Eosinophil peroxidase oxidizes isoniazid to form the active metabolite against *M. tuberculosis*, isoniazid-NAD⁺

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ABSTRACT

The formation of isonicotinyl-nicotinamide adenine dinucleotide (INH-NAD⁺) by the mycobacterial catalase-peroxidase enzyme, KatG, was known to be the major component of the mode of action of isoniazid (INH), an anti-tuberculosis drug. However, there are other enzymes that may catalyze this reaction. We have previously reported that neutrophil myeloperoxidase (MPO) is capable of metabolizing INH through the formation of INH-NAD⁺ adduct, which could be attributed to being a possible mode of action of INH. However, eosinophilic infiltration of the lungs is more pronounced and characteristic of granulomas in *Mycobacterium tuberculosis*-infected patients. Thus, the aim of the present study is to investigate the role of eosinophil peroxidase (EPO), a key eosinophil enzyme, during INH metabolism and the formation of its active metabolite, INH-NAD⁺ using purified EPO and eosinophils isolated from asthmatic donors. UV–Vis spectroscopy revealed INH oxidation by EPO led to a new product ($λ_{max} = 326$ nm) in the presence of NAD⁺. This adduct was confirmed to be INH-NAD⁺ using LC-MS analysis where the intact adduct was detected ($m/z = 769$). Furthermore, EPO catalyzed the oxidation of INH and formed several free radical intermediates as assessed by electron paramagnetic resonance (EPR) spin-trapping; a carbon-centred radical, which is considered to be the reactive metabolite that binds with NAD⁺, was found when superoxide dismutase was included in the reaction. Our findings suggest that eosinophilic EPO may also play a role in the pharmacological activity of INH through the formation of INH-NAD⁺ adduct, and supports further evidence that human cells and enzymes are capable of producing the active metabolite involved in tuberculosis treatment.

1. Introduction

Isoniazid (INH) was introduced into tuberculosis (TB) therapy in 1952 and is still the primary drug of choice for treatment of both active and latent TB [1,2]. INH (Fig. 1A) has been proposed to act as a synthetic antibiotic, which inhibits mycolic acid biosynthesis, an essential cell wall component of *Mycobacterium tuberculosis* (Mtb). The bioactivation of INH to the isonicotinyl radical (INH⁺) through peroxidation by a bacterial catalase-peroxidase enzyme, KatG, is widely accepted to be the prerequisite for INH anti-mycobacterial activity [3]. INH⁺ reacts with the oxidized form of nicotinamide adenine dinucleotide (NAD⁺) producing the INH-NAD⁺⁺ adduct. This resultant adduct blocks an essential enzyme named enoyl acyl-carrier-protein reductase inhibiting the mycolic acid biosynthesis of the Mtb cell wall [4–6]. However, the understanding of the mode of action of INH still remains incomplete.

Besides the bacterial KatG, there are numerous human peroxidases that may catalyze the formation of INH-NAD⁺⁺ adduct. The four major homologous members of human peroxidase family are myeloperoxidase (MPO), eosinophil peroxidase (EPO), lactoperoxidase (LPO), and thyroid peroxidase (TPO). The primary physiological function of all these enzymes (except TPO) is to contribute to innate immunity through the generation of oxidants and reactive oxygen species; these enzymes may also be involved in the metabolism of drugs and other xenobiotics [7]. Our group has recently shown that INH can be bioactivated to form the INH-NAD⁺⁺ adduct via neutrophil MPO [8]. However, the presence of eosinophils in lung granulomas is more typical of...
2. Materials & Methods

2.1. Chemicals and kits

Nicotinamide adenine dinucleotide free acid form (NAD⁺) was procured from Santa Cruz Biotechnology, Inc. (Dallas, TX). Human purified neutrophil MPO (180–220 units per mg lyophilized protein) and human purified EPO (1400 units per mg lyophilized protein) were purchased from Athens Research & Technology (Athens, GA, USA) and Lee Biosciences (St. Louis, MO, USA), respectively. 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO), manufactured by Dojindo Molecular Technologies, Inc. was purchased from Cedarlane Laboratories Ltd (Burlington, ON). Amplex Red, Amplex Red hydrogen peroxide/peroxidase assay kit and resorufin were purchased from Invitrogen Co. (Carlsbad, CA). Superoxide dismutase (SOD), hydrogen peroxide (H₂O₂), INH, and all other chemicals (unless otherwise noted) were purchased from Sigma-Aldrich Canada Co (Oakville, ON).

2.2. UV-Vis analysis for covalent adduct formation and other interactions

To study the reaction between INH, EPO, and NAD⁺, 500 μM NAD⁺ was mixed with 500 μM INH, 1 μM EPO, and 500 μM H₂O₂ for 30 min and the UV-Vis kinetic spectrum was analyzed. The absorbance at specific adduct peak (λ = 326 nm) was also monitored. In all experiments, Chelex-100-treated 0.1 M sodium phosphate buffer (pH 7.4) containing 100 μM DTPA was used.

2.3. Isolation of eosinophils from asthmatic donors

(a) Donor recruitment

Donors used for the study were healthy, nonatopic individuals with elevated eosinophils, or individuals with atopy that associated with self-reported allergic asthma, eczema, or allergic rhinitis. Recruitment of donors took place at the University of Alberta campus from poster and student newsletter advertising calling for donors with asthma and/or allergies that were not taking oral or inhaled corticosteroids. Voluntary donors had > 3–5% eosinophilia and were added to our established blood donor program, as approved by the University of Alberta's Human Research Ethics Office.

(b) Human peripheral eosinophil purification

Human peripheral blood was collected from donors using EDTA-coated blood collection tubes. Eosinophils were isolated and purified by negative immunomagnetic separation following the manufacturer's protocol for MACSxpress Human Eosinophil Isolation kits (Miltenyi Biotec, Auburn, CA) [10]. Eosinophil-rich supernatants were collected and serum-free RPMI-1640 ( Gibco Life Technologies) cell media was added for washing and resuspension of cells. Isolated eosinophils were diluted in serum-free RPMI to a final concentration of 1 × 10⁶ eosinophils per mL. The purity of eosinophils following immunomagnetic separation was 70–90%, and eosinophil viability > 99% as determined by trypan blue exclusion.

2.4. INH-NAD⁺ extraction and LC-MS analysis

For INH-NAD⁺ adduct identification, freshly isolated eosinophils from an asthmatic patient’s blood (see above) were diluted in serum-free RPMI to a final concentration of 1 × 10⁶ eosinophils per mL in a micro test tube and exposed to 500 μM of INH and 500 μM of NAD⁺. 5 μM of platelet-activating factor (PAF) and 0.8 μM of phorbol 12-myristate 13-acetate (PMA) were added subsequently for eosinophil activation, and the tube was incubated at 37 °C for 1 h on a ThermoMixer R (Eppendorf). After 1 h, the reaction content was centrifuged at 300 g for 5 min at RT, and the supernatant was passed through an Oasis® HLB 1 cc extraction cartridge followed by washing with deionized water. The final eluent was extracted using methanol. LC-MS was performed on an Agilent 1200 UHPLC with an Agilent 6130 single quadrupole mass spectrometer equipped with an electrospray source. Samples were injected onto a 2.1 × 50 mm Agilent Zorbax SB-C18 column with 1.8 μm silica particles and separated using a water/acetonitrile gradient with 0.1% formic acid added as a solvent modifier at a flow rate of 0.5 mL/min. After holding at 1% acetonitrile for 0.5 min, the gradient is ramped linearly to 60% acetonitrile in 5 min. Column eluent is first monitored by an Agilent G4212B diode array detector, monitoring the UV signal at 326 nm followed by the mass spectrometer giving a small delay between the UV and MS signals of 0.02 min. The mass spectrometer is run in both positive and negative ion modes, switching modes continuously between scans. Exact mass LC-MS was performed on an Agilent 6220 time-of-flight mass spectrometer equipped with an ESI source to provide compound formulae in positive ion mode.

2.5. Electron paramagnetic resonance (EPR) spin trapping and characterization

The free radical species were detected by spin trapping, where the free radical species covalently bind to the nitroene spin trap (DMPO) to produce a relatively stable paramagnetic adduct. Reactions were prepared by adding a final concentration of 2 mM of INH and 100 mM DMPO in a 200 μL volume of Chelex-100-treated 0.1 M sodium phosphate buffer (pH 7.4) containing 100 μM DTPA to a micro test tube containing either 0.1 μM EPO, or 1 × 10⁵ eosinophils (freshly isolated from an asthmatic patient’s blood – see above). Reactions were initiated as follows: 100 μM of H₂O₂ was used in reactions with EPO, whereas 15 min incubation with 5 μM of PAF and 0.8 μM of PMA were used for eosinophil activation. SOD (2.5 μM) was used to dismutate superoxide radical rapidly. 2 mM of NAD⁺ was used in reactions to study trapping of free radicals from INH. Reactions were briefly vortexed prior to transferring to a Suprasil quartz ESR flat cell (Bruker Canada, Milton, ON) for spectrum recording. EPR spectra were obtained with a Bruker Elexsys E 9500 spectrometer (Billericia, MA) equipped with an ER 4122 SHQ cavity operating at 9.78 GHz and 100 kHz modulation field at room temperature with the following parameters: power = 20 mW, scan rate = 0.47 G/s, modulation amplitude = 1 G, and receiver gain = 6.32 × 10⁶. Spectra were recorded as a single scan.

2.6. Statistical analysis

All experiments were carried out at least three times (n ≥ 3) using freshly prepared reagents.

3. Results

3.1. UV-Vis study for INH-NAD⁺ adduct formation by MPO and EPO

In order to confirm that INH-NAD⁺ adduct formation would occur by EPO as we have previously reported for MPO [8], we carried out UV-Vis spectroscopy. In Fig. 1B, we observed the characteristic absorption maximum for INH-NAD⁺ (λ = 326 nm) after 30 min when INH, EPO, and NAD⁺ were incubated in Chelex-100-treated 0.1 M sodium phosphate buffer (pH 7.4) containing 100 μM DTPA. The peak was not as prominent compared to MPO [8] because less EPO was used (on a unit/mg basis) than MPO.
3.2. Identification of INH-NAD⁺ adduct formation catalyzed by EPO

High resolution (HR) LC-MS was used to characterize the UV-Vis peak reported in section 3.1 (Fig. 1B). HR LC-MS was used to confirm that the 326 nm peak observed in the UV-Vis spectrum was the INH-NAD⁺ adduct. In Fig. 2, the absorption intensity at this wavelength was shown. The peak for INH eluted early (1.8 min), but the peak(s) of interest occurred between 8 and 10 min. Further analysis demonstrated that the peak with retention time of 8.8 min was the INH-NAD⁺ adduct. Interestingly, two different m/z were found for the same structure since it occurred in two different forms (see Fig. 2C–D, F–G). The proposed structures of the INH-NAD⁺ fragments are shown in Fig. 2H.

3.3. EPR studies for INH in human isolated EPO system

In order to identify the possible INH intermediate produced from EPO (and likely involved in forming a covalent adduct with NAD⁺), we carried out EPR spectrometry using spin trapping (using DMPO). The spectra were acquired after mixing the reactants and transferring to a flat cell for acquisition. The spectrum from the mixture of DMPO, EPO, and INH, produced a 4-line signal (Fig. 3A), which we have observed using MPO [8]. When H₂O₂ was added to initiate the peroxidase cycle of EPO, a composite spectrum was produced where multiple free radicals were trapped (Fig. 3B) as we and others have previously observed with MPO [8,11] or horseradish peroxidase [12]. When SOD was included in the reaction, the spectrum produced contained two detectable species. (The simplification of the spectrum was due to the removal of superoxide by SOD). Fig. 3C was then simulated using Winsim 2002 (Public EPR Software Tools, NIEHS/NIH), based on the assumption that two species were formed (i.e., two different free radicals were trapped).

The larger species (square symbols, area = 72%) had an = 15.1 G, aH = 14.9 G, which we assigned to DMPO/·OH (hydroxyl radical). The smaller species (asterisks, area = 28%) had an = 15.0 G, aH = 21.7 G, which correlates to a DMPO/·C (carbon radical). The simulation was well-correlated (r = 0.96).

3.4. EPR studies for INH in human primary eosinophils

In order to extend the isolated EPO studies, further EPR spectrometry studies were carried out with eosinophils from asthmatic donors. Reactions were analyzed after 15 min incubation at 37 °C and all included DMPO. Eosinophils incubated with 100 mM DMPO did not exhibit any notable free radical trapping (Fig. 4A); however, the incubation of INH with eosinophils produced an intense 4-line spectrum (Fig. 4B). Activation of eosinophils (respiratory burst and EPO release) was facilitated by using PMA and PAF. The latter produced an intense DMPO/·O₂⁻ detection (Fig. 4C). The inclusion of INH with PAF and PMA (Fig. 4D) did not produce a spectrum that we could discern from Fig. 4C. This is likely due to the overwhelming DMPO/·O₂⁻ spectrum.

When SOD was included in the reaction, the spectrum was simplified considerably (due to superoxide not being trapped, but being converted rapidly to H₂O₂). The resulting spectrum in Fig. 4E resembled the spectrum found from isolated EPO (Fig. 3C), and we scaled up the spectrum to highlight the peaks (species) of interest.

4. Discussion

In the current study, using purified EPO and eosinophils isolated from asthmatic donors, we identified the involvement of EPO in the bioactivation of INH with the formation of INH-NAD⁺ adduct. INH oxidation by EPO led to a new product in the presence of NAD⁺. This adduct was confirmed to be isonicotinyl-NAD⁺ (INH-NAD⁺) using LC-MS analysis where the intact adduct was detected (m/z = 769). It is noteworthy to mention that two separate LC peaks corresponding to two different forms were found for the same structure of INH-NAD⁺ adduct, which could be due to the formation of cyclized diastereomers in solution [13–15]. This study highlighted that the activated eosinophils are able to form INH-NAD⁺, which is considered an essential step in anti-Mtb activity.

We found that isolated EPO formed INH-derived free radicals. The most intense radical, based on the area for the peaks in the EPR spectrum, was for DMPO/·OH (hydroxyl radical). This is similar to the effect of MPO, another peroxidase enzyme in neutrophils, which also resulted in the formation of DMPO/·OH with INH [8]. The mechanism for the formation of a hydroxyl radical has been reported previously based on benzoate hydroxylation [16]. Hydroxyl radicals are the most reactive radicals formed in biological systems, but the flux of hydroxyl radicals must be low in this case with INH. This is based on a previous report of INH-treated cells, which contain high amounts of MPO, but do not demonstrate cytotoxicity at concentrations higher than 10 mM [17]. We also found superoxide formation in this reaction; this is reasonable since inhibition of MPO by INH could form this species [18]. The mechanism of INH-NAD⁺ covalent adduct formation most likely proceeded by a radical addition mechanism, where the isonicotinyl radical formed from EPO peroxidase activity adds to NAD⁺. A radical addition reaction is plausible; by analogy, diphenylelenediammonium, the commonly used NADPH oxidase inhibitor, forms a covalent adduct with flavin adenine dinucleotide after the former is converted to a radical species [19]. In our studies, we found that a six-line DMPO adduct was formed from isolated EPO and eosinophils, which we assigned to be a carbon-centred radical. For INH, the carbon-centred radical is likely the carbonyl carbon.

Though it has been reported that neutrophils are the predominant phagocyte in the lungs of Mtb-infected individuals [20], the role of eosinophils is also essential. It was recently reported that both neutrophil and eosinophil plasma proteins are increased in Mtb infection. However, neutrophil activation may contribute to pathology because of overactivation; eosinophils don’t appear to contribute to pathology, but important enzymes including EPO were detected in infected individuals [21]. Eosinophil infiltration of the lungs and eosinophilia has been.
associated with *Mtb* infection [22,23]. Indeed, EPO has been shown to catalyze killing of *Mtb* H37Rv with a similar potency as MPO. However, EPO did not require H$_2$O$_2$ as a cofactor [24]. This suggests that the collateral damage that occurs from neutrophil activation may be absent with eosinophils during infection. It is noteworthy to mention that *Mtb* can synthesize NAD$^+$ using the *de novo* biosynthesis pathway or the salvage pathway. On the other hand, as eosinophilic infiltration of the lungs and recruitment of neutrophils are more pronounced at the site of TB infection, NAD$^+$ may leak to the extracellular environment in situations leading to chronic airway inflammation [9,25]. Thus, the availability of NAD$^+$ in the extracellular infectious milieu still could facilitate the reaction of NAD$^+$ with INH free radical to form an INH-NAD$^+$ adduct. Taken together, it is possible that the interaction of INH with EPO can occur and lead to activate INH to form the active metabolite, INH-NAD$^+$. Recently, a characteristic metabolite, 4-isonicotinoylnicotinamide (4-INN), was observed in urine samples from both TB patients and healthy mice treated with INH which was proposed to be derived from the hydrolysis of INH-NAD$^+$ adduct.

Fig. 2. HR LC-UV-MS study of INH-NAD$^+$ adduct formation by EPO. In (A), the UV absorption intensity at $\lambda = 326$ nm is shown; the EIC for the INH-NAD$^+$ structure is shown in (B) and (C). The EIC for molecules of interest are shown in (C) for m/z 767 and (D) for m/z 769. The ions detected at specific retention times were also shown for confirmation, where at retention time = 1.81 min, isoniazid was detected (E), at 8.82 min INH-NAD$^+$ was detected (F), and at 8.90 min INH-NADH was detected (G), respectively. The formation and structures of the adducts are shown in (H). Experimental details are described in Materials & Methods.
Moreover, the INH-NAD\(^+\)-derived 4-INN adduct has been detected in *Mtb* culture-negative TB patients after 8 weeks of anti-TB therapy, further indicating that human metabolic pathways can be involved in drug activation [15]. This seminal study involving the urine collected from TB patients showed a direct evidence of INH activation by both the pathogen and the host, and clearly implies that the bioactivation of INH can still occur in the absence of *Mtb* infection in a KatG-independent manner suggesting a non-*Mtb* mechanism, perhaps via the contribution of host immune cells. This suggests that upon INH treatment, host immune cells may use an alternative pathway to kill *Mtb* through the production of the postulated anti-bacterial component, INH-NAD\(^+\) adduct. Interestingly, lactoperoxidase, another mammalian peroxidase, has been previously proposed to be involved in INH activation \[26,27\]. Thus, observations in the present study along with the previous data \[8,15\] indicate that in addition to the effect of bacterial KatG, the human peroxidase enzymes (like MPO and EPO) present in the immune cells hold the potential to contribute to host INH activation.

In summary, we have shown in this study that isolated or cellular EPO was able to catalyze the oxidation of INH to its anti-*Mtb* active metabolite. Further studies with eosinophils, INH, and *Mtb* should be carried out to determine the effectiveness of EPO in enhancing the anti-*Mtb* activity of INH.

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