Cytoprotective effect of isoniazid against H₂O₂ derived injury in HL-60 cells

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A R T I C L E   I N F O

Article history:
Received 23 September 2015
Received in revised form
20 November 2015
Accepted 23 November 2015
Available online 30 November 2015

Keywords:
Isoniazid
Cytoprotection
Myeloperoxidase
Covalent binding
HL-60 cells
Hydrogen peroxide

A B S T R A C T

To combat tuberculosis (TB), host phagocytic cells need to survive against self-generating oxidative stress-induced necrosis. However, the effect of isoniazid (INH) in protecting cells from oxidative stress-induced necrosis has not been previously investigated. In this in vitro study, the cytotoxic effect of H₂O₂ generation using glucose oxidase (a model of oxidative stress) was found to be abrogated by INH in a concentration-dependent manner in HL-60 cells (a human promyelocytic leukemia cell). In cells treated with glucose oxidase, both ATP and mitochondrial membrane potential were found to be decreased. However, treatment with INH demonstrated small but significant attenuation in decreasing ATP levels, and complete reversal for the decrease in mitochondrial membrane potential. Quantitative proteomics analysis identified up-regulation of 15 proteins and down-regulation of 14 proteins which all together suggest that these proteomic changes signal for increasing cellular replication, structural integrity, ATP synthesis, and inhibiting cell death. In addition, studies demonstrated that myeloperoxidase (MPO) was involved in catalyzing INH-protein adduct formation. Unexpectedly, these covalent protein adducts were correlated with INH-induced cytoprotection in HL-60 cells. Further studies are needed to determine whether the INH-protein adducts were causative in the mechanism of cytoprotection.

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1. Introduction

Isoniazid (INH), a synthetic antibiotic, was first used against active Mycobacterium tuberculosis (Mtb) infection in 1952, and was later recommended as a monotherapy in prevention and treatment of latent tuberculosis (TB) in 1967. Currently, it is a first line drug of choice in both active and latent TB [1]. However, INH is also known for idiosyncratic adverse drug reactions resulting in toxicity such as hepatotoxicity (approximately 5% patients) and autoimmune toxicity, e.g., systemic lupus erythematosus and few case reports of agranulocytosis. Although the mechanisms of these idiosyncratic drug reactions are not fully elucidated, it is suspected that INH reactive metabolites and the adaptive immune responses are involved (http://livertox.nih.gov/Isoniazid.htm). Interestingly, the pharmacological mechanism of action of INH also appears to involve reactive metabolites.

The postulated bactericidal action of INH, based on in vitro studies, is through enzymatic oxidation of INH by Mtb catalase-peroxidase (KatG) to generate a proposed isonicotinoyl acyl radical metabolite; the latter rapidly reacts with reduced form of nicotinamide adenine dinucleotide (NAD⁻) forming an isonicotinyl acyl-NAD (INH-NAD⁺) adduct, which is a potent inhibitor of mycolic acid biosynthesis (inhibits enoyl acyl-carrier-protein reductase) preventing Mtb cell wall formation [2,3]. However, this proposed mechanism of action has yet to be proven in either animal models or human [3].

Recent studies of Mtb pathophysiology reveal that macrophage-dominant phagocytic cells are the host’s first-line immune defense against Mtb. After entering into lung airways, Mtb is engulfed by resident alveolar macrophages, and form granuloma by recruiting other immune cells. However, Mtb that escape the granuloma enter...
into the bloodstream and will encounter neutrophils as the dominant phagocytic cell [4,5]. Neutrophils express myeloperoxidase (MPO), and monocytes can be induced to express MPO during infection [6]. MPO is an enzyme that shares some catalytic resemblance to *Mtb* catalase-peroxidase (KatG) to produce reactive metabolites (through the peroxidation cycle) [7], but additionally generates hypochlorous acid (HOCl, through the chlorination cycle) [8].

For pathogen killing during phagocytosis, macrophages mainly rely on their reactive nitrogen species (RNS) production [9], whereas neutrophils rely on reactive oxygen species (ROS) production [10]. Each type of phagocytic cell usually can produce nanomolar (nM) amounts of their respective reactive species (RNS/ROS). If the bacterial load is high, the surrounding activated macrophages and CD4+ T cells (within granuloma) release extrinsic death signals (TNF-α, IFN-γ) to initiate apoptosis, leading to successful eradication of *Mtb* [11]. However, *Mtb* has extra-ordinary survival strategies to escape ROS/RNS mediated killing and can induce necrosis of phagocytic cells [12]. *Mtb* possesses very high tolerance for ROS (up to 50 mM H2O2) whereas tolerance against RNS is relatively lower (few mM to 5 mM of reactive nitrogen species is bacteriostatic; above 5 mM is bactericidal) [13].

From extensive studies on TB pathophysiology, it is well-known that virulent *Mtb* strains induce necrosis in infected phagocytic cells via mitochondrial damage due to excessive reactive species production by phagocytic cells themselves [14]. To combat *Mtb*, phagocytic cells need to survive first by preventing the oxidative stress-induced self-destruction (oxidative necrosis). However, the effect of INH against oxidative necrosis has never been investigated. Moreover, if INH is cytoprotective against oxidative necrosis, this phenomenon can justify another mechanism for its effectiveness against TB. Therefore, we hypothesize that INH increases oxidative stress tolerance of immune cells. In this study, we performed *in vitro* experiments by using human promyelocytic leukemia (HL-60) cells which have a high content of MPO and have potential to differentiate into various phagocytic cells such as neutrophils, monocytes and macrophages [15]. As *Mtb* has been described to withstand oxidative or nitrosative injury, we therefore used a surrogate system to produce redox imbalance in HL-60 cells to simulate events that phagocytic cells would be exposed to during *Mtb* infection by using glucose oxidase and glucose system (GOx), which produces a flux of H2O2 per unit time.

Studies have shown that both GOx and HOCl trigger the intrinsic pathway for cell death where mitochondrial damage is the common feature [16,17]. This mitochondrial damage is usually characterized by a loss in mitochondrial membrane potential (ΔΨm) and depletion of ATP due to disruption in the electron transport chain. Depending on the severity of mitochondrial damage, the intrinsic cell death pathway follows either apoptosis (caspase-dependent or independent) or necrosis. In general, a high burden of oxidative stress similar to that induced by *Mtb*-infected phagocytic cells can cause excessive mitochondrial damage which usually leads to necrosis. In this study, GOx was used to induce necrosis through mitochondrial damage in HL-60 cells and evaluate the effect of INH.

2. Materials & methods

2.1. Chemicals and kits

Both heavy and light lysine (13C6L-Lysine and 12C6L-Lysine) and arginine (13C6L-arginine and 12C6L-arginine) were purchased from Invitrogen (Carlsbad, CA). FITC annexin-V was purchased from BD Biosciences (San Jose, CA). CellTiter-Glo® Luminescent Cell Viability Assay kit was purchased from Promega, (Madison, WI). All chemicals unless otherwise noted were from Sigma Chemical Co (Oakville, ON).

2.2. Antibodies and enzymes

The glucose oxidase enzyme was purchased from Sigma–Aldrich Canada Co (Oakville, ON). The horseradish peroxidase (HRP) enzyme was purchased from Amresco LLC (Solon, OH). The rabbit polyclonal anti-INH was generously donated by Dr. Uetrecht (University of Toronto). The rabbit anti-GAPDH was purchased from Santa Cruz biotechnology, Inc. Horseradish peroxidase (HRP) labeled goat anti-rabbit IgG secondary was purchased from Thermo scientific (USA).

2.3. HL-60 cells

HL-60 cells were obtained from ATCC (Cat No. CCL-240, Manassas, VA). The cells were grown in media containing RPMI-1640 medium (Gibco® Reference No. 11875-093), 10% fetal bovine serum (FBS) (Gibco® Cat No. 12483) and 1% of Antibiotic-Antimycotic (Gibco® Reference No. 15240-062). Cells were maintained in an atmosphere with 5% CO2 at 37 °C with media change occurring every 2 days. All HL-60 cells used during experiments had a passage number less than 30.

2.4. Trypan blue exclusion cytotoxicity assay

HL-60 cells were either pre-treated for 4 h with different concentrations (1 μM–10 mM) of INH and exposed to 5 mM glucose and 25 mM/L glucose oxidase (GOx) for 1 h and 3 h, or co-exposed with both different concentrations (1 μM–10 mM) of INH and GOx for 1 h and 3 h in 96-well plates at 1 × 106 cells/ml (>95% viability) at 5% CO2 and 37 °C. 5 mM glucose was always used wherever GOx is indicated. At the end of the experiment, a cell sample from each reaction and 0.4% trypan blue reagent (Lonza, Anaheim, CA) were mixed at a 1:1 ratio and the cell viability was measured by using a TC-10 automated cell counter (Bio-Rad Laboratories). Data was expressed as means ± SD.

2.5. Flow cytometry

We carried out a standard protocol which was adapted for our experimental settings [18]. Briefly, flow cytometry was performed on HL-60 cells (1 × 106/mL, 0.5 mL) pre-treated with varying concentrations (1 μM–1 mM) of INH for 4 h followed by GOx as described for trypan blue exclusion assays. We did not run the co-exposure experiments here. HL-60 cells were treated with GOx for 1 h (as a positive control for cell death) or HL-60 cells were untreated (used as a negative control) for annexin-V and PI staining (i.e. cells only treated with one and not the other). Following the reactions, cells were washed with 1 annexin-V binding buffer [0.01 M HEPES (pH 7.4), 0.14 M NaCl, and 2.5 mM CaCl2] twice at RT. Binding buffer (100 μL), 5 μL of FITC annexin-V and 10 μL of 50 μg/mL of PI were added to the cell pellet and resuspended. The samples were incubated at room temperature in the dark with constant shaking at 500 rpm for 15 min before being diluted with 400 μL binding buffer and subsequently analyzed. Fluorescence was induced with an argon laser and detected on FL1 (525 nm BP filter) and FL3 (620 nm LP filter) on a Beckman Coulter Quanta SC flow cytometer. A total of 10,000 events were collected per sample. Compensation was performed using Cell Lab Quanta analysis software to account for fluorophore spectral overlap; data was expressed as means ± SD.
2.6. MPO activity assay

A spectrophotometric assay of MPO activity was used in which guaiacol oxidation was measured by changes of absorbance at 470 nm (ε = 26.6 mM$^{-1}$ cm$^{-1}$ [33]). 5 mM of MPO (dissolved in Millipore water) was placed in each well of clear 95-well plate and different concentrations (1 μM to 1 mM) of either INH or ABAH (4-Aminobenzoic acid hydrazide) were added. Afterwards, 10 mM guaiacol was added into each well. The reactions were started by adding 500 μM of H$_2$O$_2$ into each well. Activity of MPO was calculated in nmol tetraguaiacol formed/min/5 nM of MPO. The data were acquired using SpectraMax M5.

2.6.1. H$_2$O$_2$ flux by GOx

The flux of H$_2$O$_2$ per unit time generated by GOx in this study has been measured as described elsewhere [19]. In brief, HRP was used to oxidize phenol red in the presence of various concentrations (1-60 μM) of H$_2$O$_2$. The reactions with H$_2$O$_2$ were carried out for 5 min, and the color change of oxidized phenol red was stabilized at pH 12.5 by adding 11.42 mM of sodium hydroxide. This produced a stable purple-mauve colored product. The absorbance was then measured at 610 nm followed by construction of a linear calibration curve. Afterwards, GOx was tested by replacing H$_2$O$_2$ in the same reaction over a 1 h period with readings at every 15 min (4 time points) to calculate the rate of H$_2$O$_2$ production over 1 h.

2.7. SILAC cell culture

HL-60 cells, were purchased from ATCC (Manassas, VA), were cultured in both heavy and light media. Firstly, the RPMI-1640 medium without l-lysine, l-arginine and l-leucine was purchased from Sigma Chemical Co. We added l-leucine to the RPMI-1640 medium as 0.05 mg/ml. 10% dialyzed fetal bovine serum (FBS), obtained from Invitrogen, was also added to RPMI-1640 medium. Afterward, the RPMI-1640 was divided equally into two parts to make either heavy or light media. For heavy medium, heavy l-lysine and l-arginine (13C$_6$-l-Lysine and 13C$_6$-l-Arginine) were added to make a final concentration of 0.04 mg/mL and 0.2 mg/mL respectively into one part of RPMI-1640 medium. To make light medium, we added unlabeled l-lysine and l-arginine in as same concentrations as in heavy medium into other part of RPMI-1640. Cells were then maintained in both heavy and light media separately under a humidified atmosphere with 5% CO$_2$ at 37 °C. Medium renewal was every 2–3 days depending on cell density. The HL-60 cells were cultured in both heavy and light media separately for at least 7 passages to achieve complete isotope incorporation [20].

2.8. SILAC cell treatment and lysis

Heavy and light cells were treated with GOx (control) or both GOx and 2.5 mM INH (treatment) for 4 h in an incubator with 5% CO$_2$ at 37 °C. The reactions were as follows: Sample A – the forward labeled sample was the mixture of control “GOx treated HL-60 cells treated, cultured in the light media” and the treatment “GOx and INH treated HL-60 cells, cultured in the heavy (containing 13C$_6$-l-Lysine and 13C$_6$-l-Arginine) media”. Sample B – the reverse labeled sample was the same as sample A but the labeling was reversed. The control and treatment of both sample A (forward labeled sample) and sample B (reverse labeled sample) were lysed separately by using RIPA (0.05 g sodium deoxycholate, 100 μl of Triton X-100, and 0.01 g of SDS in 10 ml of PBS). For preparing either sample A or B, the protein content in control and treatment have to be in a 1:1 ratio. This was achieved by SDS-PAGE of the samples, followed by staining the gel with Coomassie blue, destaining and scanning the gel using LI-COR Odyssey gel scanner. The intensity of protein content in each lane provided a quantitative check the ratio of protein between control and treatment of each sample.

2.9. SDS-PAGE & gel-digestion

2 × loading buffer (0.5 M Tris–HCl pH 6.8, 10% SDS, 1.5% Bromophenol Blue, 20% Glycerol and 5% β-mercaptoethanol) was mixed with cell lysates and the heavy and light labeled samples were combined in a 1:1 ratio. The samples were then heated to 95 °C for 5 min and then resolved on a 1.0 mm thick 10% polyacrylamide gel. After electrophoresis, the SDS gel was stained with Coomassie Brilliant Blue and destained using 10% acetic acid solution to visualize the bands and lanes. The entire gel lanes were excised into 12 equal pieces for in-gel digestion.

2.10. In-gel digestion and LC-MS/MS analysis

In-gel digestion and LC-MS/MS analysis were carried out according to an in-house standard protocol which was described in one of our recently published paper [18]. A total of 1459 and 1712 proteins were identified in sample A and B, respectively. Proteins those were reproducibly identified in both samples (390 proteins) were taken for further analysis. The expression-changes were determined from the ratios of protein abundance followed by log2 calculation. The proteins which were also reproducibly either down- or up-regulated by 0.5 on a log2 scale (over 40% changes in abundance) in both samples were reported here.

2.11. Relative cellular ATP analysis

Cellular ATP levels were determined by using CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, WI, USA) as per their protocol. In brief, CellTiter-Glo® reagent was prepared by mixing substrate and buffer (which was thawed by storing at room temperature 24 h). Run all the reactions in PBS as same as before. 100 μl (approximately 5 × 10$^6$ cells) of each reaction with three replica were placed into each well of opaque-walled 96-well plate. 100 μl of freshly prepared CellTiter-Glo® reagent was added to each well and shake the plate for 2 min on a shaker. After 10 min of incubation at RT, luminescent signals were recorded using a plate reader (SpectraMax M5).

2.11.1. Mitochondrial membrane potential change (ΔΨm) analysis

The mitochondrial membrane potential was measured by using JC-1 dye according to a previous protocol [21]. In brief, HL-60 cells were suspended in 1.5 ml micro test tubes at a density of 0.5 × 10$^6$ cells/ml of serum free RPMI 1640 media and the reactions were prepared as described before. After completion of reactions, cells were centrifuged at 500 g to discard the supernatant. The same volume of PBS containing 0.3 μM of JC-1 was added to the cells, which were resuspended, and incubated for 30 min at 37 °C. The sample was then washed twice at 200 g for 5 min to discard the unincorporated free dye. The cells were resuspended in PBS and incubated at 37 °C again for 20 min, and 300 μl of cell suspension were added to a black 96-well polystyrene microplate. The fluorescence intensity was measured as ratio of red aggregates/green monomers. For red aggregates, the parameter setting of the plate reader (SpectraMax M5) was fixed at the excitation 490 nm and emission 595 nm whereas green monomers were measured by changing only the emission to 535 nm. The ratio of red aggregates/green monomers was then calculated. Known mitochondrial toxins 5 μM of antimycin A and 50 μM of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were used as positive controls for the assay [22].
2.12. SDS-PAGE and anti-INH immunoblots

The amount of protein in the lysed samples was determined by the BCA assay and equal amounts of lysate from each reaction were loaded onto an SDS-PAGE (1.0 mm 10% polyacrylamide gel). Before loading, lysates were reduced by 2× loading buffer (0.5 M Tris–HCl pH 6.8, 10% SDS, 1.5% Bromophenol Blue, 20% Glycerol and 5% β-mercaptoethanol), and proteins were denatured for 5 min at 95 °C with shaking at 600 rpm following by cooling to room temperature. After completion of gel running, the proteins were transferred from the gel onto a nitrocellulose membrane which was blocked overnight at 4 °C with 5% bovine serum albumin (BSA) in washing buffer (TBS-T). The membrane was then treated with a rabbit anti-INH (1:1000) or rabbit anti-GAPDH (1:1000) for 1 h at room temperature (RT) with constant shaking. Afterwards, the membrane was washed and treated with goat anti-rabbit HRP-conjugated secondary antibody (1:5000 in TBS-T) for 1 h at RT. The membrane was again washed and treated with chemiluminescence HRP substrate (Millipore Corp., Cat No. WBKLS0500) reagent for 2 min and the signals were measured by using luminescent image analyzer (GE ImageQuant LAS 4000 mini).

2.13. Statistics

Data were expressed as mean ± standard deviation of mean (SD) of separate experiments (n ≥ 5) performed on separate days using freshly prepared reagents. Statistical significance was performed by a one-way pairwise multiple comparison ANOVA followed by Holm-Sidak post-hoc analysis using SigmaPlot 11.0 software.

3. Results

3.1. Cytoprotective effects of INH

In this study, HL-60 cells which were treated with GOx (glucose/glucose oxidase as described in Materials & Methods) for 3 h were either pre-incubated for 4 h or co-exposed with different concentrations of INH (1 μM–1 mM). GOx was added to the reactions to provide continuous production of H2O2. The rate of H2O2 production by GOx was calculated as 2.928 ± 0.072 μmol U−1 ml−1 h−1 (see Materials & Methods). One hour and 3 h after adding GOx, cell viability was determined by trypan blue exclusion. The trypan blue exclusion assay results (Fig. 1) showed that GOx significantly decreased the cell viability (φ p < 0.001), and both INH pre-incubation and co-exposure showed concentration dependent cytoprotection. INH co-exposure with GOx (Fig. 1a) showed significant INH cytoprotection from 20 μM after 1 h (*P < 0.001) and 50 μM INH after 3 h (*P < 0.001). However, INH pre-incubation for 4 h prior to adding GOx (Fig. 1b) showed significant cytoprotection from 1 μM (*P < 0.001) at both time points. Therefore, INH pre-incubation was found to be more cytoprotective than INH co-exposure at micro molar concentrations of INH (up to 100 μM). However, there was no significant difference between pretreatment or cotreatment of INH when concentrations more than 100 μM were used.

3.2. Isoniazid attenuates necrosis/late apoptosis induced by GOx

A representative dot plot of the cell population shown in Fig. 2a illustrates a large proportion of cells in Q2 (PI+ and annexin-V+) when treated with GOx; this was attenuated with a 4 h pre-incubation of 20 μM INH (Fig. 2b). INH showed a concentration-dependent cytoprotective effect against H2O2 (GOx) from 1 μM based on cell population in Q3 (PI– and annexin-V–; φ P < 0.005) (Fig. 2c). The cytoprotection of INH was statistically more significant from 10 μM to 1 mM (*P < 0.001) (Fig. 2c). The flow cytometry analysis also confirmed the type of cytoprotection. GOx induced mainly late apoptosis/necrotic-like cell death (PI+, annexin-V+) (Fig. 2e) since cells in the early apoptosis phase (PI–, annexin-V+) were not detected with this treatment (Fig. 2d). However, INH cytoprotection against GOx was prominent against necrosis/late apoptosis (Fig. 2e) in a concentration dependent manner. The cytoprotective capacity of INH was detected from 1 μM (φ P < 0.005) and was enhanced with 10 μM to 1 mM of INH (*P < 0.001). The statistical analysis also showed 100 μM and 1 mM of INH were significantly more protective against oxidative stress in comparison to 1 μM of INH.

3.3. INH is a relatively poor MPO inhibitor

To assay the effect of INH on MPO activity, we used four different concentrations (1 μM, 10 μM, 100 μM and 1 mM) of INH or the well-known peroxidase inhibitor, 4-aminobenzoic acid hydrazide (ABAH), as a positive control for MPO inhibition. The IC50 of INH was found 253.7 ± 7.83 μM. Our results for INH inhibition were similar to those published by Huang et al., which reported INH IC50 = 277.10 ± 6.65 μM [23]. Forbes et al. compared the loss in specific MPO activity of MPO inhibited by INH and reported a 30% loss in MPO specific activity for the former; however, ABAH completely inactivated MPO [7]. Collectively, these findings indicate that INH is a relatively poor inhibitor of MPO.

3.4. INH protein addsucts and role of MPO

HL-60 cells pretreated with INH (1 μM–1 mM) for 4 h were exposed to GOx for an additional 1 h after which they were lysed and probed for protein bound covalent adducts using an anti-isonicotinyl antibody (anti-INH). In Fig. 3, anti-INH Western blots showed that the INH-protein covalent adducts were increased with INH concentration from 10 μM to 1 mM in GOx treated cells. However, we did not find any adducts in absence of either GOx and/or INH during this time period (Fig. 4, lane 1, 2, 7 & 8). Moreover, anti-INH Western blots showed that MPO inhibition by ABAH (lanes 5 & 6) attenuated INH-protein adduct (Fig. 4). Taken together, these findings suggest that MPO activity is a catalyst for the formation of INH-derived reactive metabolites which apparently bind with proteins to form covalent adducts.

3.5. INH induced protein expressions

The replicate SILAC analysis of the INH and GOx treated HL-60 cells in comparison to the GOx treated control cells resulted in a total of 390 proteins were reproducibly identified and quantified using highly stringent data analysis. The quantified data of the ratios of protein abundance shown in Fig. 5A reveals that the majority of proteins do not significantly change in abundance upon treatment. Only 29 proteins were identified for their reproducibly significant change in abundance either down- or up-regulated by 0.5 on a log2 scale (−over 40% changes in abundance) upon treatment. These 29 proteins were listed in Table 1. String (version 9.1) analysis revealed that both upregulated and downregulated proteins demonstrated associations through co-expression analysis (Fig. 5B and C). This suggests that these associated proteins are likely to be involved in specific signaling pathways.

In case of down-regulated proteins, the majority were ribosomal proteins (10 in total) along with some nucleosomal proteins such as RHA-binding protein FUS and three histone proteins (Histone H2A type 1-H, Histone H2B type 1-K and Histone H4). The ribosomal proteins were associated with one another (Fig. 5C) and the overall trends appeared to be downregulation of protein expression.
However, the downregulation of those nucleosomal proteins could be interpreted as either DNA damage followed by cell death or the preparatory stage for replication. In this study, it has already shown that INH is cytoprotective. Therefore, the down regulation of those nucleosomal proteins might involve in replication process. The up-regulated proteins were found to involve one of the following functions: replication process (DNA replication licensing factor MCM2, Ran-specific GTPase-activating protein and proliferating cell nuclear antigen), structural integrity maintenance activity (tubulin beta chain, tubulin alpha-1B chain, alpha-actinin-1, stathmin, actin-cytoplasmic 1, T-complex protein 1 subunit eta and Prostaglandin E synthase 3), ATP synthesis (ATP synthase subunit alpha, isoform 2 of 3-hydroxyacyl-CoA dehydrogenase type-2) and blocking of cell death signals (isoform 2 of transcription intermediary factor 1-beta and programmed cell death 6-interacting protein).

3.6. Effect of INH on ATP levels

The ATP assay was performed based on proteomic findings that ATP synthesis was upregulated in response to INH treatment. In Fig. 6a, the relative ATP levels of GOx challenged cells were significantly decreased ($\Phi P < 0.001$) within 1 h of exposure. In the INH co-exposure group, 50 $\mu$M and 1 mM of INH treated cells showed a significant increase in relative ATP levels versus GOx challenged cells ($\Phi P < 0.005$, $\Phi P < 0.05$ respectively). In the INH 4 h preincubated group, both 50 $\mu$M and 1 mM INH increased ATP level with similar significance ($* P < 0.001$). At the 3 h timepoint (Fig. 6b), only the higher concentration (1 mM) of INH preincubated for 4 h significantly increased ATP levels ($\Phi P < 0.05$).

3.7. Effect of INH on mitochondria membrane potential

The mitochondria membrane potential assay (Fig. 7) showed that GOx treatment significantly reduced the mitochondrial membrane potential compared to untreated cells ($\Phi P < 0.001$). Positive controls for decreasing the mitochondria membrane potential reveal that HL-60 cells produced expected responses toward mitochondrial poisons such as the complex-III inhibitor, antimycin A, or the uncoupler, CCCP. Interestingly, INH significantly attenuated the drop in mitochondrial membrane potential of GOx challenged HL-60 cells ($* P < 0.001$) from 1 $\mu$M. This capacity of INH was also found to be significantly greater ($* P < 0.001$) for 100 $\mu$M and 1 mM INH compared with 1 and 10 $\mu$M INH.
4. Discussion

In this study, we have reported the cytoprotective effect of INH in HL-60 cells challenged with H₂O₂ (produced from GOx); this effect was observed with a concentration as low as 1 μM in 4 h preincubation group while relatively higher concentrations (20–50 μM) of INH were needed for the co-exposed group. The cytoprotective effect in both groups was in concentration dependent manner. The predominant mechanism of cell death induced by GOx treatment was late apoptosis/necrosis. This study was not able to examine the INH-induced cytoprotection capacity against early apoptosis induced by GOx, since this form of cell death was not observed for the conditions used in this study.

The involvement of mitochondria was investigated through both the relative ATP analysis and mitochondrial membrane potential assays. Both ATP and mitochondrial membrane potential play important roles in the intrinsic pathway of both apoptosis and necrosis. In this study, the increase in ATP by INH was minor. As HL-60 cells are cancer cells, their ATP is derived mostly from glycolysis; as such, the role of mitochondrial ATP is expected to be minor in relation to the mechanism of cell death; however mitochondrial membrane potential appeared to be a major factor in cell death. In this study, the effect of INH on mitochondrial membrane potential was quite marked. Therefore, INH effects on mitochondria played an important role to counteract H₂O₂/HOCI injury in HL-60 cells.

Several studies have demonstrated a different effect of INH on mitochondria [24]. It was shown that mitochondrial dysfunction induced through mitochondrial complex I and II poisoning was enhanced in the presence of INH and proposed that mitochondrial dysfunction was an underlying cause of INH-induced toxicity [24]. However, the effect of INH against mitochondrial poison-induced dysfunction does not appear related with the effect of INH against ROS-induced mitochondrial damage in our study. It is possible that the cell type can dictate the response caused by INH since we used HL-60 cells, but the aforementioned studies were carried out in mouse hepatocytes.

The role of MPO in cytoprotection/cytotoxicity was also explored in this study. MPO is a peroxidase enzyme found abundantly in HL-60 cells. In the presence of H₂O₂, resting or native MPO (Fe⁴⁺) is oxidized into its active form, compound I (Fe⁵⁺), which can be either reduced back to its resting form (chlorination cycle) or form compound II (Fe⁶⁺) through one electron redox reaction where it produces free radical metabolite of favorable 1-electron donor substrates (peroxidase cycle). Compound II can go through another step of 1 electron reduction back to its native form by generating more free radical metabolites [23,25,26].

These free radical species/metabolites may have deleterious effects [27], but this depends on the specific donor substrate. Many xenobiotics and electrophilic (non-radical) metabolites can form covalent protein adducts with electron dense regions of proteins through substitution or addition reactions [28]. Covalent adducts which interfere with biological pathways by disrupting the function of the involved proteins, may be linked with diseases or toxicity [28]. However, it is possible that adduct formation may protect a biomolecule from possible oxidation or degradation which can induce beneficial effects.

For example, it has been shown that the beneficial effect of olive oil is derived from oleuropein (an electrophilic catechol quinone metabolite of olive oil) which hydrolyzes to the catechol hydroxytyrosol quinone and functions as a hydrophobic phenolic antioxidant. It can readily form Michael adducts with thiol nucleophiles in glutathione and proteins. During redox cycling, they can be easily
oxidized and back to their native forms (catechol quinone and proteins) [29]. A comparison between large dose of acetaminophen (4'-hydroxyacetanilide) and its $m$-hydroxy isomer (3'-hydroxyacetanilide) showed that the latter produced more covalent protein adducts, yet only acetaminophen produced hepatotoxicity [30]. Studies showed that the difference between the toxicity profiles of these two isomers of acetaminophen were related with the difference of reactive metabolite localization and protein targets [31,32]. In brief, reactive species also can play a crucial role for cell survival and regeneration through protein oxidation or covalent binding. A review on post-transcriptional oxidative modification by reactive species and the beneficial effects on health and disease has been recently published [33].

From INH-induced liver injury and autoimmunity perspectives,
the INH-protein covalent adduct is of great interest [34]. A study on patients under prophylaxis treatment with INH showed the presence of INH-protein adducts in their blood serum if they had liver failure. However, in the case of mild to moderate liver injury, this adduct was not detected [35]. Another study showed that INH treatment on rats and mice up to 5 weeks did not produce liver injury. However, their liver microsomes showed INH-protein adducts [36]. A recent study showed a paradoxical effect of INH. Mice were first induced with mild autoimmune hepatitis, were

Table 1
Up and Down-regulated proteins observed upon INH treatment.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
<th>Unique peptides sample A</th>
<th>Unique peptides sample B</th>
<th>Average change (control/treated) (\pm) SD</th>
</tr>
</thead>
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<td>Q99714</td>
<td>Isoform 2 of 3-hydroxyacyl-CoA dehydrogenase type-2</td>
<td>7</td>
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<td>0.44 (\pm) 0.11</td>
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<td>P80723</td>
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<td>7</td>
<td>0.54 (\pm) 0.03</td>
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Fig. 6. INH attenuated ATP decrease induced by GOx at 1 h, but not at 3 h. (a) Relative ATP levels after 1 h of GOx exposure on 0 h and 4 h INH pre-incubated HL-60 cells; \(\phi P < 0.001\) compared to untreated HL-60 cells. \(\phi P < 0.001\) compared to GOx treated cells. \(\eta p < 0.05\) and \(\eta m p < 0.005\) compared to GOx treated cells. (b) Relative ATP levels after 3 h between 0 h and 4 h INH pre-incubated HL-60 cells; \(\phi P < 0.001\) compared to untreated cells. \(\phi P < 0.001\) compared to GOx treated cells. \(\phi P < 0.001\) compared to lower INH concentrations (0.001–0.01 mM).

Fig. 7. Effect of INH on mitochondrial membrane potential of GOx challenged HL-60 cells. Cells were pretreated with INH for 4 h and then treated with GOx for 1 h. Mitochondrial membrane potential was then assessed using JC-1 fluorescence as described in Materials and methods followed by converted into percentage by taking the fluorescence measurement of untreated cells as 100%. Each measurement represents \(n = 6\). \(\phi P < 0.001\) compared to untreated cells. \(\phi P < 0.001\) compared to GOx treated cells. \(\phi P < 0.001\) compared to lower INH concentrations (0.001–0.01 mM).
subsequently treated with INH for 5 weeks. It was expected that this would enhance liver injury, however, INH treatment markedly attenuated hepatitis [37]. Therefore, the role of INH-protein adduct are still elusive.

In this study, anti-INH immunoblots were used to detect covalently bound protein adducts (INH-protein adduct). We found that INH increased the formation of protein adducts concentration dependently. Interestingly, INH induced cytoprotection against GOx concentration dependently also. Therefore, there was a correlation observed between cytoprotection and INH covalent protein binding in this study. Additionally, ABAH (a potent MPO inhibitor) showed a significant decrease in covalent adduct formation. Therefore, MPO activity played a major role in the adduct formation through oxidation of INH.

A recent study demonstrated that INH auto-oxidation for 16 h leads to covalent adducts with lysine, human serum albumin, and human plasma in cell free system [38]. Thus, MPO appeared to act as a true catalyst in HL-60 cells as it greatly accelerated the process that leads to covalent protein binding. The exact relationship between the latter and cytoprotection (i.e., whether covalent protein binding is causative or correlated with cytoprotection) is beyond the scope of this study; future studies should be conducted to identify the proteins involved in the adduct formation.

One possible mechanism of INH-induced cytoprotection could be related to its inhibition of MPO. A study compared the MPO inhibition capacity of different inhibitors including INH. INH ranked at the bottom of the list with an IC₅₀ of 277.10 ± 6.65 μM, whereas ABAH ranked most potent, with an IC₅₀ of 0.5 ± 0.03 μM [23]. This finding was similar with our experimental results (data not shown). The reason behind the INH’s poor inhibitory capacity was explained by its slow production of superoxide in aerobic conditions through auto-oxidation [39]. Superoxide converts MPO into compound III through a one-step oxidation [39]. Compound III is very unstable and converts into native MPO within a few minutes at room temperature [26,27]. In presence of reducing agent such as ascorbic acid, it also can be converted into the native enzyme [40]. Therefore, given the potency and mechanism of INH inhibition of MPO, it is highly unlikely that the low micro molar concentrations of INH exerted cytoprotection by MPO inhibition. It is possible that the higher concentrations of INH have the added effect of MPO inhibition, particularly when using concentrations beyond its IC₅₀ for MPO. Other mechanisms, however, must be at play for cytoprotection at low micro molar concentrations. This may involve covalent binding to proteins which may provide protection against oxidative damage and/or upregulation of some protective proteins.

Several studies have previously shown that GOx-induced cell death in various systems is not via classical caspase-dependent apoptosis; rather caspase-independent apoptosis or necrosis depending on the GOx concentration [41–43]. In brief, the cell death signal is initiated through oxidative stress-induced mitochondrial damage. It causes mitochondrial membrane depolarization and loss of potential which disrupts electron transport system.

**Fig. 8.** Summary of INH-induced cytoprotection against ROS. ROS (H₂O₂) generated through GOx activated MPO which can oxidize INH to INH⁻. INH has dual effects on activated MPO. At high concentrations (~1 mM), INH can inhibit MPO as well as change the expression of numerous proteins. These proteins’ functions are involved in increasing the cell replication process, ATP synthesis and structural maintenance, and inhibition of apoptosis. At low concentration (~100 μM), INH can be oxidized into INH⁻ and form covalent adducts with various proteins. There was an apparent correlation between INH-protein adducts and cytoprotective activity; however, it is not known if INH-protein adducts were causative in this effect.
and ultimately reduces ATP synthesis markedly [44]. However, cancer cells can be overcome the reduction of ATP synthesis, since they mainly rely on glycolysis for ATP production but not for mitochondria. The mitochondrial damage releases AIF to execute apoptosis through caspase-independent pathway [41]. Again, if the mitochondrial damage is extensive due to oxidative stress, it can lead to necrosis [44]. In our study, it is possible to follow either caspase-independent apoptosis or necrosis due to nature of G0X and its concentration. We made many attempts to detect AIF in our studies, but were unsuccessful likely due to technical difficulty in isolating mitochondria from these cells (personal communication, Dr. Paige Lacy, University of Alberta).

In quantitative global protein expression experiments (using SILAC) we found 29 proteins that had significantly changed expression. Using a bioinformatic tool, String 9.1, we found protein–protein associations between these proteins through co-expression/association analysis. These associations suggested that the interacting proteins were involved in certain biological pathways, and most likely are involved with replication process, structural integrity maintenance activity, ATP synthesis and blocking of cell death signals.

In conclusion, our study showed that the detrimental effect of a flux of H2O2 was abrogated through both ATP generation and mitochondrial membrane potential recovery, which could involve the upregulation of multiple protective proteins. Besides, replication process, structural integrity maintenance activity and blocking of cell death signals may also play important role. MPO was found essential in INH-protein adduct formation. The role of INH-protein adducts may be either causative or correlated with INH-induced cytoprotection, which is currently unknown. To determine the role of protein covalent adducts, the identification of involved proteins is necessary. The summary of the findings have been illustrated in Fig. 8. In case of TB, we have already discussed that the prevention of necrosis of Mtbi-infected phagocytic cells has an important role in the prevention of the disease. The Mtbi-induced necrosis process is executed mainly through oxidative stress and subsequent mitochondrial damage. In this study, we showed INH has a protective role in this perspective. Therefore, INH-induced cytoprotection is plausible mechanism of action of INH though further research is required in appropriate cell models of TB.

Acknowledgments

The authors are grateful to Dr. Jack Uetrecht (University of Toronto) and Dr. Imir Metushi (La Jolla Institute) for generously providing the anti-isonicotinyl antibody. Saifur R. Khan (supervised by Arno G. Siraki) is supported by an Alberta Innovates Graduate Scholarship of Alberta Innovates Technology Futures (AITF). Mr. N. Aljuhani was supported by Taibah University through Saudi Arabian Cultural Bureau in Canada. This work was supported by the Natural Sciences and Engineering Research Council (#RGPIN-2014-04878).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cbi.2015.11.026.

References


