Paradoxical Attenuation of Autoimmune Hepatitis by Oral Isoniazid in Wild-Type and N-Acetyltransferase–Deficient Mice

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ABSTRACT

Isoniazid (INH) treatment can cause serious liver injury and autoimmunity. There are now several lines of evidence that INH-induced liver injury is immune mediated, but this type of liver injury has not been reproduced in animals, possibly because immune tolerance is the dominant response of the liver. In this study, we immunized mice with isonicotinic acid (INA)-modified proteins and Freund’s adjuvant, which led to mild experimental autoimmune hepatitis (EAH) with an increase in cells staining positive for F4/80, CD11b, CD8, CD4, CD45R, and KI67. We expected that subsequent treatment of mice with oral INH would lead to more serious immune-mediated liver injury, but paradoxically it markedly attenuated the EAH caused by immunization with INA-modified hepatic proteins. In addition, patients of the slow acetylator phenotype are at increased risk of INH-induced liver injury. Treatment of arylamine N-acetyltransferase-deficient Nat1/2(−/−) mice with INH for up to 5 weeks produced mild increases in glutamate and sorbitol dehydrogenase activities, but not severe liver injury. Female Nat1/2(−/−) mice treated with INH for 1, 3, or 7 days developed steatosis, an increase in Oil Red O staining, and abnormal mitochondrial morphology in the liver. A decrease in M1 and an increase in M2a and M2b macrophages was observed in female Nat1/2(−/−) mice treated with INH for 1, 3, or 7 days; these changes returned to baseline levels by day 35. These data indicate that INH has immunosuppressive effects, even though it is also known to induce autoantibody production and a lupus-like autoimmune syndrome in humans.

Introduction

Isoniazid (INH)-induced liver injury remains a significant clinical problem. The mechanism of this idiosyncratic drug reaction is controversial. Previous studies implicated bioactivation of INH to acetylhydrazine or hydrazine as being responsible for the liver injury (Mitchell et al., 1976; Nelson et al., 1976; Timbrell et al., 1980; Sarich et al., 1996). However, these studies involved acute treatment of rats or rabbits with high doses of INH (100 mg/kg per hour for 6 hours and one 50-mg/kg dose followed by three 35-mg/kg doses at 3-hour intervals, respectively), and the characteristics of this toxicity were quite different from the persistent delayed onset of liver injury that is observed in patients. Furthermore, we demonstrated that the metabolism of INH in mice is closer to that in humans than in rats, and we determined that direct bioactivation of INH forms a reactive metabolite that binds to hepatic macromolecules (Metushi et al., 2012). Therefore, the mechanism of liver injury in these models is unlikely to be the same as in humans.

For decades, INH-induced hepatotoxicity was referred to as “metabolic idiosyncrasy,” suggesting that its idiosyncratic nature resulted from differences in metabolism of the drug and, further, that it is not immune mediated. This belief was supported by the fact that in many cases of INH-induced liver injury, there is no rapid onset on rechallenge with the drug, no fever, rash, or eosinophilic infiltrate in the liver, and no anti-INH antibodies (Black et al., 1975; Mitchell et al., 1975). In addition, slow acetylators appear to have a higher incidence of liver injury, which is consistent with the postulated metabolic idiosyncrasy. However, the relative risk is small, and it is insufficient to explain its idiosyncratic nature (Huang et al., 2002).

We have also argued that there is now strong evidence to suggest that INH-induced liver injury is immune mediated (Metushi et al., 2011). In particular, studies reported by Warrington and colleagues have shown that patients who had mild INH-induced liver injury also had a positive lymphocyte transformation test when patient lymphocytes were incubated with INH and not with INH itself (Warrington et al., 1978, 1982). However, more severe cases of INH-induced liver injury had a positive lymphocyte transformation test when patient lymphocytes were incubated with INH-modified protein and with INH itself (Warrington et al., 1978, 2012). Therefore, the mechanism of liver injury in these models is unlikely to be the same as in humans.
1982; Maria and Victorino, 1997). Also, there are cases of INH-induced hepatotoxicity that are associated with the presence of fever, rash, and an infiltration of eosinophils in the liver (Maddrey and Boitnott, 1973; Black et al., 1975). Most recently, we have identified anti-INH and anti-cytochrome P450 (P450) autoantibodies in the serum of patients with INH-induced liver failure (Metushi et al., 2014). These data strongly suggest that INH-induced liver injury involves an immune response against INH-modified proteins rather than direct cytotoxicity of acetylhydrazine.

A valid animal model would greatly facilitate studies of the mechanism of INH-induced liver injury. Given the fact that metabolism in mice, especially to the reactive metabolite, is more similar to that in humans than that in rats, mice should be a better species to test. In particular, arylamine N-acetyltransferase (NAT)-deficient mice should be more susceptible since they should not eliminate INH as rapidly as wild-type mice. Our previous attempts to develop an animal model of INH-induced liver injury by oral administration of INH to mice or rats have failed (Metushi et al., 2012; Metushi and Uetrecht, 2014). If the mechanism is immune mediated, immunization with INH-modified proteins should increase the immune response. Lohse and colleagues (1990) described a model of experimental autoimmune hepatitis (EAH) in which i.p. immunization of mice with the 100,000g supematant of

**Fig. 1.** Western blot of S100 hepatic proteins and S100 proteins after reaction with an activated ester of isonicotinic acid (S100-INA) and visualized by an anti-INH antibody.

**Scheme 1. Immunization of Female C57BL/6 Mice With S100**

**Scheme 2. Immunization of C57BL/6 Mice (male + female) With S9**

**Scheme 3. Immunization of Female C57BL/6 Mice With S100**

**Fig. 2.** Schedule for immunization of mice with hepatic protein (S100 or S9) or INH-modified hepatic protein to mimic covalent binding of INH in the liver (S100-INA or S9-INA). Scheme 1: Immunization of mice with S100. C57BL/6 mice were immunized with S100 or S100-INA in CFA once a week for 3 weeks. INH was given orally to C57BL/6 mice immediately after the first immunization; INH was stopped after 6 weeks of treatment, mice were taken off drug for 4 weeks to allow CYP450 synthesis to recover in case it was inhibited by CFA, and then mice were put back on the drug for another 6 weeks. In C57BL/6 mice, at the end of the first 6 weeks, one mouse was sacrificed from each group to look for evidence of autoimmune hepatitis. Scheme 2: Mice were immunized twice with S9 or S9-INA in CFA and once with S9 or S9-INA in IFA. The INH was started 4 weeks after the last immunization and mice were kept on INH for 3 weeks. Scheme 3: Mice were immunized once with S100 or S100-INA in CFA and twice with S100 or S100-INA in IFA. Then, after 4 weeks of no treatment, mice were treated with INH in food for 5 weeks.
a syngeneic liver homogenate (S100) plus Freund’s adjuvant resulted in mild hepatic inflammation. Other investigators have been able to induce more severe EAH when S100 was modified by reactive metabolites of drugs such as halothane or alcohol (Njoku et al., 2005; Thiele et al., 2010). In this study, we adapted this strategy by immunizing mice with hepatic proteins modified by the reactive metabolite of INH to initiate an immune-mediated response against INH-modified proteins that could lead to an animal model of liver injury. In addition, we treated NAT-deficient (Nat1/2−/−) mice with INH to mimic human slow acetylators, which are well known to be at increased risk of INH hepatotoxicity (Huang et al., 2002). We hoped these strategies would lead to a chronic immune response against INH-modified hepatic proteins that would represent a model of the INH-induced liver injury that occurs in patients.

Materials and Methods

Animals. Inbred C57BL/6NCrl mice, 6–8 weeks old, were purchased from Charles River Laboratories (Montreal, QC) and allowed to acclimatize for 1 week before initiation of treatment. Nat1/2−/− mice are on a C57BL/6 background (Sugamori et al., 2003) and are bred inhouse. All animal experiments were approved by the University of Toronto animal care committee.

Treatment. INH (Sigma-Aldrich, Oakville, ON, Canada) was ground to a fine powder, thoroughly mixed with food, and given to rodents at a dose of 0.2% INH by weight (w/w) in food. Food was provided to the animals in small jars ad libitum, and the amount consumed was measured. The average amount of drug consumption was from 200–300 mg/kg daily, based on animal body weight and daily average food intake. This INH dose leads to INH blood levels between 2–6 µg/ml (Metushi et al., 2012), which is similar to the Cmax of INH in humans (Fukino et al., 2008). Alternatively, INH was dissolved in saline and given by oral gavage to female Nat1/2−/− mice at a dose of 100 mg/kg per day. Saline was administered as the vehicle control.

Western Blotting and Subcellular Fraction Preparation. Livers were perfused with cold phosphate-buffered saline (PBS) (pH 7.4), removed, and homogenized on ice. When the proteins were used for Western blotting, the homogenization was done in the presence of protease inhibitors (Sigma) and cell lysis buffer (Cell Signaling, Danvers, MA) according to the manufacturer’s instructions. The hepatic S100 cytosolic fraction was prepared according to the method described by Lohse et al. (1990). The S9 fraction was prepared by centrifugation of the liver homogenate at 2500g for 10 minutes, and the supernatant was centrifuged again at 9000g for 20 minutes. Other subcellular fractions involved differential centrifugation as previously described (McGill et al., 2012). Briefly, cell debris was separated by 2500g centrifugation for 10 minutes, the supernatant was centrifuged at 20,000g for 10 minutes to collect the mitochondria, and centrifugation at 110,000g for 1 hour precipitated the microsomes. This last supernatant was collected as the cytosolic fraction. Protein concentrations were determined by using the bicinchoninic acid kit (Pierce, Ottawa, ON, Canada). The proteins were separated by electrophoresis in an 8% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Bio-Rad, Mississauga, ON, Canada). Rabbit anti-INH antibody was produced as previously described and was used as the primary antibody (Metushi et al., 2012) and detected by goat anti-rabbit IgG-peroxidase (Sigma). Bound peroxidase was detected using Supersignal West Pico Chemiluminescent Substrate (Fisher Scientific, Ottawa, ON, Canada). Mouse monoclonal anti-GAPDH (Sigma) was used as the loading control and detected by goat anti-mouse IgG-peroxidase (Jackson ImmunoResearch, West Grove, PA). Super-signal-enhanced molecular weight markers were used to determine the approximate molecular weight (Thermo Scientific, Rockford, IL).

Preparation of Haptenated S100-INA or S9-INA. Modification of the S100 and S9 fractions was accomplished by adding 35 mg of the hydroxysuccinimide ester of isonicotinic acid to 25 mg of protein in PBS and stirring for 1 hour at room temperature, which mimics the covalent binding of the reactive metabolite of INH to lysine groups. The synthesis of the hydroxysuccinimide ester and the procedure for reaction with proteins were reported previously (Metushi et al., 2012). The modification of S100 with the reactive ester of isonicotinic acid (INA) was confirmed by Western blotting (Fig. 1). These products are abbreviated S100-INA and S9-INA, respectively.

Induction of Experimental Autoimmune Hepatitis. Induction of EAH was initiated by i.p. immunization of mice with S100 or S100-INA at a concentration of 2.5 mg/mouse (4 mg/mouse was used for S9 or S9-INA) in 150 µL of PBS emulsified with 150 µL of Freund’s adjuvant. Immunization was performed weekly for up to three times. The first immunization always involved complete Freund’s adjuvant (CFA); subsequent immunizations involved incomplete Freund’s adjuvant (IFA). In contrast to Lohse et al., we used IFA for subsequent immunizations to reduce the discomfort to animals. The schedule of immunization and drug treatment are shown in Fig. 2; the experiment was performed three times.

Histology. At the endpoint, animals were sacrificed, and their livers were perfused and placed in 10% neutral buffered formalin solution (Sigma) overnight. For preparation of frozen sections, liver tissue was placed in optimal cutting temperature (OCT) medium (VWR International, Radnor, PA) and immediately frozen using liquid nitrogen. Formalin-fixed, paraffin-embedded liver sections were stained with H&E by the department of pathology at the Hospital for Sick Children (University of Toronto, Toronto, ON, Canada). Paraffin and frozen nonstained slides were also sectioned by the department of pathology at the Hospital for Sick Children (University of Toronto). Oil Red O staining was performed on frozen slides by the department of pathology at the Hospital for Sick Children (University of Toronto). Sections for electron microscopy were prepared and analyzed by the Microscopy Imaging Laboratory at the Medical Sciences Building (University of Toronto).

Immunohistochemistry. Rat monoclonal primary antibodies against mouse CD11b (clone M1/70), F4/80 (clone CLA3-1), CD45R (clone RA3-6B2), and rabbit polyclonal antibody against mouse Ki67 were purchased from Abcam (Cambridge, MA). Rat monoclonal antibodies against mouse CD4 (clone GK 1.5) and CD8 (clone YTS169) were donated by Pamela Ohashi’s laboratory.
Polyclonal rabbit secondary antibody anti-rat IgG-biotinylated and streptavidin-peroxidase were purchased from Dako (Burlington, ON, Canada). Goat anti-rabbit IgG-peroxidase was purchased from Sigma. Each experiment was repeated at least twice, and the signal was developed using 3,3'-diaminobenzidine for paraffin-embedded slides or NovaRed for frozen slides (Vector, Burlington, ON, Canada) with Mayer’s hematoxylin (Sigma) as the counterstain. Antibodies against F4/80, CD45R, and KI67 were used on paraffin-embedded slides. Antibodies against CD11b, CD4, and CD8 were used on frozen slides. Grading for immunohistochemical analysis and EAH was blinded, and the number of cells was counted in five fields under a microscope for two slices of tissue (3–6 mm²).

Biochemical Measures of Liver Injury. Liver enzyme activities were measured in blood collected from the saphenous vein. As biomarkers of liver injury, the activity of alanine aminotransferase (Thermo Scientific, Middle-town, VA) and sorbitol dehydrogenase (SDH; Catachem, Oxford, CT) were measured as described by the manufacturer. The method for glutamate dehydrogenase (GLDH, Randox, Crumlin, UK) was slightly modified as previously described (Metushi and Uetrecht, 2013).

Flow Cytometry. Animals were sacrificed, and the lymph nodes from the neck were collected. Lymph nodes were disaggregated by meshing the lymph node with a 70-µm cell strainer. A single cell suspension was prepared with 1 × 10⁶ cells/ml. Cells were incubated with red cell lysis buffer for 5 minutes and washed with fluorescence-activated cell sorter (FACS) buffer, which consisted of 10% fetal calf serum in PBS, pH 7.4. Aliquots of 5 × 10⁵ cells were placed in conical bottom-microplate wells; nonspecific binding was blocked by incubating cells with anti-mouse CD32 antibody (eBioscience, San Diego, CA); and cells were incubated with anti-mouse CD11b-AF700 (Biolegend, San Diego, CA), anti-F4/80-PE (Life Technologies, Burlington, ON, Canada) at 4°C for 30 minutes. Cells were washed three times with cold FACS buffer, fixed, and permeabilized with cold Perm/Fix buffer (eBioscience) for 30 minutes. Cells were washed again and incubated for 1 hour at 4°C with anti-interleukin (IL)-10-PB (Cedarlane, Burlington, ON, Canada), anti-tumor necrosis factor (TNF)-α-APC (eBiosciences), or anti-CCL2-FITC (Cedarlane) in combinations to allow the correct measurement of each cell marker. Cells were washed three times and analyzed in a flow cytometer (LSR II; BD Biosciences, Mississauga, ON, Canada) recording 50,000 events per duplicate sample. Data were analyzed using the FlowJo software (Treestar, Ashland, OR).

Cytokine Quantification. Serum cytokines in 25 µl of serum were measured using Bio-Rad’s Bio-Plex Pro Mouse Cytokine 23 Plex according to the manufacturer’s instructions. Cytokines measured include IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, and IL-17A; eotaxin, granulocyte cell-stimulating factor, granulocyte-macrophage colony-stimulating factor, interferon-γ, chemokine (C-X-C motif) ligand 1 (CXCL1/KC), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, RANTES, and TNF-α.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Data were analyzed using...
two-way analysis of variance (ANOVA), one-way ANOVA, or Mann-Whitney’s U test. A P value < 0.05 was considered significant (*P < 0.05; **P < 0.01; ***P < 0.001).

Results

Immunization of C57BL/6 Mice with S100/S9. Our most recent attempts to develop an animal model of INH-induced hepatotoxicity were unsuccessful (Metushi et al., 2012; Metushi and Uetrecht, 2013, 2014). To induce immune-mediated liver injury, we immunized mice with hepatic protein to initiate an immune-mediated response against the liver, or we immunized mice with INA-modified hepatic protein to sensitize the immune system to the reactive metabolite of INH. Each of these strategies was followed by oral treatment with INH, which we know binds in the livers of mice in vivo (Metushi et al., 2012), to produce ongoing liver injury that could represent a model of INH-induced liver injury in humans.

Similar to previous reports, our preliminary results suggested that immunization of mice with S100 resulted in induction of EAH (Supplemental Fig. 1A) (Lohse et al., 1990). Histologically, EAH was present at 6 and 16 weeks after initial S100 immunization, with week 6 being more severe than week 16 (data not shown for all slides; refer to Fig. 2, Scheme 1A, for methods). However, a marked reduction in the degree of EAH was observed when S100 immunization was followed by treatment with INH, which we know binds in the livers of mice in vivo (Metushi et al., 2012), to produce ongoing liver injury that could represent a model of INH-induced liver injury in humans.

In the initial experiments, we treated animals with INH during the immunization schedule; later, we waited for 4 weeks from the last immunization with hepatic protein in IFA before starting INH (Fig. 2, Schemes 2 and 3). We confirmed that at the end of experiment (indicated as endpoint in Fig. 2, Scheme 3), a high degree of covalent binding of INH was seen in the livers of mice (Supplemental Fig. 2); therefore, bioactivation of the drug was not a significant issue when we delayed the onset of INH treatment.

S100 does not contain P450s. We have shown that INH binds to P450s, and most of the covalent binding is localized around the central vein, where most P450s are located (Metushi et al., 2012). Therefore, we tried replacing S100 with S9, which does contain P450s. Mice immunized with S9 could not be maintained for more than 7 weeks after the last immunization because they developed peritonitis (Fig. 2, Scheme 2, for the immunization schedule). Oral administration of INH to S9- or S9-INA-immunized mice prevented autoimmune hepatitis in a similar way as in S100-treated mice (Supplemental Fig. 1, D and E).

It is clear that oral treatment with INH attenuated EAH induced by either S100/S9 or S100-INA/S9-INA immunizations. To determine the effects of S100-INA alone in addition to S100-INA + oral INH, we performed an additional experiment with all the necessary control groups (schematic shown in Fig. 2, Scheme 3). The body weight of animals exposed to INH was slightly reduced after 1 week of treatment, but no other differences were observed between groups (Supplemental Fig. 3). Alanine aminotransferase activity is inhibited by oral administration of INH (O’Brien et al., 2002; Metushi et al., 2012); therefore, GLDH and SDH activities were measured as biomarkers of liver injury in addition to H&E slides. INH alone did not result in any changes in liver enzyme activities, but GLDH/SDH activities were significantly increased in the S100- and S100-INA− immunized groups; the S100-INA group had the greatest elevation in enzyme activities (Fig. 3, A and B). Immunization with S100-INA produced a higher grade of EAH compared with the group receiving S100 alone (Fig. 4, Fig. 6A); however, oral INH...
administration once again reduced the severity of EAH induced either by S100 or S100-INA. Along with the increase in severity of EAH in the S100-INA treatment group, there was an increase in cells that stained positive for F4/80, CD11b, CD8, CD4, CD45R, and KI67 markers (Figs. 4, 5, 6; full figures are shown in Supplemental Figs. 4–7); most of these changes were attenuated by oral treatment with INH. In the spleen, a more prominent increase in CD11b was observed in the group of mice immunized with S100-INA (Supplemental Fig. 8). Representative micrographs of spleen are shown in Supplemental Figs. 4 and 8–10. Serum cytokine measurements revealed small increases in eotaxin, interferon-γ, IL9, and IL13 in the S100-INA group; however, the large variation between samples made any possible differences statistically not significant (Supplemental Fig. 11).

**Chronic Treatment of Nat1/2(−/−) Mice with INH.** We have shown that mice treated with INH have higher blood levels of INH and more covalent binding than rats (Metushi et al., 2012). Thus, mice appear to be a better model for human slow acetylators who are at increased risk of hepatotoxicity (Huang et al., 2002). We went one step farther and treated Nat1/2(−/−) mice, which should not eliminate INH by acetylation, expecting this to result in greater covalent binding of INH in the liver and more hepatotoxicity. Treatment of Nat1/2(−/−) mice with INH resulted in mild elevations in GLDH and SDH, but only in females (Fig. 7, A–D). The increase in SDH occurred within the first week and returned to baseline by week 5. The pattern was less clear for GLDH; there may have been an early increase. However, it was not statistically significant until week 3, and it did not appear to decrease, although again lack of statistical power prevents a clear conclusion. Treatment with INH reduced the body weight gain in both male and female Nat1/2(−/−) mice (Fig. 7, E and F), with female mice being more sensitive than male mice. In contrast to expectations, female Nat1/2(−/−) mice had similar covalent binding to female C57BL/6 mice (Fig. 8A). Interestingly, male Nat1/2(−/−) mice had higher covalent binding than females (Fig. 8 B), although female Nat1/2(−/−) mice had higher GLDH elevations.

**Acute Treatment of Female Nat1/2(−/−) Mice with INH.** Only a small increase in GLDH, indicating mild liver injury, was observed when treating female Nat1/2(−/−) mice with INH for up to 5 weeks; therefore, we looked for signs of an immune response after acute treatment. We determined the amount of INH covalent binding as a function of time and found that INH covalent binding to liver proteins was higher in Nat1/2(−/−) mice treated for 7 days than in mice treated for only 1 or 3 days (Supplemental Fig. 12); however, there was no significant increase in GLDH or SDH levels (Supplemental Table 1). Hepatic steatosis was observed as early as day 1 of treatment and was sustained until day 7 (Fig. 9; data not shown for all time points). There was a considerable increase in lipid infiltration in the liver after 3 days of treatment with INH as shown by Oil Red O staining. In addition, electron microscopy revealed depletion of glycogen, an increase in lipid vesicles, and abnormal mitochondria and endoplasmic reticulum shape (Fig. 9). There were no changes in the proportion of F4/80 positive cells in the liver at this...
early time point (Fig. 9). A comparison of INH covalent binding in different fractions of liver homogenate revealed that the highest covalent binding was to microsomes, followed by mitochondria, and the lowest was to the cytosol. However, binding occurred to all fractions, and the differences were small (Fig. 10). This is consistent with the potential for INH to cause mitochondrial damage.

Phenotyping of M1 versus M2 Macrophages in Female Nat1/2(−/−) Mice Treated with INH. Flow cytometry was used to phenotype macrophages in the cervical nodes of INH-treated Nat1/2(−/−) mice as shown in Fig. 11. The markers used were adapted from the literature (Benoit et al., 2008; Fairweather and Cihakova, 2009; Laskin, 2009; Pulendran et al., 2010). Monocytes were first gated by forward and side scatter and then by the membrane expression of CD11b and/or F4/80 versus the intracellular expression of TNF-α and/or IL-10. In these plots, we also evaluated the presence of intracellular CCL2. With this combination of cell markers, we determined the phenotype and the proportion of activated macrophages present in peripheral lymph nodes. Treatment with INH led to an increase in the percentage of macrophages after 1, 3, and 7 days of treatment, but these returned to baseline levels by day 35 despite continued treatment (Fig. 11A). Further analysis of macrophage markers suggested that INH treatment increased the proportion of M1 macrophages at days 1–7, indicating the specific recruitment of M1 cells, whereas at day 35 of treatment, a dramatic decrease in M1 macrophages was observed (Fig. 11B). The number of M2 macrophages may have decreased slightly, but the increase in M1/M2 ratio was unequivocal. Further analysis using the presence of intracellular cytokines indicated that although the M1/M2 ratio increased, the ratio of activated M1/M2 macrophages actually decreased with a significant increase in activated M2a and M2b macrophages (Fig. 11C). These changes returned to baseline levels by day 35 of treatment (Fig. 11C).

Discussion

The mechanism of INH-induced liver injury remains unknown, and the lack of valid animal models has greatly hampered mechanistic studies. Previously, we demonstrated that mice are a better species than rats to model the metabolism and covalent binding of INH in humans (Metushi et al., 2012). However, efforts to develop an animal model of INH-induced hepatotoxicity by treating mice orally with INH were unsuccessful (Metushi et al., 2012). More recently, we found a higher incidence of mild liver injury in various knockout mouse strains that have impaired immune tolerance relative to wild-type (C57BL/6) mice (Metushi and Uetrecht, 2013). This finding...

Fig. 7. GLDH, SDH, and body weights in male (A, C, and E) and female (B, D, and F) Nat1/2(−/−) mice from control or treated for 5 weeks with 0.2% INH w/w (n = 4). (A, B) GLDH activity in Nat1/2(−/−) mice. (C, D) SDH activity in Nat1/2(−/−) mice. (E, F) Body weight in Nat1/2(−/−) mice. Mean ± S.E. *P < 0.05; **P < 0.01; ***P < 0.001, Two-way ANOVA.

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**Figure Captions:**

- **Figure 7:** GLDH, SDH, and body weights in male (A, C, and E) and female (B, D, and F) Nat1/2(−/−) mice from control or treated for 5 weeks with 0.2% INH w/w (n = 4). (A, B) GLDH activity in Nat1/2(−/−) mice. (C, D) SDH activity in Nat1/2(−/−) mice. (E, F) Body weight in Nat1/2(−/−) mice. Mean ± S.E. *P < 0.05; **P < 0.01; ***P < 0.001, Two-way ANOVA.
of INH was also associated with a decrease in immune cell infiltration of macrophages/dendritic cells and T and B lymphocytes (Figs. 4–6), which suggests suppression of an immune response in the liver. The most dramatic change was the marked attenuation of F4/80+ macrophage and CD8+ T cells infiltration induced by the immunization with INH-modified S100 hepatic proteins.

We also tried to develop a model of INH-induced liver injury by treating Nat1/2(−/−) mice, which should have impaired acetylation of INH (Sugamori et al., 2003). The rationale for using acetylator knockout mice is the observation that patients who are slow acetylators are at increased risk of INH-induced hepatotoxicity (Huang et al., 2002). We expected greater covalent binding of INH in these animals and possibly an increase in liver injury; however, the amount of INH covalent binding in female C57BL/6 and Nat1/2(−/−) mice was similar. It is paradoxical that male Nat1/2(−/−) mice had greater hepatic covalent binding of INH compared with female Nat1/2(−/−) mice (Fig. 8), but GLDH and SDH were elevated only in the female Nat1/2(−/−) mice (Fig. 7). This is consistent with the observation that women appear to be more susceptible to (idiosyncratic drug-induced liver injury) caused by many drugs (Sgro et al., 2002; De Valle et al., 2006). However, given the lack of abnormal histology, this does not represent a model of significant INH-induced liver injury. Treatment of Nat1/2(−/−) mice with INH for 1–7 days also produced steatosis and an increase in Oil Red O staining, which indicates lipid accumulation, and this was followed by a reduction of glycogen and abnormal mitochondrial morphology (Fig. 9). Other reports have indicated that INH can cause mitochondrial injury in mice and rats (Sodhi et al., 1997; Chowdhury et al., 2006), and more recently, in a mouse diversity panel of 34 strains, microvesicular steatosis was observed when mice were treated with 100 mg/kg daily INH for 3 days (Eaddy et al., 2012). In addition, changes in the expression of several genes related to the mitochondria dysfunction were observed (Eaddy et al., 2012), suggesting that in animals, treatment with INH can cause mitochondrial damage at early time points. We also found that there is significant binding of the reactive metabolite of INH to the mitochondrial fraction of a liver homogenate (Fig. 10), suggesting that the reactive metabolite of INH can directly bind to mitochondrial protein, and this may be responsible for the induction of steatosis. Steatosis has also been observed in a few clinical cases of INH-induced liver injury (Pessayre et al., 1977; Pilheu et al., 1979, 1981). However, these cases involve administration of INH together with other drugs such as rifampin, ethambutol, and pyrazinamide, and these cases do not involve the common histologic feature of hepatocellular necrosis, which is observed in most cases of INH-induced liver failure (Maddrey and Boitnott, 1973).

In our studies, steatosis appeared to resolve despite continued treatment after 5 weeks of INH treatment. It is possible that mitochondrial injury is an early event that helps to initiate an immune response, but it is no longer evident at later times when liver biopsies are performed on patients with INH-induced liver failure. However, there is also a difference in the mode of INH administration between the acute and chronic studies; specifically, in the acute studies, the INH was administered by gavage, which would be expected to produce higher peak levels of INH compared with administration of INH in food.

In INH-treated Nat1/2(−/−) mice, we observed a decrease in the percentage of activated M1 macrophages, which are thought to be proinflammatory, and an increase in the proportion of activated M2a and M2b macrophages, which are characterized as anti-inflammatory (Fig. 11) (Laskin, 2009). This is also consistent with an immunosuppressive effect of INH in mice and may prevent most patients from developing significant INH-induced liver injury. This is in contrast to suggested that immune tolerance may be the major barrier to inducing liver injury. However, attempts to convert these models of mild hepatotoxicity into models of INH-induced liver failure have been unsuccessful. In this study, we used a previously reported model of EAH in mice to try to overcome immune tolerance (Lohse et al., 1990). Specifically, we induced an immune response against INH-modified proteins by immunizing mice with S100 or S9 hepatic proteins that had been modified to mimic the in vivo covalent binding of INH, followed by treatment with INH, which we know modifies hepatic proteins (Metushi et al., 2012). We expected that prior immunization with INH-modified proteins would lead to a brisk immune response to the INH-modified proteins that were formed during metabolism of the oral INH and result in significant liver injury. Modification of these hepatic proteins with INH increased their ability to induce EAH relative to native proteins (Figs. 4–6), but paradoxically, subsequent treatment with INH markedly decreased the liver injury caused by this immunization. The inhibition of EAH occurred independent of whether it was induced by native hepatic proteins or INH-modified hepatic proteins. This finding implies that in mice, INH acts as an immunosuppressant. The prevention of autoimmune hepatitis by oral administration of INH in mice treated with 0.2% INH w/w in food for 5 weeks. (A) Female C57BL/6 or Nat1/2(−/−) mice (n = 4); (B) male or female Nat1/2(−/−) mice (n = 4).
the fact that INH treatment commonly induces antinuclear antibodies, and in some cases, a lupus-like autoimmune syndrome (Salazar-Paramo et al., 1992).

So what do these data mean? Just as treatment of most patients with drugs that can cause serious liver injury does not result in significant liver injury, the present studies highlight the complex immune response to drugs such as INH. In humans, there are several lines of evidence to suggest that INH-induced hepatotoxicity involves an immune mechanism. In particular, binding of the parent drug appears to induce an immune response that can lead to idiosyncratic liver injury as evidenced by the specificity of lymphocyte transformation tests in patients with INH-induced liver injury (Warrington et al., 1978, 1982). In addition, we have identified anti-INH and anti-P450 antibodies in the serum of patients with INH-induced liver failure (Metushi et al., 2014), and the major cell type in liver biopsies from cases of INH-induced hepatotoxicity are CD8+ cells (Foureau et al., 2012). Together, these data provide strong evidence that INH-induced liver injury is immune mediated, and in particular there is an immune response against INH rather than the N-acetylhydrazine metabolite. However, the observation that oral treatment with INH prevented EAH and induced M2 macrophages is consistent with our previous observations that in animals, INH can cause immune suppression.

Fig. 9. Steatosis as an early hepatic response to treatment of Nat1/2(−/−) mice with INH (100 mg/kg/d by gavage for 3 days). Stains were H&E, Oil Red O, transmission electron microscopy (TEM, n = 3), or anti-F4/80 staining in the liver (n = 4).
(Metushi and Uetrecht, 2013, 2014); this could explain why it is so difficult to develop an animal model of severe INH-induced liver injury similar to the idiosyncratic reaction in humans that can lead to liver failure.

**Authorship Contributions**

**Participated in research design:** Metushi, Cai, Vega, Uetrecht.

**Conducted experiments:** Metushi, Cai, Vega.

**Contributed new reagents or analytic tools:** Metushi, Cai.

**Performed data analysis:** Metushi, Vega.

**Wrote or contributed to the writing of manuscript:** Metushi, Uetrecht, Grant.

**References**


Huang YS, Chen JD, Su WJ, Wu JC, Lai SY, Chang FY, and Lee SD (2002) Covalent binding of INH to subcellular liver fractions. Female C57BL/6 were treated for 7 days with INH in food at 0.2% w/w. C, Supernatant of liver homogenate from control mouse liver after 2500 g centrifugation; 2500 g, supernatant after 2500 g centrifugation from INH-treated mice. The mitochondria, microsomes, and cytosol fractions are from INH-treated mice and were isolated as outlined in the methods section.

Fig. 10. Covalent binding of INH to subcellular liver fractions. Female C57BL/6 were treated for 7 days with INH in food at 0.2% w/w. C, Supernatant of liver homogenate from control mouse liver after 2500 g centrifugation; 2500 g, supernatant after 2500 g centrifugation from INH-treated mice. The mitochondria, microsomes, and cytosol fractions are from INH-treated mice and were isolated as outlined in the methods section.

Fig. 11. Macrophage phenotyping by flow cytometry in the cervical lymph nodes of Natl2α–/– mice. Day 0 is before INH treatment (n = 4) while for days 1, 3, and 7, INH was given by gavage at a dose of 100 mg/kg daily (n = 3); and for the 35-day data, INH was administered in food at 0.2% w/w (n = 4). (A) Percentage of total events from the monocye gate after 1, 3, 7, or 35 days of INH treatment, (B) Phenotyping of macrophages into M1 (F4/80+ only) and M2 (F4/80+CD11b+), M2a (CD11b+IL10+), M2b (IL10/TNFα+/CCL2–), and M2c (IL10/TNFα+/CCL2+). Mean ± S.D. *P < 0.05; **P < 0.01; ***P < 0.001, Mann-Whitney’s U test.


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