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Expression and genotype-dependent catalytic activity of *N*-acetyltransferase 2 (NAT2) in human peripheral blood mononuclear cells and its modulation by Sirtuin 1

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Abstract

N-acetyltransferase 2 (NAT2) catalyzes the biotransformation of numerous arylamine and hydrazine drugs and carcinogens. Genetic polymorphisms of NAT2 modify drug efficacy and toxicity and susceptibility to diseases such as cancer and type 2 diabetes. Expression of NAT2 has been documented in the liver and gastrointestinal tract but not in other tissues. Deacetylation of cytosolic proteins by sirtuins is a post translational modification important in regulatory networks of diverse cellular processes. The aim of the present study was to investigate NAT2 expression in peripheral blood mononuclear cells (PBMC) and the effects of NAT2 genotype and Sirtuin 1 (SIRT1). Both NAT2 and SIRT1 proteins were expressed on PBMC. Their expression was more prevalent on CD3⁺ compared to CD19⁺ and CD56⁺ cell populations. *N*-acetylation capacity of PBMC exhibited an NAT2 gene-dose response toward the *N*-acetylation of isoniazid. Subjects with rapid NAT2 genotype showed an apparent $V_{\max}$ of 42.1 ± 2.4 ; intermediate NAT2 genotypes an apparent $V_{\max}$ of 22.6 ± 2.2 ; and slow acetylator NAT2 genotypes an apparent $V_{\max}$ of 19.9 ± 1.7 nM acetyl-isoniazid/24h/million cells. The *N*-acetylation capacity of NAT2 in the presence of SIRT1 enhancer was significantly decreased ($p < 0.001$), conversely, the transient silencing of SIRT1 resulted in an increase of *N*-acetylation capacity ($p < 0.001$). These findings are the first report of NAT2 genotype-dependent expression on PBMC and post-translational modification by SIRT1. These findings constitute a substantial advance in our understanding of human *N*-acetyltransferase expression and a new much less invasive method for measurement of human NAT2 expression and phenotype.

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Conflict of interest

The authors declare that they have no conflict of interest.

Keywords

N-acetyltransferase 2; Sirtuin 1; Acetylator phenotype; Peripheral blood mononuclear cells; Post-translational modifications

1. Introduction

N-acetyltransferases (E.C. 2.3.1.5) catalyze the *N*-acetylation of numerous arylamine and hydrazine drugs [1]. The administration of isoniazid (INH) for the treatment of tuberculosis revealed different responses of the patients to the treatment, which led to the understanding that *N*-acetyltransferase 2 (NAT2) gene is subject to genetic polymorphisms resulting in rapid, intermediate and slow acetylator phenotypes [1]. Human NAT2 catalyzes *NAT2* genotype-dependent *N*-acetylation and *O*-acetylation of xenobiotic compounds and carcinogens [2]. *NAT2* genetic polymorphisms have been linked to decreased enzymatic activity and also variable stability, leading to an imbalance on the xenobiotic detoxification and increased susceptibility of different forms of cancer, including urinary bladder [3], colon [4] and breast [5, 6]. Recent reports have also shown the involvement of NAT2 as an insulin sensitivity gene [7] suggesting that NAT2 genetic polymorphisms modify susceptibility to type 2 diabetes [8]. These studies highlight the importance of NAT2 polymorphisms as a risk factor for the development of a complex disease like type 2 diabetes suggesting new roles for NAT2 previously described only as a drug and xenobiotic metabolizing enzyme.

Cell signaling networks are information-processing pathways that control most cellular functions. They are regulated by reversible protein post-translational modifications that allow cells to adapt and respond to internal and external signals. Acetylation-mediated cellular signaling involves three types of proteins: lysine acetyltransferases, which add acetyl groups to proteins; lysine deacetylases, that remove acetyl groups from proteins; and acetyl-lysine binders, which selectively interact with acetylated proteins [9].

Lysine deacetylases identified in the human and mouse genomes belong to two distinct families with different catalytic mechanisms: Zn²⁺-dependent histone deacetylases (HDAC1–11) [10] and NAD⁺-dependent sirtuin deacetylases (SIRT1–7) [11]. Sirtuin proteins are present in the nucleus, cytoplasm and mitochondria. Although initially identified as deacetylases, sirtuins also have shown deacylase and *O*-ADP-ribosylase activity [12]. SIRT3, SIRT4, and SIRT5 are mitochondrial proteins, while SIRT1, SIRT6 and SIRT7 are nuclear enzymes and can take part in the epigenetic regulation of cell phenotype. SIRT2 can be shuttled between nucleus and cytoplasm, depending on the phase of the cell cycle. However, some of these proteins can translocate from their typical compartments under specific circumstances [13, 14]. Sirtuins deacetylate a multitude of targets including histones, transcription factors, and metabolic enzymes. Therefore, sirtuins have been implicated in numerous cellular processes including stress response, DNA repair, energy metabolism, and tumorigenesis [15, 16]. The best characterized and widely studied protein of this family is SIRT1; it has a crucial role in both metabolism and immune response [17, 18] by regulation directly through deacetylation of some critical transcriptional factors or indirectly through metabolic pathways [19].

One of the best-characterized substrates of NAT2 is INH, which is one of the first-line drugs for the anti-tuberculosis therapy. NAT2 genotype and phenotype have been found to be susceptibility factors in INH hepatotoxicity [20, 21]. On the other hand, the effect of resveratrol, a SIRT1 activity modulator, can prevent hepatotoxicity caused by INH in mouse models [22]. Since SIRT1 and NAT2 are intracellular cytoplasmic proteins we hypothesized that a NAT2 regulation mechanism includes acetylation/deacetylation equilibrium by sirtuins. A regulation mechanism proposed for N-acetyltransferase 1 suggests a role for acetylation/deacetylation equilibrium that confers stability to the protein and prevents lysine deacetylation and ubiquitin degradation [23]. A recent report provided preliminary data in support of Sirtuin modulation of NAT2 [24].

As recently reviewed [21], the NAT2 phenotype is most commonly measured by administration of caffeine containing beverages followed by measurement of a urinary caffeine metabolite ratio. Some studies report good concordance between acetylator phenotype determined by caffeine metabolite ratio and NAT2 genotype [25, 26], however others show discordance [27–32]. An alternative non-invasive method for measuring NAT2 phenotype would substantially benefit investigations into the role of NAT2 phenotype in the efficacy and toxicity of numerous drugs and carcinogens, as well as susceptibility to various diseases including cancer and type 2 diabetes.

2. Materials and Methods

2.1 Population

Twenty (nine male; 11 female) healthy subjects, ranging between 20 and 32 years of age, were recruited for our study. The Bioethics Committee of the Autonomous University of San Luis Potosi approved the study and all participants signed a written informed consent form. Blood samples were obtained from each participant and were collected in 8 mL EDTA Vacutainer® tubes (BD Biosciences, San Jose, CA, USA) for DNA, RNA, and PBMC isolation. A non-probabilistic sampling method was used on the participants. The same cohort participated in a parallel study [33].

2.2 Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient protocol as follows; Blood was diluted 1:1 with phosphate-buffered saline (PBS) pH 7.4. This dilution was gently transferred to a 15 mL conical tube containing 3 mL of Ficoll-Histopaque (Millipore-Sigma, St. Louis, MO, USA) and centrifuged at $1375 \times g$ for 20 min at room temperature. The resulting PBMC layer was transferred to a new 15 mL conical tube and washed twice with PBS at room temperature and resuspended in Dulbecco's modified Eagle's media (DMEM) supplemented with 50 U/mL penicillin and 50 µg/mL streptomycin (Lonza, Basel, SUI). Trypan blue exclusion assay was used to assess cell viability.

2.3 NAT2 genotyping and assignment of acetylator phenotype

Genomic DNA was isolated from peripheral blood using the Wizard Genomic DNA Purification Kit® (Promega Corporation, Madison, WI, USA). DNA was quantified spectrophotometrically using a Synergy® HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA) and stored at -80°C until use. Six SNPs were analyzed:

rs1041983 (282C>T), rs1801280 (341T>C), rs1799929 (481C>T), rs1799930 (590G>A), rs1208 (803A>G) and rs1799931 (857G>A). The genotyping assays were performed as previously described [34]. Briefly, A 20X primer and probe mix was supplied for each assay, and a 1X concentration of this mix as well as a half volume of 2X TaqMan® Genotyping Master Mix (Life Technologies, Waltham, MA, USA) and 10 ng/μL of the genomic DNA sample were added to each well. The thermal conditions of the experiment were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 90 s. The PCR amplification and endpoint reading was performed on a StepOne® real-time instrument (Life Technologies, Waltham, MA, USA). In each experiment, control samples with no DNA template were run to ensure that there was no amplification of contaminating DNA. Participants possessing two NAT2 alleles associated with rapid acetylation activity were classified as rapid acetylators; individuals possessing one of these alleles and one allele associated with slow acetylation activity were classified as intermediate acetylators, and those individuals that possessed two slow acetylation alleles were classified as slow acetylators.

2.4 NAT2 and SIRT1 protein and gene expression

NAT2 and SIRT1 expression were evaluated on PBMC using different combinations of monoclonal antibodies on the T, B or NK cell subpopulations as previously reported [24, 33, 34]. Briefly, to detect CD3, CD19 or CD56 molecules on plasmatic membrane an incubation step using the corresponding monoclonal antibodies, anti-CD3-PE, anti-CD19-PE or anti-CD56-PE was performed (BD Biosciences, San Jose, CA, USA). To detect NAT2 or SIRT1, the cells were treated with the commercial Fix/Perm Buffer Kit (eBioscience, Waltham, MA, USA) according to manufacturer recommendations. Next the cells were incubated with mouse anti-NAT2 polyclonal antibody (ab88443, Abcam, Cambridge, UK) for 2 h at 4°C or anti-SIRT1 monoclonal antibody (ab32441, Abcam, Cambridge, UK) for 30 min at 4°C, protected from light. Then, the cells were incubated with anti-mouse APC or anti-rabbit APC secondary antibody (eBioscience, Waltham, MA, USA) for 30 min at 4°C protected from light. Finally, cells were fixed with 1% paraformaldehyde, and the stained cells were analyzed by flow cytometry using a FACSCanto II instrument (BD Bioscience, San Jose, CA, USA). FACSDiva software (BD Bioscience, San Jose, CA, USA) was used to analyze the data and the results were expressed as the percentage of positive cells.

To evaluate the NAT2 and SIRT1 gene expression, total RNA purification was performed using the TRIzol® method. The concentrations and integrity of RNA were determined using a Synergy® HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA) and 10 ng for each RNA sample was used for producing cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA), according to manufacturer recommendations. cDNA (100 ng) was used to perform Real-Time PCR using the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Specific primers were used to obtain an amplification of the *NAT2* gene as follows: Fw: GATCACTTCCCTTG CAGACTTT and Rv: AGGCTGAATGCAATCCTCTTG; and *SIRT1* gene Fw: TGCTGGCCTAATAGAGTGGCA and Rv: CTCAGCGCCATGGAAAATGT. An initial incubation at 50°C was carried out for 2 min and then 94°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Control reagents for quantitation of the endogenous control, GAPDH, were used to determine Ct (NAT2 or SIRT1 Ct – GAPDH

Ct). Ct was determined by subtraction of the smallest Ct and relative amounts of NAT2 or SIRT1 mRNA were calculated using 2^{-Ct} as described previously [35]. All reactions were analyzed using CFX 96 Real-Time PCR (Bio-Rad Laboratories, Hercules, CA, USA).

2.5 Measurement of INH *N*-acetylation capacity *in situ*

The *N*-acetylation capacity of PBMC was evaluated as previously reported [24, 33]. Briefly, 2×10^5 previously purified PBMC were plated on Corning® Costar® 96-Well Cell Culture Plates (Millipore-Sigma, St. Louis, MO, USA), and cultured on pre-warmed DMEM media supplemented with 50 U/mL penicillin and 50 µg/mL streptomycin (Lonza, Basel, SUI). Cells were incubated with increasing concentrations of INH (10 – 100 µM) for 24 h at 37°C in a humidified atmosphere of 5% CO₂. After this time, media was removed, and protein precipitated with the addition of 30 µL of 10% trichloroacetic acid and centrifuged at 2700 ×*g* for 10 min. The resulting supernatant was used for the HPLC quantification of acetyl-INH formation. The determination of the number of live cells per sample was determined after INH incubation was completed using Alamar Blue cell viability protocol [36]. INH *N*-acetylation was reported with live cell numbers as nM of acetyl-INH over 24 h incubation per million cells.

The amount of acetyl-INH produced was determined following separation and quantitation by high-performance liquid chromatography (HPLC). Cell culture supernatants previously protein precipitated were injected into a Waters system with a multisolvent delivery system 1525, using a 717 Plus loop injector, a UV/VIS 2487 detector and breeze software V3.2 (Waters Corporation, Milford, MA, USA). The column used was X-Terra Shield RP18, 125 Å, 3.5 µm, 3 × 150 mm (Waters Corporation, Milford, MA, USA). The guard column was an X-Terra Shield RP18, 125 Å, 5 µm, 3 × 20 mm (Waters Corporation, Milford, MA, USA). The standards and chromatographic solvents were HPLC grade. The mobile phase consisted of 2.5 mM phosphate buffer and 30 mM 1-heptansulfonic sodium salt (Millipore-Sigma, St. Louis, MO, USA), as an ion pairing agent and the pH of this solution was adjusted to 2.7; this solution was combined with acetonitrile 90:10 (v/v) ratio. The flow rate was 0.5 mL/min. Absorbance was measured at 266 nm. Retention times for INH and acetyl-INH were 5.5 and 4.1 min, respectively. The concentration of each compound in the sample was calculated using respective calibration curves in the range from 1.0 to 80.0 ng/µL.

2.5.1 Assessment of Sirtuin effect on the *N*-acetylation activity of NAT2 *in situ*—PBMC (2×10^5) were plated on Corning® Costar® 96-Well Cell Culture Plates (Millipore-Sigma, St. Louis, MO, USA), and cultured on pre-warmed DMEM media supplemented with 50 U/mL penicillin and 50 µg/mL streptomycin (Millipore-Sigma, St. Louis, MO, USA). Cells were treated with increasing concentrations of the small molecule SIRT1 activity enhancer SRT1720 (0.1 – 1 µM) (Santa Cruz Biotechnology, Dallas, TX, USA), and incubated for 3 h at 37°C in a humidified atmosphere of 5% CO₂. Once this incubation period was concluded, culture media was removed and replaced with fresh pre-warmed DMEM media and the procedure described above was used to measure INH *N*-acetylation capacity.

2.5.2 SIRT1 silencing through PBMC electroporation with siRNA—To evaluate the effect of SIRT1 on NAT2 activity, we used a specific siRNA and quantified the formation of acetyl-INH by HPLC. PBMC (4×10^6)/well were cultured in DMEM media supplemented with 10% fetal calf serum, 50 U/mL penicillin and 50 µg/mL streptomycin (Lonza, Basel, SUI), and maintained at 37 °C in a humidified atmosphere of 5% CO₂ for 24 h. After this time, the PBMC were collected and washed with antibiotic and serum-free DMEM. The cells were resuspended in 200 µL of this media and incubated with a total of 6 µL of a 10 µM solution of anti-SIRT1 small interfering RNA (siRNA) (sc-40986, Santa Cruz Biotechnology, Dallas, TX, USA) or scramble siRNA (sc-37007, Santa Cruz Biotechnology, Dallas, TX, USA) for 5 min at 4°C. Afterward, the mixed siRNA-cell suspension was transferred to a previously cooled 4.0-mm electroporation cuvette. Cells were electroporated using a GenePulser Xcell electroporation device (Bio-Rad, Hercules, CA, USA): Square-Wave pulse, 300 V, 3 ms. After electroporation, PBMC were resuspended in DMEM supplemented media and cultured in 12-well plates for 24 h at 37°C in a humidified atmosphere of 5% CO₂. Transfection efficacy was verified by flow cytometry using the protocol described above. INH *N*-acetylation capacity was measured as described above.

2.5.3 CD3+ cell purification assay—CD3 purification of PBMC was performed with a CD3 MicroBeads kit (Miltenyi Biotec, Cologne, GER). Briefly, PBMC were centrifuged at $300 \times g$ for 10 min and the pellet resuspended in MACS® buffer. This suspension was incubated with CD3 MicroBeads antibody for 15 min at 4°C and then the cells were washed and centrifuged. The pellet was resuspended and magnetic separation performed using an MS MACS Column (Miltenyi Biotec, Cologne, GER) for a positive selection (CD3+ cells attach magnetically to the column whereas CD3- cells pass through the column). CD3+ and CD3- fractions were plated and cultured for INH *N*-acetylation capacity as described above.

2.6 Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). The results are expressed as mean $\pm$ the standard error (SEM) of the percentage of positive cells. Differences were analyzed for significance by Student's t-test or One-way ANOVA followed by Tukey-Kramer multiple comparisons tests. Linear regression analysis was used to identify correlations. Enzymatic kinetics parameters (K_M and V_{max}) were calculated using nonlinear regression analysis. Values of $p < 0.05$ were considered statistically significant. Unless specified in the corresponding section, data represent twenty independent experiments performed in duplicate.

3. Results

3.1 NAT2 genotyping and assignment of acetylator phenotype

A six NAT2 SNP panel was evaluated on our sample. Previous studies in Mexican population have demonstrated the accuracy of this panel to predict the acetylator phenotype [34, 37]. These SNPs allowed the identification of *NAT2*4* (reference), *NAT2*12A*, *NAT2*5B*, *NAT2*5C*, *NAT2*6A*, and *NAT2*7B*; according to the consensus nomenclature (<http://nat.mbg.duth.gr/>). *NAT2*4* and *NAT2*12A* are rapid acetylator alleles, whereas, *NAT2*5*, *NAT2*6* and *NAT2*7* families of haplotypes are slow acetylator alleles.

Individuals possessing two rapid acetylation alleles were classified as rapid acetylators, participants having one rapid and one slow allele were classified as intermediate acetylators, and individuals possessing two slow acetylation alleles were classified as slow acetylators. The frequency of NAT2 genotypes were 25% rapid, 30% intermediate and 45% slow acetylators. Three individuals (15%) showed a *NAT2*4/*4* genotype, two of the participants (10%) had *NAT2*4/*12A* genotypes. Three intermediate acetylator genotypes *NAT2*4/*7B*, *NAT2*5B/*12A* and *NAT2*4/*5B* were found, corresponding to 3 (15%), 2 (10%) and 1 (5%) of the individuals respectively. Three subjects (15%) had a *NAT2*5B/*6A* genotype, two participants (10%) showed *NAT2*5B/*5B* genotype, three individuals (15%) possessed a *NAT2*5C/*5C* genotype and 1 participant (5%) had a *NAT2*5B/*7B* genotype.

3.2 NAT2 and SIRT1 protein and mRNA expression on PBMC

NAT2 and SIRT1 protein expression on PBMC and subsets of lymphocytes as T lymphocytes (CD3+), B cells (CD19+) and NK cells (CD56+) were detected using flow cytometry. The percentage of cells expressing NAT2 was 41.7 (± 2.7) of the total PBMC. The NAT2 expression breakdown according to the subpopulations evaluated was as follows, 33.9 % (± 2.15) were NAT2+CD3+, 3.2 % (± 0.34) were NAT2+CD19+, and 4.6 % (± 0.51) were NAT2+CD56+. NAT2 protein levels were predominantly on T lymphocytes compared to B cells or NK cells ($p < 0.001$) (Fig. 1a). When we analyzed the protein expression related to NAT2 genotype, we found 56.6 % (± 2.02) NAT2 expression in the rapid acetylator individuals, 44.7 % (± 1.39) in the intermediate acetylators, and 31.4 % (± 2.86) in the slow acetylators. Differences were significant between rapid and intermediate acetylators ($p < 0.05$) and between intermediate and slow acetylators ($p < 0.01$) (Fig. 1b).

SIRT1 protein expression on PBMC was 55.6 % (± 2.00) of the total cells. Analyzing the subsets of cells, T cells expressing SIRT1 were 45.3 % (± 1.72); B cells expressing SIRT1 protein were 4.08 % (± 0.28), and NK cells expressing SIRT1 were 6.17 % (± 0.62). Significant statistical differences were found between the CD3+ cells expressing SIRT1 vs. the CD19+ and CD56+ cells ($p < 0.001$) (Fig. 1c). No significant ($p > 0.05$) differences in SIRT1 expression were noted among the rapid, intermediate, and slow acetylator NAT2 acetylator genotypes (Fig. 1d). No significant ($p > 0.05$) differences in NAT2 mRNA expression levels were noted among the rapid, intermediate, and slow acetylator NAT2 acetylator genotypes (Fig. 2).

3.3 Measurement of INH *N*-acetylation capacity *in situ*

PBMC were cultured in DMEM media for up to 24 h in the absence or presence of increasing concentrations of INH, *N*-acetylation capacity was NAT2-genotype dependent between 10 and 300 μM INH. The rapid acetylators showed an apparent K_M of 14.1 ± 2.7 μM and a V_{max} of 42.1 ± 2.4 nM Ac-INH/24h/million cells; the intermediate acetylators showed an apparent K_M of 11.7 ± 3.6 μM and a V_{max} of 22.6 ± 2.2 nM Ac-INH/24h/million cells; finally, the slow acetylators showed an apparent K_M 19.7 ± 5.1 μM and a V_{max} of 19.9 ± 1.7 nM Ac-INH/24h/million cells (Fig. 3). Significant differences were found between the apparent V_{max} of rapid vs. intermediate acetylators ($p < 0.001$), and between rapid vs. slow acetylators ($p < 0.0001$).

N-acetylation capacity correlated very highly with NAT2 protein expression across the 20 individual samples ($r^2 = 0.9075$, $p < 0.0001$, Fig. 4). This high significant correlation was observed within the rapid ($r^2 = 0.9151$, $p = 0.011$), intermediate ($r^2 = 0.8443$, $p = 0.009$), and slow acetylation phenotypes ($r^2 = 0.9318$, $p < 0.0001$).

NAT2 was primarily expressed on T cells, therefore CD3+ and CD3- fractions were obtained and assessed for NAT2 expression. Correlations between NAT2 protein expression and INH *N*-acetylation capacity were significantly correlated across all PBMC ($r^2 = 0.846$; $p < 0.0001$), as well as with the CD3+ ($r^2 = 0.91$; $p < 0.0001$) and CD3- ($r^2 = 0.783$; $p < 0.0001$) fractions.

3.4 Sirtuin 1 effect on *N*-acetylation capacity

Since the most abundant isoform of the Sirtuin family is SIRT1, the possible effect of this protein on the INH *N*-acetylation capacity in PBMC was examined in the presence and absence of the specific Sirtuin 1 activator (SRT1720) and by transient silencing of SIRT1 expression using a siRNA. In presence of increasing concentrations of SRT1720, INH *N*-acetylation capacity decreased in comparison to the untreated cells (Fig. 5a); treatment with 0.5 μM SRT1720 reduced apparent V_{max} to 20.4 ± 1.9 nM Ac-INH/24h/million cells (Fig. 5b, $p < 0.0001$) and the treatment with 1.0 μM SRT1720, decreased the apparent V_{max} of INH *N*-acetylation to 12.7 ± 0.8 nM Ac-INH/24h/million cells (Fig. 5b, $p < 0.0001$). SRT1720 did not result in significant ($p > 0.05$) effects in apparent K_M . The rapid acetylators showed an apparent K_M of 12.4 ± 1.9 μM and a V_{max} of 30.0 ± 1.0 nM Ac-INH/24h/million cells; the intermediate acetylators showed an apparent K_M of 8.78 ± 4.53 μM and V_{max} 9.93 ± 1.22 nM Ac-INH/24h/million cells, and the slow acetylators showed an apparent K_M of 10.6 ± 4.0 μM and V_{max} 8.55 ± 0.83 nM Ac-INH/24h/million cells. Statistical differences were found between the apparent V_{max} of rapid vs. intermediate acetylators ($p < 0.0001$), and rapid vs. slow acetylators (Fig. 5c, $p < 0.0001$). No significant ($p > 0.05$) effects were found for the apparent K_M .

Transient silencing of SIRT1 was achieved using a specific siRNA and the efficacy of the silencing is shown in Fig. 6a; after 24h of incubation of the PBMC in the presence of the siRNA for SIRT1, the protein was expressed in less than 10% of the cells; at 48h of incubation, the protein expression doubled. Thus, subsequent experiments were carried out after 24h of SIRT1 siRNA incubation. INH *N*-acetylation capacity following SIRT1 siRNA incubation was increased compared to the untreated cells ($p < 0.01$, Fig. 6b). Treated cells showed an apparent K_M of 20.8 ± 7.2 μM , and V_{max} of 58.5 ± 6.8 nM Ac-INH/24h/million cells which differed significantly from the untreated cells ($p = 0.0002$). No significant effects were observed in apparent K_M .

4. Discussion

NAT2 is a cytosolic protein that can acetylate a wide variety of arylamine and hydrazine compounds [38]. Differences in response to the anti-tuberculosis therapy between patients [39, 40] led to the description of rapid and slow acetylation phenotypes. Several genetic polymorphisms have been described, resulting in a trimodal distribution of the acetylator phenotype [41], classifying each subject as a rapid, intermediate or slow acetylation

phenotype based on the presence of specific single nucleotide polymorphisms (SNPs). The relevance of the presence of SNPs resides in the fact that increased risk for drug toxicity, therapeutic failure or carcinogenesis have been correlated with the presence of this genetic polymorphisms and acetylation phenotype [20] including genetic heterogeneity within the NAT2 slow acetylator phenotype [42]. The presence of slow acetylation activity SNPs has been linked to the risk of developing type 2 diabetes [7, 8]. The sample of healthy volunteers participating on this study showed a high prevalence of a slow acetylation phenotype consistent with previous reports with larger samples of Mexican [34, 37] and Central American populations [43]. This can be attributed to the admixture effect present on American mestizo groups [44, 45].

Whereas NAT1 is widely expressed in different tissues, NAT2 expression is thought to be limited to the liver, small intestine and colon [46]. Previous reports on blood cells have found only NAT1 enzymatic activity, but not NAT2 [47, 48]; one report demonstrated the *N*-acetylation activity of sulfamethazine on whole blood [49] however the investigators did not find a difference between rapid and slow acetylators.

NAT2 protein expression was measured on PBMC and identified on 41% of the cells. The vast majority of NAT2 expression (81%) was present on T cells, consistent with previous reports [34]. Significant differences in NAT2 protein expression were associated with *NAT2* genotype (rapid>intermediate>slow acetylators). This constitutes the first report of NAT2 protein expression in PBMC correlated to the NAT2 acetylator genotype. Although, NAT2 mRNA expression did not correlate with *NAT2* genotype. Approximately 82% of SIRT1 protein was expressed on the T cells consistent with one of the functions of SIRT1 during T cell differentiation [50]. As expected, SIRT1 protein expression was not associated with NAT2 acetylation phenotype.

NAT2 genotype-dependent *N*-acetylation of INH [51] and hydralazine [52] has been described in cell cultures of cryopreserved human hepatocytes. We found that INH *N*-acetylation capacity on PBMC was time, dose, and NAT2 genotype-dependent in cultures of PBMC.

In mammals, seven sirtuins (SIRT1–SIRT7), have been identified. The first sirtuin protein characterized was Silent Information Regulator 2 (Sir2) from *Saccharomyces cerevisiae*. First described as a chromatin-silencing component, several studies demonstrated its beneficial effects on calorie restriction, health, and longevity in different species [53]. SIRT1 is the human ortholog of Sir2, a nuclear/cytosolic enzyme with an important role on the metabolism, immune response, cell differentiation and cancer, where different studies suggest a role for SIRT1 as a tumor suppressor or as an oncogenic factor on different physiological conditions [12, 50, 54–56]. SIRT1 acts as a lysine deacetylase which has been shown to be an important post-translational modification for cytosolic proteins [57]. The SIRT1 function can be modified in the presence of specific inhibitors and enhancers. In the present study, deacetylase activity of SIRT1 was enhanced using SRT1720, a specific SIRT1 activator [58]. We found that by activation of SIRT1 deacetylation activity, the INH *N*-acetylation capacity was decreased in a dose-dependent manner with increasing concentrations of SRT1720. The effect of SRT1720 was observed in rapid, intermediate, and

slow acetylator genotypes. On the other hand, when SIRT1 deacetylation activity was silenced using specific siRNA an increase in the INH *N*-acetylation capacity was observed.

Our results suggest NAT2 is a target for SIRT1 with potential implications for different physiological processes as well as tumorigenesis or toxicity following drug and carcinogen exposures. Similar SIRT1 deacetylation regulation mechanisms have been described for other proteins or transcription factors such as Foxp3 [59] and PGC-1 α [60]. Our data show that INH *N*-acetylation capacity on PBMC is NAT2 genotype-dependent and is primarily carried out by CD3+ cells.

In summary, the results presented here are the first report of NAT2 genotype-dependent expression on PBMC and post-translational modification by SIRT1. These findings constitute a substantial advance in our understanding of human *N*-acetyltransferase expression and a new much less invasive method for measurement of human NAT2 expression and phenotype.

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Fig. 1.

NAT2 expression on PBMC. **a.** NAT2 total protein expression is plotted as percentage of NAT2-positive cells for all PBMC (filled circle), T cells (filled square), B cells (filled triangle), NK cells (inverted filled triangle). The data presented are the mean $\pm$ SEM of twenty independent experiments, *** $p < 0.001$ (One-way ANOVA, Tukey-Kramer multiple comparison post-hoc test). **b.** NAT2 protein expression according to NAT2 predicted phenotype; rapid acetylators (filled circle), intermediate acetylators (filled square), and slow acetylators (filled triangle). The data presented are the mean $\pm$ SEM of twenty independent experiments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (One-way ANOVA, Tukey-Kramer multiple comparison post-hoc test). **c.** SIRT1 total protein expression is plotted as percentage of SIRT1-positive cells for all PBMC (filled circle), T cells (filled square), B cells (filled triangle), NK cells (inverted filled triangle). The data presented are the mean $\pm$ SEM of twenty independent experiments, *** $p < 0.001$ (One-way ANOVA, Tukey-Kramer multiple comparison post-hoc test). **d.** SIRT1 protein expression according to NAT2 predicted phenotype; rapid acetylators (filled circle), intermediate acetylators (filled square), and slow

acetylators (filled triangle). The data presented are the mean $\pm$ SEM of twenty independent experiments (One-way ANOVA, Tukey-Kramer multiple comparison post-hoc test).

Fig. 2. NAT2 mRNA expression. Relative expression of NAT2 mRNA in rapid (filled square), intermediate (filled triangle), and slow acetylators (inverted filled triangle) PBMC. The data presented are the mean $\pm$ SEM of twenty independent experiments (One-way ANOVA, Tukey-Kramer multiple comparison post-hoc test).

Fig. 3.

NAT2 genotype-dependent *N*-acetylation of INH *in situ*. PBMC were cultured in the presence of INH as described in materials and methods. Quantification of acetyl-INH formation on PBMC culture media supernatants were detected by HPLC. The data presented are the mean $\pm$ SEM of twenty independent experiments for NAT2 rapid (filled circle), intermediate (filled square), and slow (filled triangle) subjects. The rapid acetylator subjects showed an apparent INH K_M of $14.1 \pm 2.7 \mu\text{M}$ and a V_{max} of $42.1 \pm 2.4 \text{ nM Ac-INH/24h/million cells}$; the intermediate acetylator subjects showed an apparent K_M of $11.7 \pm 3.6 \mu\text{M}$ and a V_{max} of $22.6 \pm 2.2 \text{ nM Ac-INH/24h/million cells}$; the slow acetylator subjects showed an apparent K_M $19.7 \pm 5.1 \mu\text{M}$ and a V_{max} $19.9 \pm 1.7 \text{ nM Ac-INH/24h/million cells}$. The apparent V_{max} in NAT2 intermediate and slow acetylator subjects was significantly lower compared to rapid acetylators, *** $p < 0.001$ (Non-linear regression).

Fig. 4.
Correlation of NAT2 protein expression vs INH *N*-acetylation in PBMC. (Linear regression).

Fig. 5.

Effect of SIRT1 activity enhancing on INH *N*-acetylation activity. **a.** PBMC were treated in presence of SRT1720 and cultured in presence of INH as described in materials and methods. Quantification of acetyl-INH formation on PBMC culture media supernatants were detected by HPLC. The data presented are the mean $\pm$ SEM of twenty independent experiments, *** $p < 0.001$ (One-way ANOVA, Tukey-Kramer multiple comparison post-hoc test). **b.** Comparison between untreated cells (filled circle), cells treated with 0.1 μ M SRT1720 (filled square), 0.5 μ M SRT1720 (filled triangle), and 1.0 μ M SRT1720 (inverted filled triangle). Cells treated with 0.5 μ M SRT1720 showed an apparent $V_{\max}$ of 20.4 ± 1.9 nM Ac-INH/24h/million cells; cells treated with 1.0 μ M of SRT1720, decreased as well as the $V_{\max}$ for INH *N*-acetylation was reduced to 12.7 ± 0.8 nM Ac-INH/24h/million cells. The data presented are the mean $\pm$ SEM of twenty independent experiments, **** $p < 0.0001$ (Non-linear regression). **c.** INH *N*-acetylation in rapid (filled square), intermediate (filled triangle) and slow (inverted filled triangle) acetylators subjects following treatment with 1.0 μ M SRT1720. Rapid acetylators showed an apparent $V_{\max}$ of 30.0 ± 1.3 nM Ac-INH/24h/million cells; intermediate acetylators showed an apparent $V_{\max}$ of 9.93 ± 1.22 nM Ac-INH/24h/million cells; slow acetylators showed an apparent $V_{\max}$ of 8.55 ± 0.83 nM Ac-INH/24h/million cells. The apparent $V_{\max}$ in NAT2 intermediate, and slow acetylators was significantly lower compared to rapid acetylators ($p < 0.0001$). The data presented are the mean $\pm$ SEM of twenty independent experiments, **** $p < 0.0001$ (Non-linear regression).

Fig. 6.

Effect of SIRT1 activity silencing on INH *N*-acetylation. **a.** SIRT1 expression silencing on PBMC using siRNA. Protein expression was significantly lower after 24h treatment. The data presented are the mean $\pm$ SEM of three independent experiments, *** $p < 0.001$ (One-way ANOVA, Tukey-Kramer multiple comparison post-hoc test). **b.** PBMC were treated with SIRT1 siRNA and cultured in presence of INH as described in materials and methods. Quantification of acetyl-INH formation on PBMC culture media supernatants were detected by HPLC. Increased INH *N*-acetylation was observed for the treated cells vs the untreated. The data presented are the mean $\pm$ SEM of three independent experiments, ** $p < 0.01$ (One-way ANOVA, Tukey-Kramer multiple comparison post-hoc test).