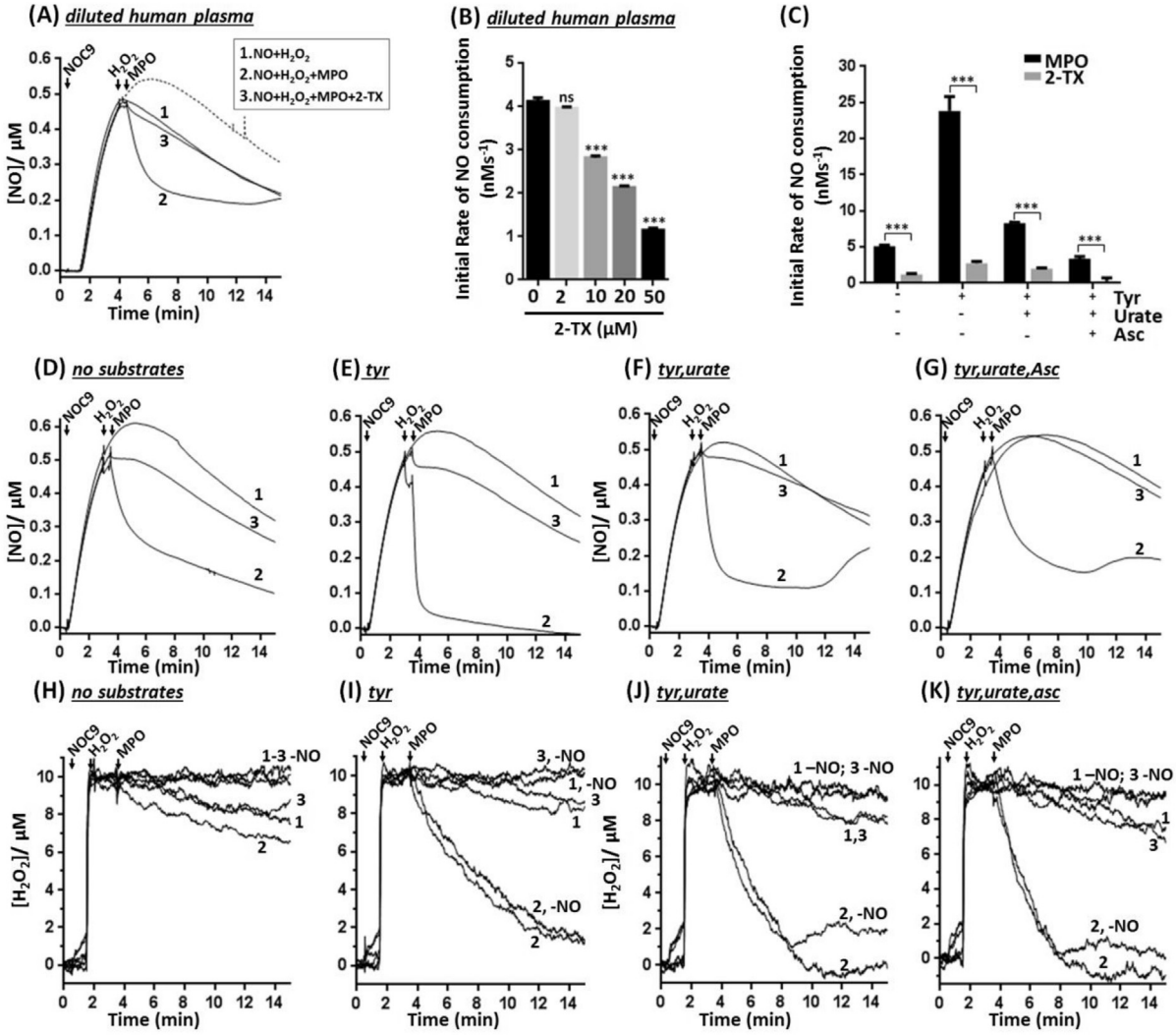

Fig. 6. Effect of 2-Thioxanthine on MPO-catalyzed NO and H_2O_2 consumption. (A) Representative trace of the effect of 2-thioxanthine (2-TX; 50 µM) on NO consumption in diluted human plasma (see panel B for initial rate data; dotted line is NO-released by NOC-9 without H_2O_2). (B) Effect of 2-TX (2–50 µM) on the initial rates of NO consumption in diluted human plasma, $^{***}P < 0.001$ (Data represent the mean ± SEM, n = 3; see panel A for a representative trace). (C) Effect of 2-TX (50 µM) on the initial rates of NO consumption in protein-free model systems in the absence (no substrates) or presence of 50 µM tyrosine (Tyr), 200 µM urate and/or 50 µM ascorbate (Asc); $^{***}P < 0.001$ (Data represent the mean ± SEM, n = 3; see panels D–G for representative traces). (D–G) Representative traces of the effect of 2-TX (50 µM) on NO consumption in protein-free model systems containing 50 µM Tyr, 200 µM urate and/or 50 µM Asc (see Panel C for initial rate data). (H–K) Representative traces of the effect of 2-TX (50 µM) on H_2O_2 consumption in protein-free model systems in the absence or presence of Tyr, urate and/or Asc in the absence (−NO) and presence of ~500 nM NO (from NOC-9; i.e. under identical conditions as Panels D–G). The H_2O_2 measurements are representative of 3 independent determinations.

3.3. Hydroxamic acid

Peroxidase-catalyzed catabolism of hydroxyurea to NO, involving a complex 3-electron oxidation process, has been reported for horseradish peroxidase (HRP) in simple model systems [37]. On the basis of these observations it has been proposed that peroxidases, such as MPO, could contribute to the hydroxyurea-dependent NO generation in vivo and beneficial pharmacological actions of hydroxyurea in sickle cell disease [35]. Peak plasma concentrations of hydroxyurea can reach up to 350 µM [93]. The effects of hydroxyurea on MPO turnover and MPO-dependent NO consumption under physiological conditions have yet to be examined.

3.3.1. Hydroxyurea

Hydroxyurea (50 µM) markedly stimulated the rates of MPO-catalyzed NO consumption in diluted human plasma (6-fold) and in all protein-free model systems examined (Fig. 7A–G). These increases in the rate of NO consumption (Fig. 7D–G) correlated with marked increases in the rate of MPO turnover (Fig. 7H–K). After the cessation of MPO turnover due to the depletion of H_2O_2, a recovery in NO levels was apparent that is consistent with the continual release of NO from residual NOC-9.

Whilst a previous study reports that HRP-mediated oxidation of hydroxyurea can yield NO (using chemiluminescent detection of NO, a sensitive method that can detect NO concentrations <1 ppb) [37], other studies report that HRP is an NO oxidase [21]. Employing in-solution electrochemical detection (limit of detection, 1 nM NO) we found that oxidation of hydroxyurea (50 or 500 µM) by MPO or HRP did not generate detectable levels of NO (Fig. 8B). Indeed, similar to the situation with MPO, in the presence of NO (500 nM), hydroxyurea (50 µM) stimulated HRP-mediated NO consumption (Fig. 8A, B).

We next addressed the potential mechanistic basis for hydroxyurea-stimulated NO consumption. Although rate constants for the reactions of hydroxyurea with MPO Compound I and MPO Compound II have yet to be determined, both reactions are

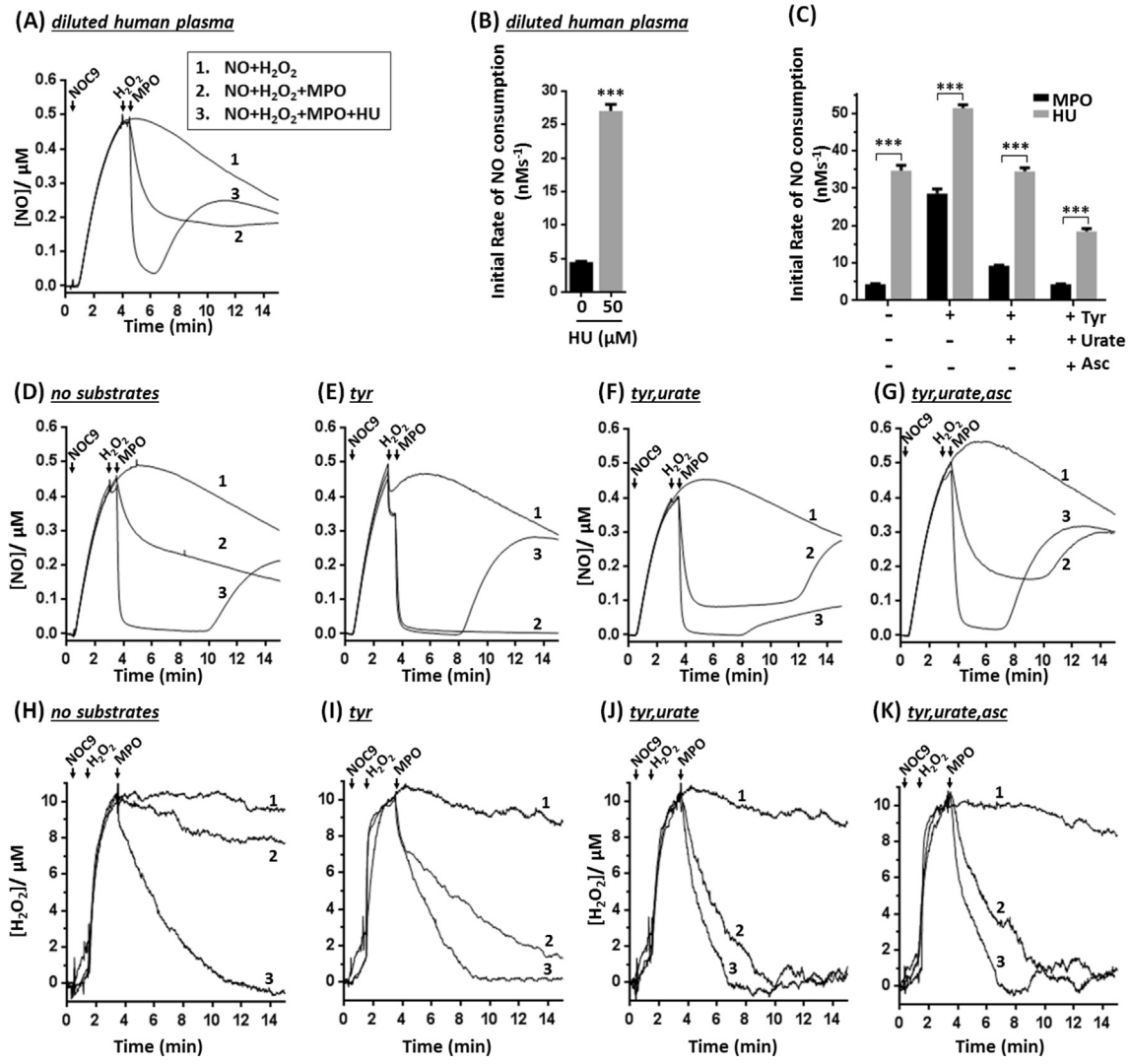

Fig. 7. Effect of Hydroxyurea (HU) on MPO-catalyzed NO consumption and H_2O_2 consumption. (A) Representative trace of the effect of HU (50 µM) on NO consumption in diluted human plasma (see panel B for initial rate data). (B) Effect of HU (50 µM) on the initial rates of NO consumption in diluted human plasma, $^{***}P < 0.001$ (Data represent the mean ± SEM, n = 3; see panel A for a representative trace). (C) Effect of HU (50 µM) on the initial rates of NO consumption in protein-free model systems in the absence (no substrates) or presence of 50 µM tyrosine (Tyr), 200 µM urate and/or 50 µM ascorbate (Asc), $^{***}P < 0.001$ (Data represent the mean ± SEM, n = 3; see panels D–G for representative traces). (D–G) Representative traces of the effect of HU (50 µM) on NO consumption in protein-free model systems in the absence (no substrates) or presence of 50 µM Tyr, 200 µM urate and/or 50 µM Asc (see Panel C for initial rate data). (H–K) Representative traces of the effect of HU (50 µM) on H_2O_2 consumption in protein-free model systems in the absence or presence of Tyr, urate and/or Asc in the presence of ~500 nM NO (from NOC-9; i.e. under identical conditions as Panels D–G). The H_2O_2 measurements are representative of 3 independent determinations.

thermodynamically favourable [94] and our data identify that hydroxyurea is an efficient MPO Compound II substrate as it stimulated enzyme turnover under all conditions examined (Fig. 7H–K). Increases in MPO turnover due to MPO Compound II reduction by hydroxyurea will drive increased NO consumption by MPO redox intermediates as well as MPO-derived radicals, possibly including hydroxyurea radicals or secondary radicals derived from these (scavenging of hydroxyurea radicals by urate and ascorbate is thermodynamically favourable; $E^o(HU^{\cdot}/HU) = 751$ mV [94]; E^o(urate-H$^{\cdot}$/urate-H) = 590 mV; $E^o(Asc^{\cdot}/Asc^{-}) = 282$ mV [95]).

Overall, the data obtained with MPO and HRP identify that rather than serving as a 'peroxidase-activated' NO donor hydroxyurea enhances peroxidase turnover and peroxidase-catalyzed NO consumption under biologically-relevant conditions by acting as an efficient substrate for Compound II.

3.4. Indoles

Indole derivatives are typically 'poor' peroxidase substrates for MPO and related peroxidases; i.e., whilst they are good substrates for MPO Compound I, the rate constants for their reaction with MPO Compound II are typically very slow [84,85,96]. These properties allow indoles to reversibly inhibit MPO by promoting the accumulation of MPO compound II when the availability of other substrates for this redox intermediate is limited. Here, we studied the indoles melatonin and L-tryptophan (L-Trp), which both inhibit the activity of purified MPO [43,97]. Melatonin has also been proposed to exert vascular protection by inhibiting MPO-catalyzed oxidative reactions [40,98]. Melatonin typically reaches peak plasma concentrations of up to 3 nM in humans [99], whilst L-Trp can reach 30–100 µM in humans [97,100].

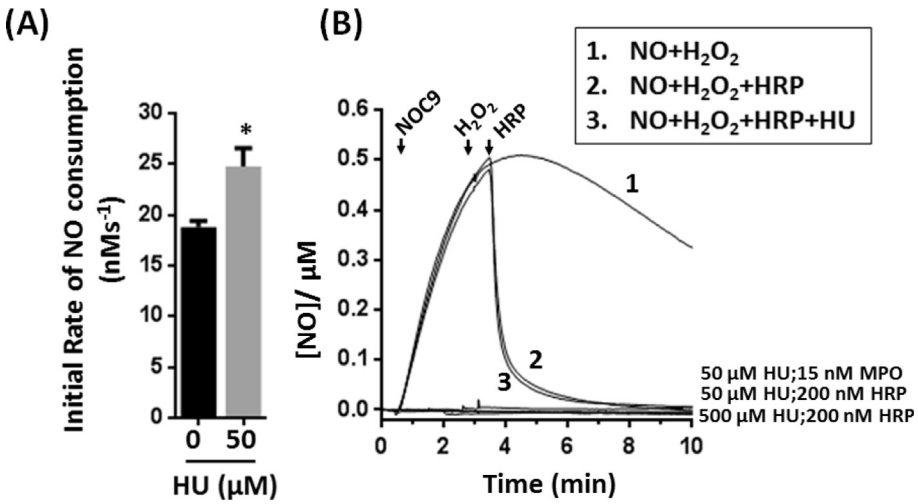

Fig. 8. HRP-catalyzed NO consumption in the presence of hydroxyurea (HU). HRP-catalyzed reactions were initiated 3 min after the addition of NOC-9 by adding HRP (200 nM) in the presence of H_2O_2 (10 μM) and the absence or presence of HU (50 μM). The effect of the addition of MPO (15 nM) or HRP (200 nM) to HU (50–500 μM) in phosphate buffer (containing no substrates) in the absence of NOC-9 and presence of H_2O_2 (10 μM) was also measured to test for a potential NO-producing action of HU. (A) Effect of HU (50 μM) on the initial rates of HRP-catalyzed NO consumption in protein-free model systems in the absence of substrates; $^*P < 0.05$ (Data represent the mean ± SEM, n = 3; see panel B for representative traces). (B) Representative traces of the effect of HU (50 μM) on NO consumption in phosphate buffer in the presence of HRP (200 nM). Additionally, representative traces of the effect of the addition of MPO (15 nM) or HRP (200 nM) to HU (50 or 500 μM) in phosphate buffer in the presence of H_2O_2 (10 μM) (and absence of NOC-9).

3.4.1. Melatonin

At supra-pharmacological concentrations, melatonin (50 μM) marginally stimulated the initial rate of MPO-catalyzed NO consumption in diluted human plasma and in all of the protein-free model systems examined (Fig. 9A–G). Measurements of H_2O_2 consumption revealed that melatonin did not detectably alter MPO turnover under any conditions examined (Fig. 9H–K). At lower, more pharmacologically-relevant concentrations, melatonin (1 μM) did not significantly alter MPO NO oxidase activity in diluted human plasma (data not shown).

Kinetic analyses predict that at a supra-pharmacological concentration of 50 μM in the presence of urate, tyrosine and ascorbate, melatonin does not alter the rates of MPO turnover as it is a very minor substrate for MPO Compound II (contributing ~0.1% to all substrates oxidized; Fig. 4). Additionally, melatonin is predicted to suppress the rate of NO consumption solely by MPO redox intermediates by 10%. This marginal attenuation of NO consumption by MPO redox intermediates may be counteracted by other NO-consuming reactions (i.e. Reaction 5, Fig. 1). At pharmacologically-relevant concentrations (<1 μM), melatonin is predicted to be an extremely minor substrate for MPO (<0.3% of total substrates oxidized by MPO in each catalytic cycle in the presence of physiological levels of tyrosine, urate and ascorbate; result not shown). The poor reactivity of melatonin with MPO compound II (Table 2) accounts for its failure to effectively alter MPO turnover (Fig. 9H–K).

3.4.2. L-Trp

At physiological levels L-Trp did not affect MPO NO oxidase activity or MPO turnover in diluted human plasma or in protein-free model systems containing physiological MPO substrates and radical scavengers (Fig. 10A). L-Trp did, however, significantly inhibit MPO NO oxidase activity in the absence of other physiological MPO substrates (Fig. 10A). The inability of L-Trp to alter MPO peroxidase turnover and hence H_2O_2 consumption (Fig. 10B–E) is consistent with the amino acids' activity as a poor MPO Compound II substrate [85,97] (Table 2).

Kinetic analyses predict that in the presence of tyrosine, urate and ascorbate, L-Trp (50 μM) will attenuate the rate of NO consumption by MPO redox intermediates by 11% (primarily due to its ability to competitively inhibit NO consumption by MPO compound I). As L-Trp failed to inhibit NO consumption in the presence of other endogenous MPO substrates and radical scavengers, the amino acids' ability to decrease NO consumption by MPO Compound I is likely to be countered by NO consumption by tryptophanyl radicals or secondary radicals generated from these: i.e. tryptophanyl radicals react with NO at a near diffusion limited rate [101] and also react rapidly with tyrosine, urate and ascorbate to generate their respective radicals [102,103].

3.5. Phenolic compounds

Phenolic compounds are typically excellent MPO peroxidase substrates and radical scavengers, with both of these properties potentially affecting MPO NO oxidase activity (see Table 1, Fig. 1). The effect of phenolic pharmacological agents on MPO NO oxidase activity under physiological conditions (i.e., in the presence of physiological levels of endogenous peroxidase substrates and radical scavengers) is currently unknown. Here we examined the effects of the phenols acetaminophen, resveratrol, apocynin and trolox. In humans, peak plasma concentrations of acetaminophen can reach up to 200 μM, with trough plasma concentrations of ~13 μM achieved during regular dosing throughout a 12-h period [104]. Peak plasma concentrations of resveratrol can reach up to 2.4 μM [105,106]. In pre-clinical models, peak plasma concentrations of apocynin reach up to 8 μM [107] whilst millimolar levels of trolox can be achieved in vivo [62].

3.5.1. Acetaminophen

Acetaminophen, at concentrations relevant to normal therapeutic dosing (10–200 μM), increased the initial rates of NO consumption in diluted human plasma (Fig. 11A–C). Acetaminophen also stimulated initial rates of MPO-dependent NO consumption in protein-free phosphate buffer systems (Fig. 11D) containing: (i) no physiological peroxidase substrates (Fig. 11E), (ii) tyrosine and urate (Fig. 11G) or (iii) tyrosine, urate and ascorbate (Fig. 11H). In the presence of tyrosine alone, acetaminophen attenuated the initial rate of NO consumption (Fig. 11D, F). Although initially

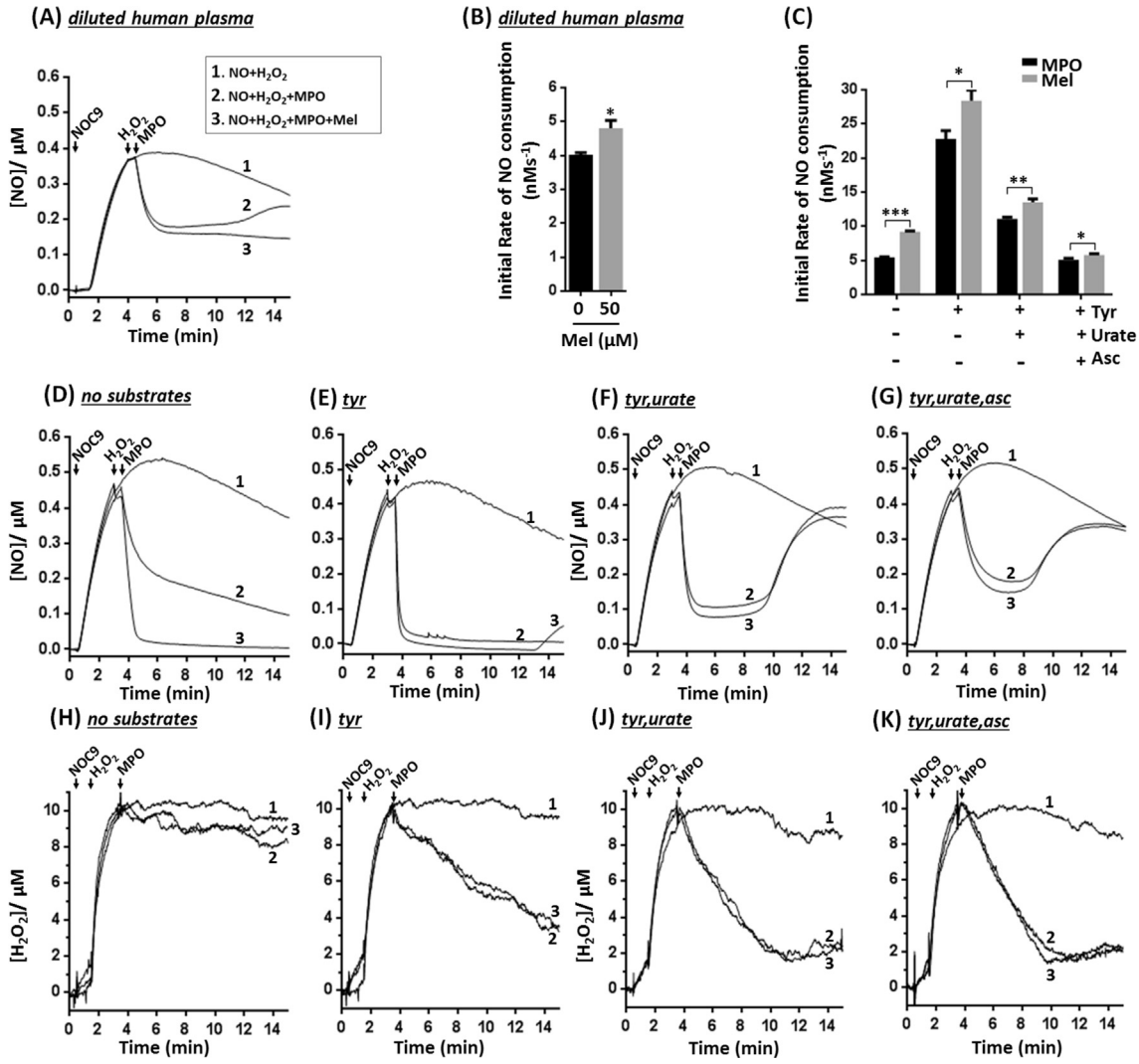

Fig. 9. Effect of Melatonin (Mel) on MPO-catalyzed NO and H_2O_2 consumption. (A) Representative trace of the effect of Mel (50 µM) on NO consumption in diluted human plasma (see panel B for initial rate data). (B) Effect of Mel (50 µM) on the initial rates of NO consumption in diluted human plasma, $^{*}P < 0.05$ (Data represent the mean ± SEM, n = 3; see panel A for representative traces). (C) Effect of Mel (50 µM) on the initial rates of NO consumption in protein-free model systems in the absence (no substrates) or presence of 50 µM tyrosine (Tyr), 200 µM urate and/or 50 µM ascorbate (Asc); $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ (Data represent the mean ± SEM, n = 3; see panels D–G for representative traces). (D–G) Representative traces of the effect of Mel (50 µM) on NO consumption in protein-free model systems in the absence or presence of 50 µM Tyr, 200 µM urate and/or 50 µM Asc (see Panel C for initial rate data). (H–K) Representative traces of the effect of Mel (50 µM) on H_2O_2 consumption in protein-free model systems in the absence or presence of Tyr, urate and/or Asc in the presence of ~500 nM NO (from NOC-9; i.e. under identical conditions as Panels D–G). The H_2O_2 measurements are representative of 3 independent determinations.

accelerating MPO-catalyzed NO consumption, acetaminophen addition inhibited the overall extent of NO consumption in all protein-free model systems examined, with NO consumption ceasing within ~0.5 min following MPO addition (Fig. 11A, E–H). This overall inhibitory action of acetaminophen was most pronounced in ascorbate-replete systems; i.e., the initial rate and the overall extent of NO consumption promoted by acetaminophen in diluted plasma (Fig. 11A, C) or in the protein-free model system containing tyrosine and urate (Fig. 11D, G, H) were decreased in the presence of ascorbate by 50% or 30%, respectively.

Parallel measurements of H_2O_2 consumption revealed that acetaminophen significantly increased MPO turnover in all protein-free model systems examined, and that H_2O_2 was depleted within ~0.5 min following MPO addition (Fig. 11I–L; H_2O_2 depletion was confirmed by a lack of electrode response to the addition of excess catalase at the end of the experiment; data not shown). The depletion of H_2O_2 in all systems correlated closely with the cessation of NO consumption (Fig. 11E–H *vs.* I–L). The increase in NO levels after 0.5 min is consistent with the continued release of NO from residual NOC9. A second addition of H_2O_2 (10 µM) at a time point following the depletion of the initial amount resulted in a second transient rapid loss of NO followed by recovery of NO levels (data not shown), further confirming that the cessation of NO consumption was due to H_2O_2 depletion.

The increase in MPO turnover promoted by acetaminophen, indexed by the rate of H_2O_2 consumption, is consistent with the ability of acetaminophen to rapidly reduce MPO Compound II (the rate constant for this reaction is estimated to be 9-fold greater than that for the reaction of tyrosine with MPO Compound II: see Table 2).

A number of lines of evidence indicate that ascorbate and acetaminophen exhibit a synergistic action that decreases the efficiency (and hence the overall extent) of NO consumption and that this reflects (i) the capacity of acetaminophen to competitively inhibit NO consumption by MPO compound I (Reaction 1 *vs.* 2, Fig. 1), coupled with (ii) the ability of ascorbate to rapidly scavenge

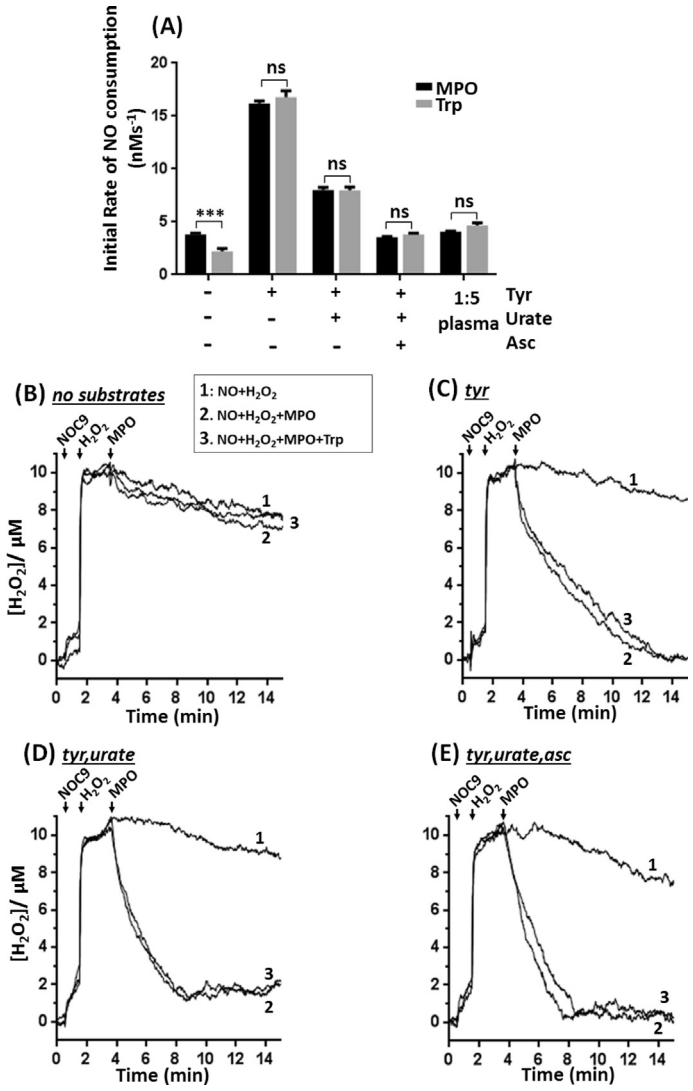

Fig. 10. Effect of L-tryptophan (L-Trp) on MPO-catalyzed NO and H_2O_2 consumption. (A) Effect of L-Trp (50 μM) on the initial rates of NO consumption in protein-free model systems in the absence (no substrates) or presence of 50 μM tyrosine (Tyr), 200 μM urate and/or 50 μM ascorbate (Asc) or in diluted plasma (1:5 plasma), $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ and ns $P > 0.05$ (Data represent the mean ± SEM, n = 3). (B–E) Representative traces of the effect of L-Trp (50 μM) on H_2O_2 consumption in protein-free model systems in the absence (no substrates) or presence of Tyr, urate, and/or Asc and in the presence of ~500 nM NO (from NOC-9; i.e. under identical conditions as Panel A). H_2O_2 measurements are representative of 3 independent measurements.

acetaminophen radicals (or secondary NO-consuming radicals derived from these, i.e. urate radicals) to yield ascorbyl radicals, which consume NO with relatively low efficiency (Reaction 5 *vs.* 6, Fig. 1). The rate constant for the reaction of acetaminophen with MPO compound I is predicted to be $\sim 3 \times 10^7$ M^{-1} s^{-1} (based on an IC_{50} value of 77 μM for the inhibition of MPO chlorination activity [47]), which is sufficiently large for acetaminophen to be an effective competitive inhibitor of MPO Compound I-mediated NO consumption. In kinetic analyses where the rate constant for the reaction of acetaminophen with MPO Compound I (k_2) was assumed (conservatively) to be identical to that for tyrosine (7.7×10^5 M^{-1} s^{-1}, Table 2), in the presence of physiological concentrations of NO, urate, tyrosine and ascorbate and pharmacologically relevant levels of acetaminophen (50 μM); acetaminophen is predicted to be the major substrate for both MPO Compound I and MPO Compound II, with acetaminophen radicals being the dominant radical produced during MPO turnover (38% of all substrates oxidized where urate is 34%; Fig. 4). Kinetic analyses corroborate that ascorbate is likely to be protective in the presence of acetaminophen due to its scavenging of acetaminophen and/or urate radicals rather than competitive inhibition of MPO-mediated NO consumption. In the presence of urate, tyrosine and acetaminophen, the addition of ascorbate is predicted to reduce NO consumption by MPO redox intermediates by 20%, which is less than the experimentally-determined decreases in NO consumption (i.e., 27% decrease in the protein-free model system containing tyrosine and urate – Fig. 11D; 50% decrease in diluted human plasma – Fig. 11C). Notably, the predicted protective effect of ascorbate is likely to be an overestimate as previous studies show that NO decreases the affinity of MPO Compound I for ascorbate (and hence limits the ability of ascorbate to competitively inhibit NO consumption by this redox intermediate) [21] and our kinetic analyses indicate that acetaminophen has a much higher k_2 than used in these calculations. It follows that the protective effect of ascorbate in the presence of acetaminophen derives instead from ascorbate's activity as a radical scavenger, involving its ability to scavenge and prevent NO consumption by acetaminophen radicals, as well as secondary NO-consuming radicals (e.g. urate radicals) generated from these.

With regard to the NO-consuming properties of acetaminophen radicals, acetaminophen decreased the rate of NO consumption in the presence of tyrosine alone (Fig. 11D, F), despite significantly

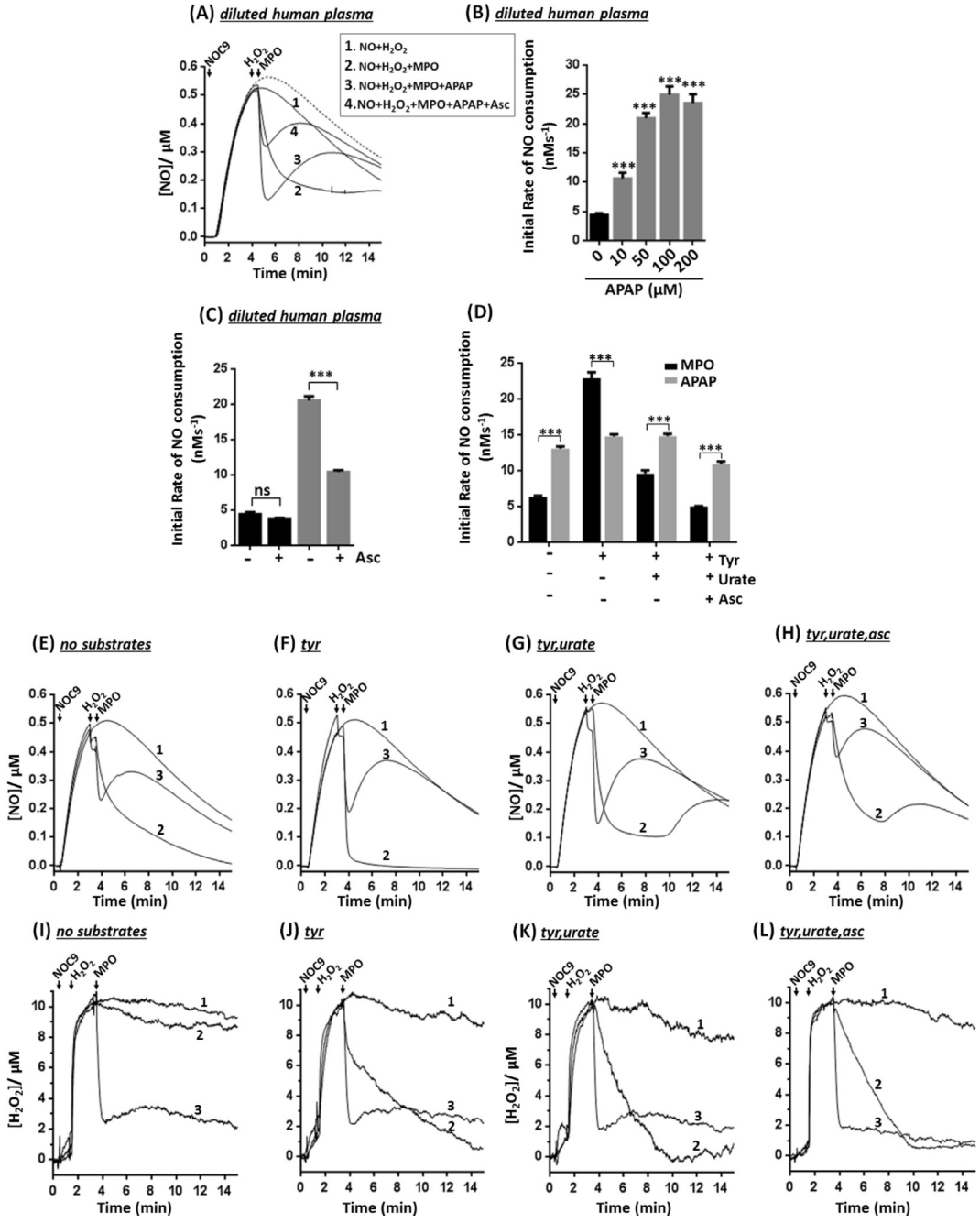

Fig. 11. Effect of acetaminophen (APAP) on MPO-catalyzed NO consumption and H_2O_2 consumption. (A) Representative trace of the effect of APAP (50 µM) on NO consumption in diluted human plasma in the absence or presence of exogenously added ascorbate (Asc, 50 µM) (see panel B and C for initial rate data). Dotted line is the addition of NOC9 without H_2O_2. (B) Effect of APAP (10–200 µM) on the initial rates of NO consumption in diluted human plasma; $^{***}P < 0.001$ relative to 0 µM APAP (Data represent the mean ± SEM, n = 3; see panel A for representative traces for 50 µM APAP). (C) Effect of Asc (50 µM) on the initial rates of NO consumption in diluted human plasma with APAP (50 µM) (see panel A for representative traces). (D) Effect of APAP (50 µM) on the initial rates of NO consumption in protein-free model systems in the absence (no substrates) or presence of 50 µM tyrosine (Tyr), 200 µM urate and/or 50 µM Asc, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ and ns $P > 0.05$ (Data represent the mean ± SEM, n = 3; see panels E–H for representative traces). (E–H) Representative trace of the effect of APAP (50 µM) on NO consumption in protein-free model systems in the absence (no substrates) or presence of 50 µM Tyr, 200 µM urate and 50 µM Asc (see Panel D for initial rate data). (I–L) Representative traces of the effect of APAP (50 µM) on H_2O_2 consumption in protein-free model systems in the absence or presence of Tyr, urate and/or Asc in the presence of ~500 nM NO (from NOC-9; i.e. under identical conditions as Panels E–H). Note: Catalase (50 µg/ml) was added to confirm complete H_2O_2 consumption at the completion of the experiment (i.e., following 14 min for Trace 3 in Panels I–L). The H_2O_2 measurements are representative of 3 independent determinations.

increasing MPO turnover under these conditions (Fig. 11F *vs.* J). This indicates that acetaminophen attenuated MPO compound I-mediated NO consumption and produced acetaminophen radicals that consume NO less efficiently than tyrosyl radicals.

In summary, the overall effect of acetaminophen and ascorbate is to divert MPO to an inefficient pathway of NO consumption (i.e., NO consumption by ascorbyl radicals) at the expense of an efficient pathway of NO consumption (NO oxidation by MPO Compound I). Whilst acetaminophen can initially increase NO consumption via its ability to reduce Compound II and accelerate MPO turnover, the overall effect of acetaminophen in the presence of ascorbate is protective due to the rapid depletion of the MPO co-substrate

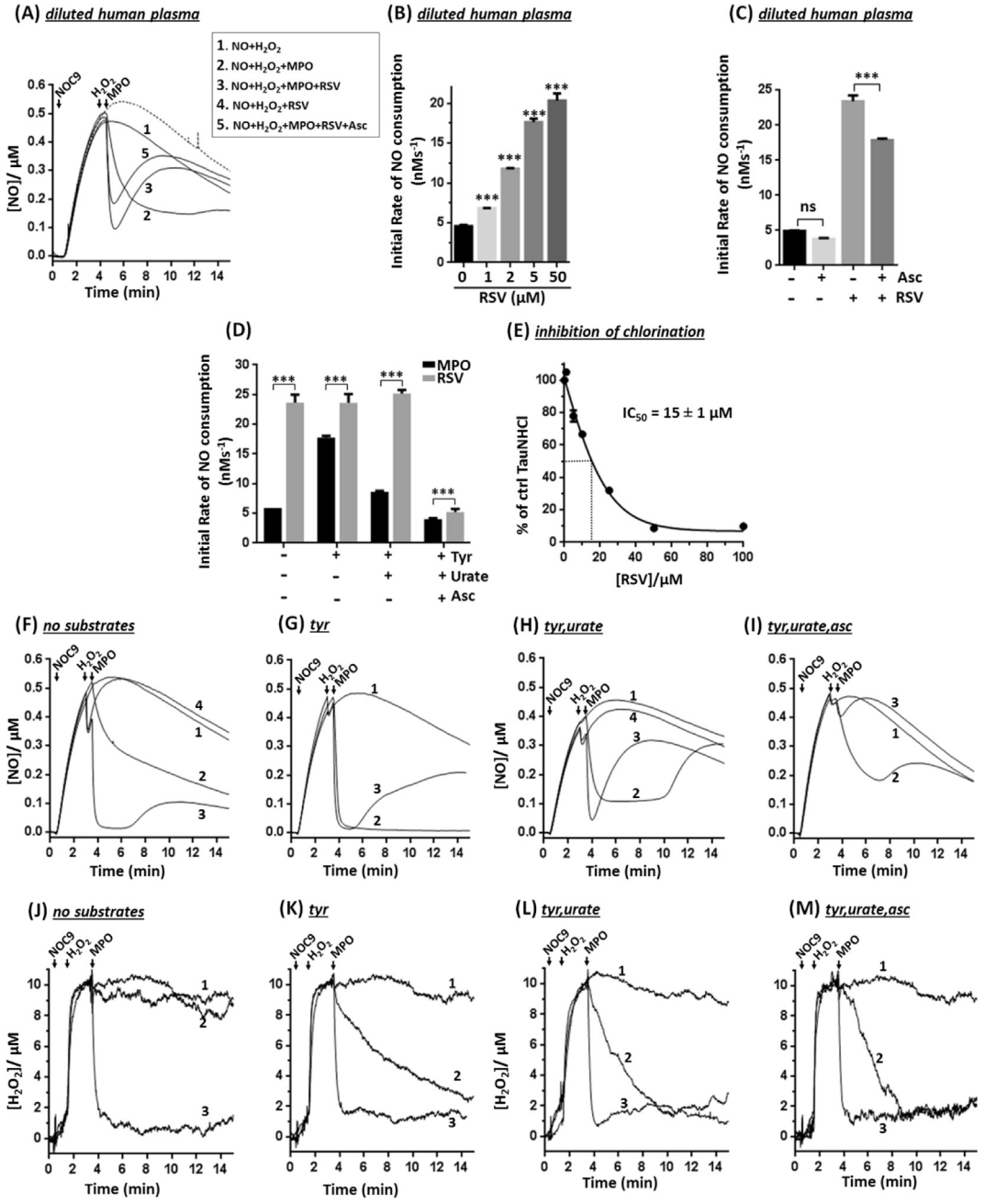

Fig. 12. Effect of Resveratrol (RSV) on MPO-catalyzed NO consumption and H_2O_2 consumption. (A) Representative trace of the effect of RSV (50 µM) on NO consumption in diluted human plasma in the absence or presence of exogenously added ascorbate (Asc, 50 µM), see panel B and C for initial rate data. Dotted line is the addition of NOC9 without H_2O_2. (B) Effect of RSV (1–50 µM) on the initial rates of NO consumption in diluted human plasma, $^{***}P < 0.001$ relative to 0 µM RSV (Data represent the mean ± SEM, n = 3; see panel A for representative trace for 50 µM RSV). (C) Effect of Asc (50 µM) on the initial rates of NO consumption in diluted human plasma with RSV (50 µM) (Data represent the mean ± SEM, n = 3; see panel A for representative traces). (D) Effect of RSV (50 µM) on the initial rates of NO consumption in protein-free model systems in the absence (no substrates) or presence of 50 µM tyrosine (Tyr), 200 µM urate and/or 50 µM Asc, $^{***}P < 0.001$ (Data represent the mean ± SEM, n = 3; see panels F–I for representative traces). (E) IC_{50} of RSV for inhibition of chlorination of taurine (20 mM) catalyzed by MPO (100 nM) in the presence of H_2O_2 (50 µM) for 5 min (Data represent the mean ± SEM, n = 3). 95% confidence intervals for the IC_{50} are 12.42–16.60 µM, and the goodness of fit (R^2) value is 0.98. (F–I) Representative traces of the effect of RSV (50 µM) on NO consumption in protein-free model systems in the absence (no substrates) or presence of 50 µM Tyr, 200 µM urate and/or 50 µM Asc (see Panel D for initial rate data). (J–M) Representative traces of the effect of RSV (50 µM) on H_2O_2 consumption in protein-free model systems in the absence or presence of Tyr, urate and/or Asc in the presence of ~500 nM NO (from NOC-9; i.e. under identical conditions as Panels F–I). The H_2O_2 measurements are representative of 3 independent determinations.

H_2O_2 and formation of ascorbyl radicals, which consume NO inefficiently. In other words, acetaminophen and ascorbate act synergistically to reduce the total amount of NO consumed over time per H_2O_2 molecule consumed as an MPO co-substrate.

3.5.2. Resveratrol

Similar to acetaminophen, resveratrol (1–50 µM) stimulated the initial rates of NO consumption in diluted human plasma (Fig. 12A–C, up to 4.7-fold) and in all protein-free model systems

examined (Fig. 12D, F–I). These resveratrol-dependent increases in NO consumption corresponded with marked increases in the rate of MPO turnover under all conditions examined, with MPO turnover ceasing due to H_2O_2 depletion within 0.5 min following the addition of MPO (Fig. 12J–M). The time point at which depletion of H_2O_2 occurred correlated closely with the observed recovery of NO levels (Fig. 12H, I *vs.* L, M), which is consistent with the release of NO from residual NOC-9.

As with acetaminophen, the overall extent of NO consumption promoted by resveratrol was less in ascorbate-replete *vs.* ascorbate-free fluids. Thus, the addition of ascorbate to diluted plasma or protein-free model systems containing tyrosine and urate significantly reduced the ability of resveratrol to promote NO consumption by MPO decreasing the initial rate of NO consumption by 20% or 80%, respectively (Fig. 12C, D). Importantly, the addition of resveratrol to ascorbate-containing systems also decreased the overall extent of NO consumption (Fig. 12I; Trace 3 *vs.* Trace 2). These data highlight that resveratrol and ascorbate act synergistically to inhibit the overall efficiency of MPO-catalyzed NO consumption.

Rate constants for the reaction of resveratrol with MPO Compound I and MPO Compound II have yet to be determined. However, our data indicate that these rate constants are likely to be comparable to or greater than those determined for acetaminophen. Thus, the ability of resveratrol (like acetaminophen) to significantly increase MPO turnover even in the presence of physiological peroxidase substrates (Fig. 12J–M), indicates that it is a dominant substrate for MPO Compound II under these conditions. To gain insight into the ability of resveratrol to act as a competitive MPO Compound I substrate, we also examined its capacity to competitively inhibit MPO-catalyzed chloride oxidation (Fig. 1, Reaction 2 *vs.* 8). Resveratrol inhibited MPO chlorination activity (100 nM MPO/50 μM H_2O_2) with an IC_{50} of 15 (±1) μM (Fig. 12E), which is less than the equivalent value for acetaminophen

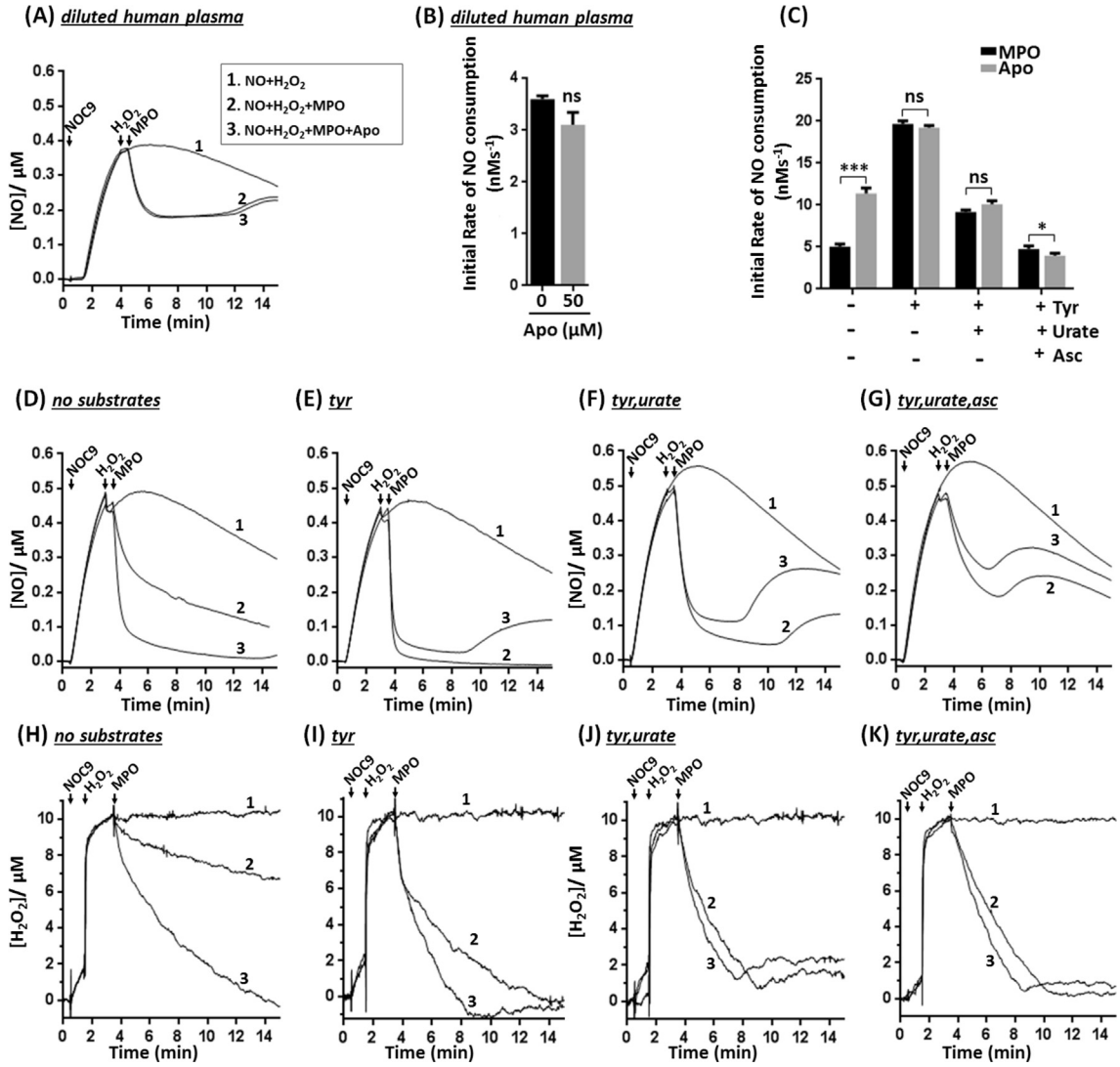

Fig. 13. Effect of apocynin (Apo) on MPO-catalyzed NO consumption and H_2O_2 consumption. (A) Representative trace of the effect of Apo (50 μM) on NO consumption in diluted human plasma (see panel B for initial rate data). (B) Effect of Apo (50 μM) on the initial rates of NO consumption in diluted human plasma, ns $P > 0.05$ (Data represent the mean ± SEM, n = 3; see panel A for representative trace for 50 μM Apo). (C) Effect of Apo (50 μM) on the initial rates of NO consumption in protein-free model systems in the absence (no substrates) or presence of 50 μM tyrosine (Tyr), 200 μM urate and/or 50 μM ascorbate (Asc), $^{***}P < 0.001$, $^{*}P < 0.05$ and ns $P > 0.05$ (Data represent the mean ± SEM, n = 3; see panels D–G for representative traces). (D–G) Representative traces of the effect of Apo (50 μM) on NO consumption in protein-free model systems in the absence (no substrates) or presence of 50 μM Tyr, 200 μM urate and/or 50 μM Asc (see Panel C for initial rate data). (H–K) Representative traces of the effect of Apo (50 μM) on H_2O_2 consumption in protein-free model systems in the absence or presence of Tyr, urate and/or Asc in the presence of ~500 nM NO (from NOC-9; i.e. under identical conditions as Panels D–G). The H_2O_2 measurements are representative of 3 independent determinations.

(IC_{50} value ~77 μM performed with the same concentrations of MPO/H_2O_2 [47]). Resveratrol is therefore an excellent substrate for MPO Compound I and (similarly to acetaminophen) it is likely to be an effective competitive inhibitor of NO oxidation by this redox intermediate. In ascorbate-depleted systems, resveratrol radicals generated by MPO Compound I may directly mediate NO consumption even in the presence of urate, as scavenging of resveratrol radicals by urate is thermodynamically unfavourable (E^o(urate-H·/urate-H) = 590 mV [95]; E^o(Resveratrol·/H^+/Resveratrol) = ~480–500 mV [108]). Where ascorbate is available, it is likely to suppress resveratrol-dependent NO consumption by scavenging both urate radicals (generated solely by urate catabolism by MPO redox intermediates; see above) and resveratrol-derived radicals.

3.5.3. Apocynin

Supra-pharmacological concentrations of apocynin (50 μM) did not significantly affect the initial rate or extent of MPO-catalyzed NO consumption in diluted human plasma and likewise failed to affect the rate of NO consumption in protein-free buffer systems containing physiological peroxidase substrates urate and tyrosine (Fig. 13A–G). Apocynin marginally attenuated the initial rate of NO consumption in the presence of urate, tyrosine and ascorbate (Fig. 13C, G). In contrast, apocynin significantly accelerated MPO-dependent NO consumption in the absence of physiological peroxidase substrates (Fig. 13C, D).

Measurement of H_2O_2 consumption showed that apocynin consistently increased the rate of MPO turnover in all protein-free model systems (Fig. 13H–K); findings are consistent with apocynin being an efficient MPO compound II substrate, as previously

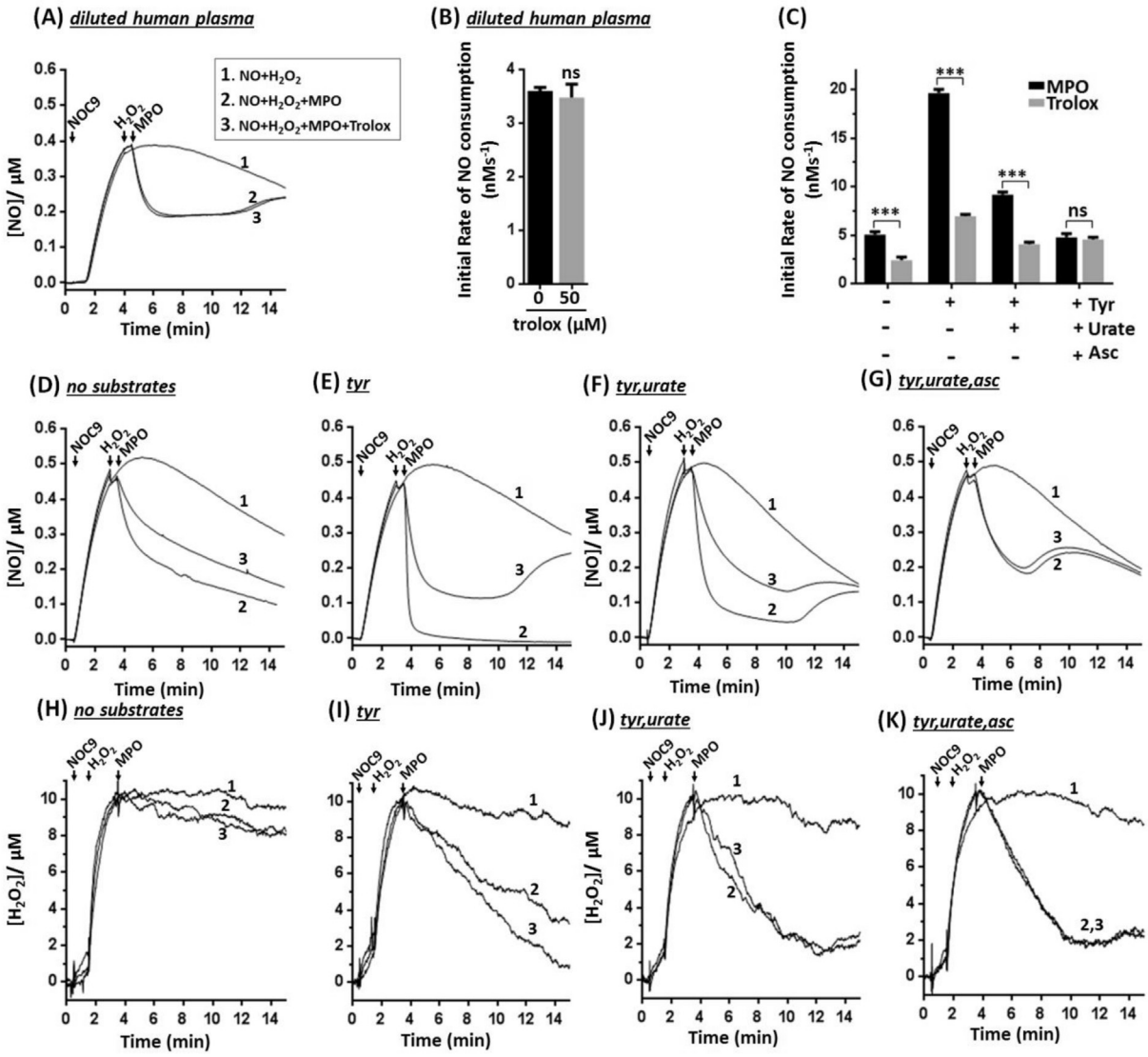

Fig. 14. Effect of Trolox on MPO-catalyzed NO consumption and H_2O_2 consumption. (A) Representative trace of the effect of trolox (50 μM) on NO consumption in diluted human plasma, see panel B for initial rate data. (B) Effect of trolox (50 μM) on the initial rates of NO consumption in diluted human plasma, ns $P > 0.05$ (Data represent the mean ± SEM, n = 3; see panel A for representative trace for 50 μM trolox). (C) Effect of trolox (50 μM) on the initial rates of NO consumption in protein-free model systems in the absence (no substrates) or presence of 50 μM tyrosine (Tyr), 200 μM urate and/or 50 μM ascorbate (Asc), $^{***}P < 0.001$ and ns $P > 0.05$ (Data represent the mean ± SEM, n = 3; see panels D–G for representative traces). (D–G) Representative traces of the effect of trolox (50 μM) on NO consumption in protein-free model systems in the absence (no substrates) or presence of 50 μM Tyr, 200 μM urate and/or 50 μM Asc (see Panel C for initial rate data). (H–K) Representative traces of the effect of trolox (50 μM) on H_2O_2 consumption in protein-free model systems in the absence or presence of Tyr, urate and/or Asc in the presence of ~500 nM NO (from NOC-9; i.e. under identical conditions as Panels D–G). The H_2O_2 measurements are representative of 3 independent determinations.

proposed [58]. The rate constants for the reactions of apocynin with MPO Compound I and MPO Compound II have yet to be determined, however our data indicate that the rate constant for the reaction of apocynin with MPO Compound II is comparable to those for tyrosine and urate (Table 2). This accounts for its ability to increase NO consumption in the absence of competing peroxidase substrates as it stimulates MPO turnover and thus consumption of NO by MPO redox intermediates. The failure of apocynin to promote an increase in NO consumption in the presence of physiological substrates despite increasing MPO turnover, indicates that apocynin attenuates NO oxidation by MPO Compound I and forms apocynin-derived radicals that are relatively inefficient at consuming NO (with this protective effect counteracting its stimulatory effect on MPO turnover). In the presence of endogenous radical scavengers, apocynin radicals may mediate NO consumption by promoting formation of secondary NO-consuming radicals (scavenging of apocynin radicals by urate and ascorbate is thermodynamically favourable; E^o(Apocynin$^{\cdot}$/Apocynin) = 785 mV [109]; E^o(urate-H$^{\cdot}$/urate-H) = 590 mV; E^o(Asc$^{\cdot}$/Asc^{-}) = 282 mV [95]).

3.5.4. Trolox

Trolox (50 μM) did not alter the rate of MPO-catalyzed NO consumption in diluted human plasma (Fig. 14A, B) or in protein-free model systems containing tyrosine, urate and ascorbate (Fig. 14C, G). Notably, in model systems where ascorbate was absent (Fig. 14C): i.e. (i) no physiological peroxidase substrates (Fig. 14D), (ii) tyrosine alone (Fig. 14E) or (iii) tyrosine and urate (Fig. 14F), trolox exerted significant protection (Fig. 14C–F). Measurements of H_2O_2 consumption revealed that trolox failed to alter MPO turnover under physiologically-relevant conditions (Fig. 14H–K), although an increase in MPO turnover was apparent in the presence of tyrosine alone (Fig. 14I).

Whilst trolox has been shown to act as a peroxidase substrate for HRP and LPO [63–65], the rate constants for the reactions of trolox with MPO Compounds I and II have not been determined. The failure of trolox to stimulate MPO turnover in the absence of peroxidase substrates (Fig. 14H) indicates that it is a poor substrate for MPO Compound II. The ability of trolox to decrease the rate of NO consumption in this setting, without decreasing MPO turnover, could reflect its ability to competitively inhibit MPO compound I mediated NO consumption (and hence reduce the efficiency of NO consumption in each catalytic cycle). In contrast, the ability of trolox to decrease the rate of NO consumption in the presence of urate and tyrosine, without altering MPO turnover, is likely to reflect its ability to rapidly scavenge and prevent NO-consumption by tyrosyl radicals and/or urate radicals (both reactions are thermodynamically favourable [95]), with the resulting trolox phenoxyl radicals unable to react with NO [110]. As ascorbate effectively reduces phenoxyl radicals [21], this likely explains the failure of trolox to exert additional protection in ascorbate-containing fluids.

3.6. Nitroxides

Nitroxides are a class of stable synthetic radicals that have been shown to enhance vascular NO bioavailability in multiple animal models of oxidative stress, a property that is widely attributed to their ability to act as superoxide dismutase (SOD) mimetics and prevent NO consumption by $O_2^{\cdot-}$ [68,111]. However, recent studies highlight that nitroxides can also potently suppress MPO-catalyzed oxidative reactions (chlorination, nitration) *via* their activity as reversible MPO inhibitors and free radical scavengers [70,71,112]. The effect of nitroxides on MPO NO oxidase activity has not previously been examined. In pre-clinical models, peak plasma concentrations of tempol can reach millimolar levels [72]. Here we examined the activity of tempol and related piperidine nitroxides.

Tempol (4-hydroxy TEMPO, 4HT; 50 μM) inhibited the initial rates of MPO-catalyzed NO consumption in diluted human plasma (Fig. 15A, B). The IC_{50} value for this inhibition in diluted human plasma was 39 (±1) μM (Fig. 15C). Tempol also inhibited MPO's NO oxidase activity in protein-free buffer systems containing (i) no physiological peroxidase substrates (Fig. 15D, E), (ii) tyrosine alone (Fig. 15D, F) and (iii) tyrosine, urate and ascorbate (Fig. 15D, H). In contrast, tempol failed to inhibit NO consumption in the presence of tyrosine and urate alone (Fig. 15D, G).

Tempol (which is small and uncharged) is a good substrate for MPO Compound I (but not MPO Compound II) [70,71] and reacts rapidly with a wide variety of radicals [113–116]. Notably, whilst urate radicals are a major species formed in biological systems (and are implicated in mediating a range of pathological reactions including NO consumption [21,22]) it is unknown whether nitroxides are capable of scavenging these radicals. In order to gain insight into whether the observed effects of tempol on MPO-catalyzed NO consumption are related to its activity as an MPO substrate or scavenger of substrate radicals, equivalent experiments were performed with the related nitroxides 4-carboxyTEMPO and 4-aminoTEMPO, which exhibit varying properties and activities as MPO substrates [70].

Previous work [70] identified that compared to tempol the negatively-charged nitroxide 4-carboxyTEMPO is a poor substrate for MPO Compound I whilst the positively-charged nitroxide 4-aminoTEMPO is a better substrate [117]. The capacity of nitroxides to inhibit the rate of MPO-catalyzed NO in ascorbate-containing systems, including diluted human plasma (Fig. 15B) and ascorbate-containing model systems (Fig. 15D), varied with 4-aminoTEMPO being the most effective, tempol (4-HT) exhibiting intermediate activity and 4-carboxyTEMPO the least effective. All the nitroxides examined failed to exert protection in the absence of ascorbate, i.e. in the presence of urate and tyrosine alone (Fig. 15D, G). However, when urate was omitted (i.e. in the presence of tyrosine only) or where no endogenous substrates were present (Fig. 15D–F), tempol and 4-carboxyTEMPO reduced the rate of NO consumption with tempol more effective than 4-carboxyTEMPO. In contrast, 4-aminoTEMPO had either no effect or enhanced NO-consumption under these conditions (Fig. 15D–F).

With respect to the effects of nitroxides on MPO turnover, in systems that contained physiological levels of tyrosine and urate (with and without ascorbate), none of the nitroxides altered the rate of enzyme turnover (Fig. 15K, L), which is consistent with their known activity as poor MPO Compound II substrates [70]. In 'substrate poor' systems, i.e. containing tyrosine alone (Fig. 15J) or no endogenous substrates (Fig. 15I), 4-aminoTEMPO, tempol and 4-carboxyTEMPO all afforded slight increases in MPO turnover.

Overall, the preceding data reveal that the inhibitory activity of tempol against MPO NO oxidase activity in substrate-replete solutions (i.e. at least containing physiological levels of tyrosine and urate) is (i) dependent on the presence of ascorbate and (ii) independent of any effects on MPO turnover. Kinetic analyses also indicate that tempol exerts protection by scavenging diffusible radicals rather than by competitively inhibiting the direct consumption of NO by MPO redox intermediates. Thus, whilst tempol is predicted to inhibit the direct consumption of NO by MPO by 8% in ascorbate-replete systems, the experimental data show that tempol inhibits the initial rate of NO consumption by 53% under these conditions. It therefore appears that tempol suppresses MPO NO oxidase activity in ascorbate-replete systems by scavenging and preventing NO consumption by ascorbyl radicals. Interestingly, the lack of efficacy of tempol and other nitroxides in ascorbate-free but urate-containing systems (Fig. 15D, G) indicates that the nitroxide moiety is inefficient at scavenging urate radicals.

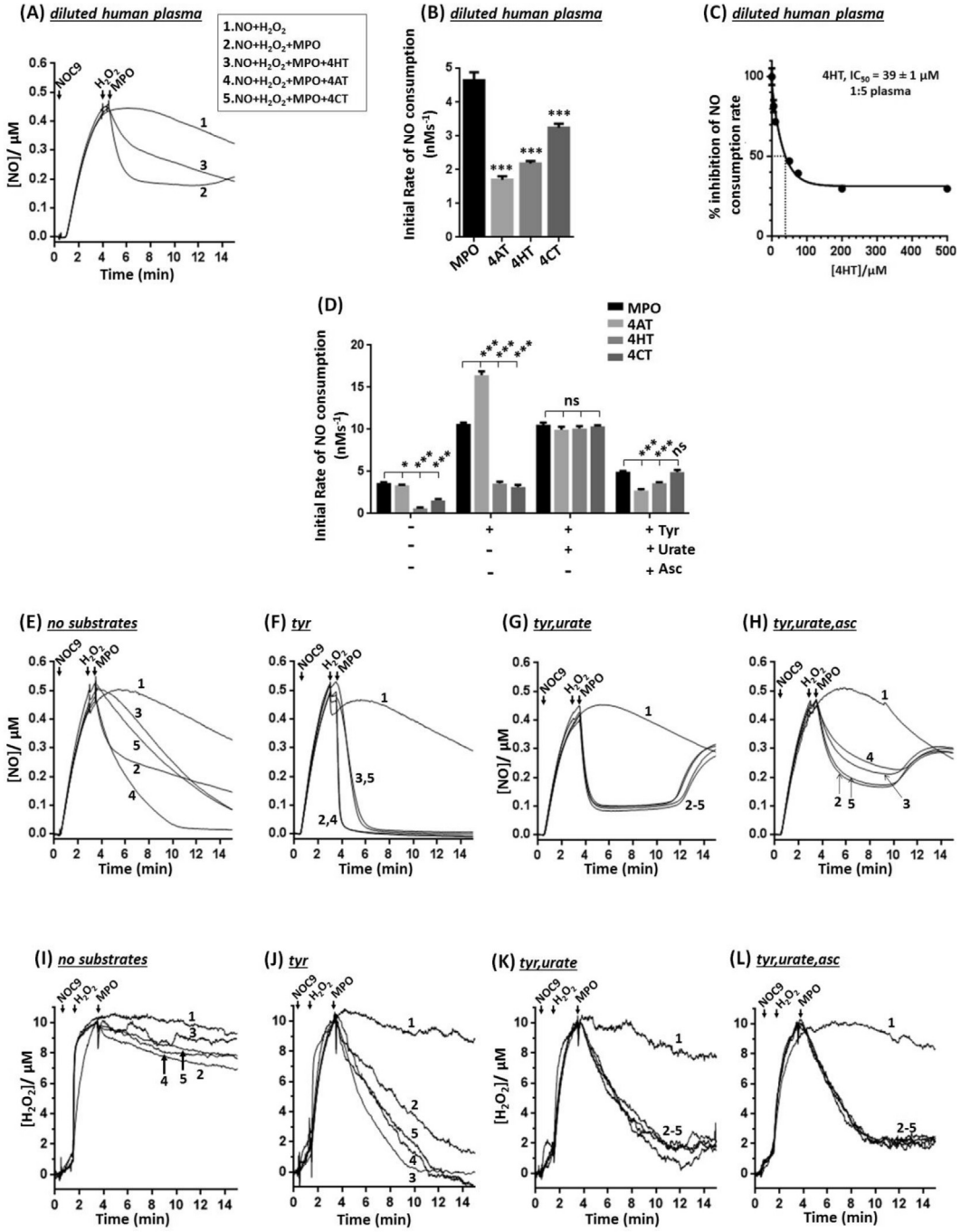

Fig. 15. Effect of Tempol (4HT) and related nitroxides on MPO-catalyzed NO and H_2O_2 consumption. (A) Representative trace of the effect of 4-HT (50 µM) on NO consumption in diluted human plasma (see panel B for initial rate data). (B) Effect of 4HT, 4-amino TEMPO (4AT) and 4-carboxy TEMPO (4CT) (50 µM) on the initial rates of NO consumption in diluted human plasma, $^{***}P < 0.001$ relative to 0 µM nitroxides (Data represent the mean ± SEM, n = 3; see panel A for representative trace for 50 µM 4-HT). (C) Determination of IC_{50} value for 4-HT inhibiting MPO NO oxidase activity in diluted human plasma. 95% confidence intervals of the IC_{50} are 29.17 to 47.64 µM, and goodness of fit R^2 value is 0.97. (D) Effect of nitroxides (4AT, 4HT, 4CT; 50 µM) on the initial rates of NO consumption in protein-free model systems in the absence (no substrates) or presence of 50 µM tyrosine (Tyr), 200 µM urate and/or 50 µM ascorbate (Asc), $^{***}P < 0.001$, $^{*}P < 0.05$ and ns $P > 0.05$ (Data represent the mean ± SEM, n = 3; see panels E–H for representative traces). Initial rates for 'no substrates' and 'Tyr' alone were measured at 225–230 s, to provide a more accurate quantitative description of the initial linear rates observed in the representative traces in Fig. 13E and F. All other initial rates were measured in line with all other figures at 215–220 s. (E–H) Representative traces of the effect of nitroxides (4AT, 4HT, 4CT; 50 µM) on NO consumption in protein-free model systems in the absence (no substrates) or presence of 50 µM Tyr, 200 µM urate and/or 50 µM Asc (see Panel D for initial rate data). (I–L) Representative traces of the effect of nitroxides (4AT, 4HT, 4CT 50 µM) on H_2O_2 consumption in protein-free model systems in the absence (no substrates) or presence of Tyr, urate and/or Asc in the presence of ~500 nM NO (from NOC-9; i.e. under identical conditions as Panels E–H). The H_2O_2 measurements are representative of 3 independent determinations.

4. Discussion

MPO is implicated in playing a significant role in impairing NO bioavailability during inflammatory conditions *via* its NO oxidase activity [9,20]; however the potential for pharmacological agents to influence this process has, to date, not been investigated. Therefore, the current study examined the effects of various relevant pharmacological agents, including agents with established activity

as MPO inhibitors, on MPO NO oxidase activity under physiological conditions.

This study reveals that a number of pharmacological agents that are widely employed in pre-clinical research or as therapeutic drugs (Fig. 2 and Table 1) significantly alter MPO NO oxidase activity in human plasma and physiological model systems at pharmacologically relevant concentrations. Kinetic analyses further revealed the mechanistic basis for the activities of these drugs and identified criteria for developing drugs with improved efficacy against MPO NO oxidase activity. Overall, our data identify previously unrecognized mechanisms by which different pharmacological agents can impact on NO bioavailability and highlight new pharmacological strategies to limit MPO-dependent NO depletion during inflammatory conditions. The biochemical and kinetic studies carried out here lay the foundation for future experiments testing the ability of these pharmacological agents to modulate MPO NO oxidase activity and hence NO bioactivity in relevant in vivo and ex vivo experimental systems, e.g., endothelium-dependent arterial relaxation.

Very similar results were obtained in experiments with human plasma and with protein-free model systems (containing physiological mixtures of tyrosine, urate and ascorbate), thus supporting the value of these model systems in informing our mechanistic analyses. However, we did not exhaustively examine how all components of extracellular fluids may modulate the effects of pharmacological agents on MPO NO oxidase activity. For example, thiocyanate (SCN^-) is a significant MPO substrate, which is oxidized by MPO in the presence of NO [78], and capable of modulating MPO NO oxidase activity in the presence of other MPO substrates, tyrosine, urate and ascorbate [21]; i.e., we recently showed that SCN^- acts as a competitive inhibitor of NO consumption by MPO Compound I [21], with up to 30% inhibition at the upper end of the range of SCN^- concentrations in normal human plasma (10–140 μM SCN^- [118–120]) and up to 60% inhibition at concentrations present in lung lining fluid (500 μM SCN^- [121]). Therefore it is plausible that in addition to tyrosine, urate and ascorbate, SCN^- also influences the actions of the pharmacological agents on MPO NO oxidase activity and this aspect warrants further investigation.

Suicide-based MPO inhibitors have attracted considerable attention as potential MPO-targeted therapeutics [96]. These drugs inhibit MPO turnover through the MPO-dependent formation of drug-derived substrate radicals that rapidly react with the MPO active site thereby irreversibly (e.g. ABAH, 2-thioxanthine) or reversibly (e.g. isoniazid) inhibiting the enzyme. For example, the hydrazide-based MPO inhibitor ABAH potently inhibits the chlorination and peroxidation activities of MPO and has been widely employed as a research tool to inhibit MPO activity in vitro and in some in vivo studies [23–28]. Accordingly, it may be presumed that such agents would also be effective inhibitors of MPO NO oxidase activity. However, our novel data show that under physiologically relevant conditions NO abrogates the ability of hydrazide-derived substrate radicals (ABAH radicals and isoniazid radicals) to inhibit MPO (Fig. 16, Reaction 5 *vs.* 7). This likely reflects the ability

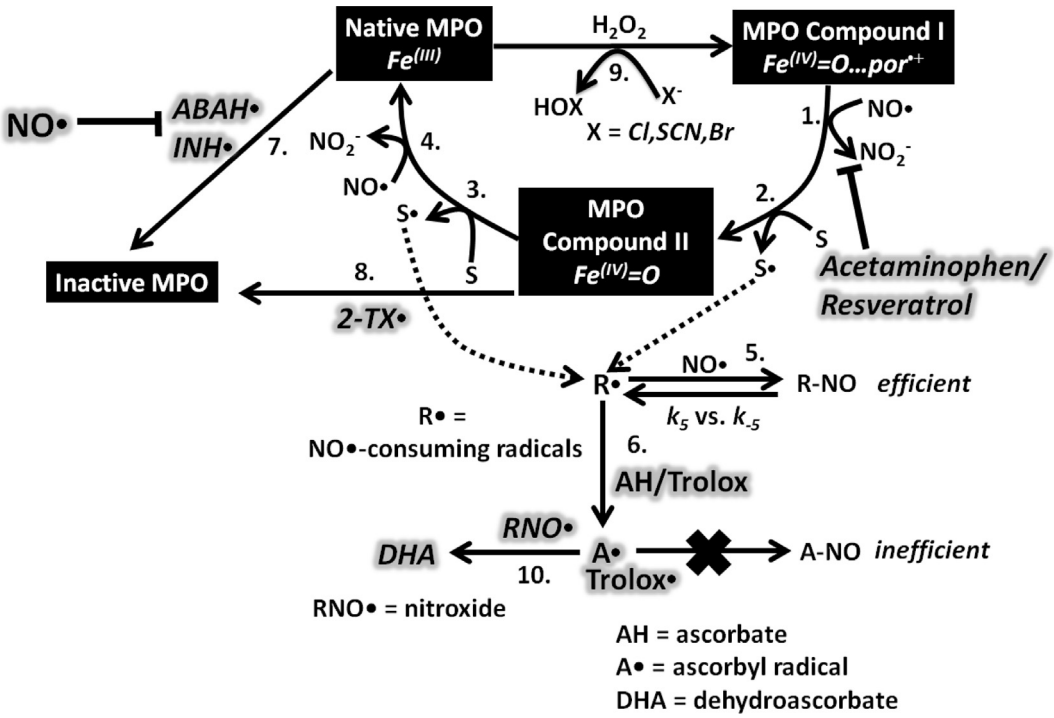

Fig. 16. Regulation of MPO NO oxidase activity by different pharmacological agents. Hydrazide derivatives (ABAH, INH) stimulated MPO-catalyzed NO consumption. Our data and kinetic analyses indicated that this was due to (i) a direct reaction between hydrazyl radicals (ABAH•, INH•) and NO which results in (ii) the NO-dependent suppression of MPO-inactivating reactions by hydrazyl radicals (Reaction 7) and thus (iii) initial/transitory stimulation of MPO turnover (Reaction 3) leading to increased consumption of NO by MPO compound I (Reaction 1) and diffusible substrate radicals (Forward Reaction 5). 2-Thioxanthines were the most protective drug-class examined and inhibited MPO catalytic activity immediately (Reaction 8). Its rapid reaction with the heme of the MPO active site is thought to be responsible for its inhibitory potency. Acetaminophen and resveratrol exhibit a synergistic action with ascorbate resulting in the enhanced inhibition of MPO-catalyzed NO consumption in the presence of the vitamin. Kinetic analyses suggest that this is due to a combination of Reaction 2 and Reaction 6; whereby acetaminophen/resveratrol inhibit NO consumption via MPO compound I (Reaction 2 *vs.* 1) due to their sufficiently high rate constant of reaction with MPO compound I, and ascorbate subsequently scavenges the resultant phenoxyl radicals to yield ascorbyl radicals, which are comparatively inefficient at consuming NO (Reaction 5 *vs.* 6). Nitroxide radicals ($RNO^•$) attenuated MPO-catalyzed NO consumption in the presence of ascorbate, urate and tyrosine; this is most likely accounted for by a reaction between ascorbyl radicals and nitroxide radicals (Reaction 10) to form the reduced form of nitroxides (hydroxylamine, RNOH) and dehydroascorbate (DHA). This highlights that the contribution of ascorbyl radicals to NO consumption is not negligible. Trolox exhibited a similar antioxidant activity to ascorbate whereby it did not affect MPO catalytic activity but scavenged urate and tyrosyl radicals, thereby inhibiting their reaction with NO (Reaction 5 *vs* 6).

of NO to rapidly react with these drug-derived substrate radicals and prevent their reactions with the MPO active site. Conversely, ABAH and isoniazid both increase MPO turnover by promoting the reduction of Compound II and in turn increase NO consumption by MPO redox intermediates and diffusible substrate radicals (Fig. 16, Reaction 1 and 5). Whilst ABAH is a good substrate for MPO Compound II, in the case of isoniazid our data suggests that isoniazid radicals, but not the parent drug itself, are responsible for reducing MPO Compound II and driving increases in MPO turnover.

The ability of NO to antagonize the MPO inhibitory activity of ABAH and isoniazid (and related hydrazide-based MPO inhibitors) resulting in stimulation of MPO turnover and the production of reactive radicals, has important implications for their utility as research tools (where ABAH is widely used) or therapeutic agents [96,122]. Thus, the availability of NO produced from endogenous or exogenous sources will be a key factor that determines the efficacy of hydrazide-based MPO inhibitors in biological systems and could account for the poor activity of ABAH as an in vivo MPO inhibitor reported in some studies [24,122].

Our findings may also have implications for the pharmacological actions of isoniazid in tuberculosis patients, where it is a first-line therapy [29] and is proposed to adversely affect innate immunity by inhibiting MPO-mediated bacterial killing [30]. Our data support that bioavailable NO in the lungs of tuberculosis patients could limit isoniazid-mediated MPO inhibition thereby preserving innate immunity. Conversely, isoniazid-mediated increases in MPO's NO oxidase activity may act to impair NO-mediated mycobacterial killing [123,124]. Therefore, the possibility that isoniazid, MPO and NO interact to influence the innate immune response during tuberculosis warrants further investigation.

Whilst isoniazid and ABAH had unexpected limitations as inhibitors of MPO NO oxidase activity, the more recently discovered 2-thioxanthine class of mechanism-based inhibitors (AZD5904, TX4) potently inhibited MPO NO oxidase activity. The inability of NO to antagonize inhibition of MPO catalytic activity by 2-thioxanthines reported in this work is indeed in line with previous studies. 2-Thioxanthines reportedly yield enzyme-inactivating radicals that once formed react avidly with the heme of MPO Compound II and do not readily exit the MPO active site and also sterically block access to the active site by other competing peroxidase substrates [32,34]. These properties thereby limit adverse reactions between the 2-thioxanthine radical and locally available reductants, such as NO and/or limit the formation of other NO-consuming diffusible substrate radicals by MPO compound II. In previous work, Tidén and co-authors [32] indicated that the mechanism of action of 2-thioxanthine enables it to minimise adverse reactions between the substrate radical and other reactive targets, such as tyrosine and $O_2^{\cdot-}$. This is supported by our findings where the 2-thioxanthine tested was unique amongst the mechanism-based MPO inhibitors in its ability to effectively inhibit both MPO NO oxidase activity and overall MPO catalytic activity in the presence of various competing peroxidase substrates at physiological concentrations including tyrosine, urate and ascorbate. Therefore, 2-thioxanthines represent the most effective class of drugs tested in this study with respect to inhibition of MPO NO oxidase activity under physiological conditions. As such, further investigations into the ability of this class of irreversible MPO inhibitors to preserve NO bioavailability in vivo are therefore warranted.

Acetaminophen and resveratrol initially enhanced MPO-dependent NO consumption, which involved their ability to rapidly increase MPO turnover by reducing compound II. Despite these acute effects, acetaminophen and resveratrol both suppressed the overall extent of NO consumption, in part, due to their ability to rapidly deplete the MPO co-substrate, H_2O_2. Acetaminophen and resveratrol also suppressed NO consumption more effectively in ascorbate-replete *vs.* ascorbate-deplete fluids by exerting a 'co-antioxidant' activity with ascorbate. This co-antioxidant activity involves the ability of acetaminophen and resveratrol to competitively inhibit NO consumption by MPO compound I (Reaction 1 vs 2, Fig. 16) generating phenolic substrate radicals that are efficiently scavenged by ascorbate thereby preventing their reaction with NO and instead forming ascorbyl radicals that react with NO with less efficiency (Reaction 5 vs 6, Fig. 16).

This mechanistic model is supported by experimental evidence, obtained here and in previous studies, that acetaminophen and resveratrol are both effective MPO Compound I substrates, with both phenolic drugs potently inhibiting MPO chlorination activity: i.e., with IC_{50} values of ~77 μM [47] and 15 μM (determined here, Fig. 12E) for acetaminophen and resveratrol, respectively. Based on these IC_{50} values, the rate constant of reaction for these substrates with MPO Compound I (k_2) is predicted to be in the order of $\geq 10^7\ M^{-1}\ s^{-1}$. Conversely, the failure of the structurally related phenol apocynin, or the indoles L-Trp and melatonin, to exhibit an equivalent 'co-antioxidant' action with ascorbate is consistent with these substrates exhibiting significantly lower reaction rates with MPO compound I (i.e., k_2 values 1–2 orders of magnitude lower at least in the case of L-Trp and melatonin; see Table 2), and hence poorer activity as competitive inhibitors of NO catabolism by MPO compound I. The lack of protection from apocynin, L-Trp and melatonin is unlikely to relate to differences in the ability of ascorbate to scavenge their radicals, e.g. ascorbate reacts with tryptophanyl and acetaminophen radicals at similar rates ($k = 1 \times 10^8\ M^{-1}\ s^{-1}$ [125,126], $k = \sim 10^8\ M^{-1}\ s^{-1}$ [127], respectively).

Notably, the protection afforded by acetaminophen and resveratrol against overall NO losses depends not only on the presence of ascorbate but also on their ability to promote MPO turnover and rapidly deplete the MPO co-substrate, H_2O_2. As such, the in vivo actions of acetaminophen and resveratrol on MPO-dependent NO consumption are likely modulated by differences in the local flux of H_2O_2 in inflammatory microenvironments; e.g., in the presence of a low flux of H_2O_2, drug-mediated rapid consumption of the oxidant by MPO may act to preserve overall NO bioactivity by removing MPO's co-substrate. However, under local conditions where ascorbate is depleted and MPO is afforded a constant high flux of H_2O_2, acetaminophen and resveratrol have the capacity to significantly accelerate MPO-dependent NO consumption. These findings are consistent with the action of acetaminophen when inhibiting MPO chlorination activity [47], where acetaminophen increased the initial rate of HOCl production by isolated MPO by increasing total MPO turnover via its reaction with MPO compound II; however the overall effect of acetaminophen was to inhibit the total yield of HOCl.

Similar to acetaminophen and resveratrol, the ability of nitroxides to protect against MPO's NO oxidase activity is potentiated by ascorbate, but by a different mechanism. Whilst protection afforded by acetaminophen and resveratrol reflects their activity as MPO Compound I substrates, the protective effect of nitroxides reflects their ability to prevent NO consumption by reacting with ascorbyl radicals (Fig. 16). This is likely to involve the reduction of nitroxides by ascorbyl radicals to yield their corresponding hydroxylamine and dehydroascorbic acid (DHA), which are both non-radical products and hence unreactive towards NO (i.e. $Asc^{\cdot-} + RR'NO^{\cdot} + H^+ \rightarrow RRNOH + DHA$) [128]. The lower activity of negatively-charged nitroxides against MPO NO oxidase activity is likely to reflect electronic barriers to this reaction [129]. Interestingly, although previous studies identify that ascorbyl radicals (compared to tyrosyl or urate radicals) are relatively inefficient in consuming NO [21], our present studies and those of others [9] highlight that they can still contribute to NO consumption and that this can be suppressed by nitroxides.

Notably, nitroxides were ineffective in attenuating MPO NO oxidase activity in ascorbate-depleted systems where urate radicals are the major source of NO-consuming species [21]. This indicates that nitroxides are unreactive towards urate radicals, possibly due to unfavourable steric interactions. In contrast, trolox protected in ascorbate-depleted systems (likely by scavenging tyrosyl and urate radicals), but failed to protect in ascorbate-containing systems. This differential reactivity of pharmacological antioxidants towards ascorbyl radicals and urate radicals has important implications for their potential impact on NO bioavailability during inflammatory conditions, where local extracellular ascorbate levels may vary considerably, e.g. due to oxidative depletion and accumulation within activated leukocytes under conditions of inflammation and oxidative stress [86,87]. Thus, the current study identifies that the local availability of ascorbate is an important determinant of the protective actions of the phenolics acetaminophen and resveratrol, as well as nitroxides against MPO-dependent NO consumption in vivo.

The modulatory functions we have identified for acetaminophen, resveratrol and nitroxides against MPO NO oxidase activity may have important implications for their biological actions on cardiovascular function during inflammatory disease conditions. For example, the acute administration of acetaminophen to critically-ill patients (who typically exhibit elevated MPO levels [13]) can significantly decrease blood pressure [45], which could reflect the ability of acetaminophen to acutely suppress MPO NO oxidase activity. In contrast, chronic high frequency use or high doses of acetaminophen are associated with increased cardiovascular event risk [130] and blood pressure in coronary artery disease patients [131], a patient group that exhibits elevated levels of circulating extracellular MPO that strongly correlates with the degree of endothelial dysfunction [15,16,132]. Whilst these putative cardiovascular actions of acetaminophen may relate to a variety of drug targets (e.g., cyclooxygenase 2), our data indicate that acetaminophen may significantly alter MPO's NO oxidase activity during a variety of inflammatory conditions. Resveratrol has consistently been shown to increase NO bioavailability in a range of animal models of inflammation and disease, with this proposed to primarily reflect the ability of resveratrol to up-regulate NO synthesis and the expression of antioxidant enzymes (e.g. superoxide dismutase) [49,133]. More direct 'antioxidant' actions of resveratrol have previously been considered to be unimportant, based on an expectation that resveratrol should not provide any additional protection against oxidative NO depletion to that afforded by ascorbate [49]. However, our data support that resveratrol can provide protection against MPO NO oxidase activity in ascorbate-replete fluids over and above that afforded by ascorbate alone, due to its ability to potently inhibit NO consumption by MPO Compound I and increase the production of (less-reactive) ascorbyl radicals.

In the case of nitroxides, it is widely assumed that the capacity of tempol and related nitroxides to preserve NO bioavailability in vivo derives principally from their superoxide dismutase activity [68]. However, our data identify that tempol may also preserve NO bioactivity in vivo by inhibiting MPO's NO oxidase activity. Importantly, in ascorbate-replete fluids, tempol inhibits MPO NO oxidase activity with an IC_{50} of 39 (±1) μM, which is well within maximum tolerated concentrations of this agent in vivo [72].

The pharmacological actions of apocynin have been linked to its action as an MPO substrate [58], inhibitor of NADPH oxidase enzymes [57] or H_2O_2 scavenger [59]. Our data identify that the ability of apocynin to preserve NO bioavailability during inflammatory conditions [134] is unrelated to its actions on MPO NO oxidase activity. Instead, these protective effects most likely reflect apocynin's ability to inhibit NADPH oxidase enzymes in the vascular wall thereby attenuating NO consumption by NADPH oxidase-derived $O_2^{\cdot-}$ [56]. Similarly, although melatonin has been proposed to exert vascular protection by inhibiting MPO-catalyzed reactions [43], our data show that melatonin and the related indole L-Trp fail to inhibit MPO turnover or MPO NO oxidase activity under physiological conditions.

The pharmacological actions of hydroxyurea have been linked to its ability to increase NO bioavailability (with hydroxyurea administration acutely improving vasodilation in sickle cell disease patients) [35,135,136]). However, we show that hydroxyurea enhances MPO NO oxidase activity under physiological conditions. We showed that hydroxyurea also enhances HRP-catalyzed NO consumption and that MPO- or HRP-mediated oxidation of hydroxyurea does not increase NO levels as previously reported [37]. Thus, whilst it has been proposed that peroxidase-mediated oxidation of hydroxyurea may be an important mechanism of NO generation in sickle cell patients [35], our data do not support this proposal.

This study identifies key mechanistic criteria to optimise pharmacological strategies to inhibit MPO NO oxidase activity during inflammatory conditions. Our data highlight that an important and previously unrecognized limitation of certain suicide-based MPO inhibitor drugs (i.e., ABAH, isoniazid) is that their enzyme-inactivating substrate radicals can be efficiently scavenged by NO, with this antagonizing their capacity to inhibit MPO. The data further indicate that the most effective mechanism-based MPO inhibitors are those whose enzyme-inactivating substrate radicals most directly and rapidly react within the MPO active site (i.e., 2-thioxanthines), with this minimising competing off-target reactions with NO. Secondly, our studies with acetaminophen and resveratrol highlight that agents which react with MPO Compound I with very high rate constants (10^7 M^{-1} s^{-1} or more) hold promise in suppressing MPO NO oxidase activity in ascorbate-replete fluids by competitively inhibiting NO consumption by MPO Compound I and diverting the enzyme to the production of ascorbyl radicals; however efficient reduction of MPO Compound II can limit the effectiveness of these drugs. Agents that inhibit MPO NO oxidase activity by outcompeting NO for reaction with MPO Compound I will provide optimal protection if they are also poor MPO Compound II substrates, as increases in MPO turnover due to MPO Compound II reduction increase the flux of NO-consuming reactions mediated by MPO. As extracellular ascorbate can become depleted during inflammation at sites of heightened oxidative stress [86], an ideal pharmacologic inhibitor of MPO-dependent NO consumption will also yield drug-derived substrate radicals that consume NO inefficiently and hence are able to exert protection in the absence of ascorbate.

Thirdly, direct scavenging of NO-consuming substrate radicals also remains a viable therapeutic approach to suppress deleterious reactions catalyzed by MPO. Tempol and related nitroxides exhibited a unique 'co-antioxidant' action with ascorbate that was independent of their activity as MPO substrates, but dependent on their ability to scavenge and prevent NO consumption by ascorbyl radicals. However, tempol was ineffective in ascorbate-depleted systems where urate radicals are the major NO-consuming species [21]. In direct contrast, trolox significantly suppressed MPO NO oxidase in ascorbate-depleted systems by effectively scavenging tyrosyl and urate radicals, but was ineffective in ascorbate-replete systems. Agents that can react rapidly with both urate and ascorbyl radicals to form species with lower reactivity towards NO (ideally non-radical products) could have optimal activity in inflammatory microenvironments where ascorbate can be depleted from physiological fluids whilst urate remains. Whilst it is unclear why tempol and related nitroxides display poor reactivity towards urate radicals, development of nitroxides that effectively scavenge both urate and ascorbyl radicals will yield drugs with optimised activity against MPO NO oxidase activity and therapeutic potential.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

SLM, MDR and SRT contributed to study conception and design; SLM performed the experiments and analyzed the data; SLM, JCM, MDR and SRT contributed to the interpretation of the data; SLM, JCM, MDR and SRT contributed to the drafting/revision of the work for intellectual content and context. All Authors certify the originality and integrity of work and gave final approval for the work; MDR and SRT assume overall responsibility for the published work.

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