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

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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.

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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.,