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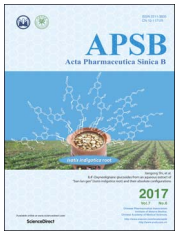

ORIGINAL ARTICLE

A pilot study of the modulation of sirtuins on arylamine *N*-acetyltransferase 1 and 2 enzymatic activity

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Abstract Arylamine *N*-acetyltransferase (NAT; E.C. 2.3.1.5) enzymes are responsible for the biotransformation of several arylamine and hydrazine drugs by acetylation. In this process, the acetyl group transferred to the acceptor substrate produces NAT deacetylation and, in consequence, it is susceptible of degradation. Sirtuins are protein deacetylases, dependent on nicotine adenine dinucleotide, which perform post-translational modifications on cytosolic proteins. To explore possible sirtuin participation in the enzymatic activity of arylamine NATs, the expression levels of NAT1, NAT2, SIRT1 and SIRT6 in peripheral blood mononuclear cells (PBMC) from healthy subjects were examined by flow cytometry and Western blot. The *in situ* activity of the sirtuins on NAT enzymatic activity was analyzed by HPLC, in the presence or absence of an agonist (resveratrol) and inhibitor (nicotinamide) of sirtuins. We detected a higher percentage of positive cells for NAT2 in comparison with NAT1, and higher numbers of SIRT1+ cells compared to SIRT6 in lymphocytes. *In situ* NAT2 activity in the

Abbreviations: Ac-INH, acetyl-Isoniazid; Ac-PABA, acetyl-*p*-aminobenzoic acid; APC, allophycocyanin; CHO, Chinese hamster ovary cells; DMEM, Dulbecco's modified Eagle's medium; E2F1, E2F transctriptios factor 1; ER81, ETS-related protein 81; FITC, fluorescein IsoTioCyanate; FOXO1, forkhead box protein O1; HeLa, adenocarcinoma epithelial cells; HPLC, high performance liquid chromatography; INH, isoniazid; NAD, nicotinamide adenine dinucleotide; NAM, nicotinamide; NAT, arylamine *N*-acetyltranferase; PABA, *p*-aminobenzoic acid; PAS, *p*-aminosalicilic acid; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PGAM1, phosphoglycerate mutase 1; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator 1α; RSV, resveratrol; RUNX3, runt-related transcription factor 3; SIRT, sirtuin; SMZ, sulfamethazine; SREBP1a, sterol regulatory element-binding protein 1a; SREBP2, sterol regulatory element-binding protein 2

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presence of NAM inhibitors was higher than in the presence of its substrate, but not in the presence of resveratrol. In contrast, the activity of NAT1 was not affected by sirtuins. These results showed that NAT2 activity might be modified by sirtuins.

1. Introduction

Acetylation is the major biotransformation pathway for arylamine and hydrazine drugs with pharmacological/toxicological relevance, and it is catalyzed by arylamine *N*-acetyltransferases (NATs; E.C. 2.3.1.5) enzymes[1,2]. NAT1 and NAT2 are phase II cytosolic enzymes that transfer an acetyl group from acetyl CoA to a xenobiotic substrate (arylamine, aromatic, heterocyclic or hydrazine compounds)[3]. In spite of the high similarity at genetic structure and protein level, these two enzymes have different specificity of substrate and tissue expression. *p*-Aminobenzoic acid (PABA), *p*-aminosalicylic acid (PAS) and *p*-aminobenzoyl glutamic acid are specific substrates for human NAT1, and show wide tissue distribution[4,5]. Conversely, NAT2 has a more restricted distribution and expression to the liver, intestinal epithelium and colon. The known targets of this enzyme are: sulfamethazine (SMZ), isoniazid (INH), procainamide and dapsone. Moreover, other compounds function as substrates for both enzymes, *e.g.* 2-aminofluorene[6].

NATs can be regulated at transcriptional, post-transcriptional and post-translational levels. These enzymes share three domains, the domains I and II are more conserved between NAT enzymes than domain III and have a conserved catalytic triad composed of three residues: Cysteine, Histidine and Aspartate; in their functional structure, which forms part of the active site[1,2]. Regarding the post-translational regulation of NATs, the active site Cysteine of these enzymes is acetylated in the absence of substrate, which makes it more resistant to proteasomal degradation[1]. In contrast, the non-acetylated form of the protein is sensitive to polyubiquitination that leads to its degradation by the proteasome[7]. It has been reported that acetylation of the active site cysteine (Cys68) defines the stability of NAT1. On the other hand, acetylation of NAT, like many cytosolic proteins, occurs in different amino acid residues across the entire structure, this prevents the protein from being degraded or having a greater half-life. Nevertheless, the acetyl groups have to be removed from the protein in order to maintain the equilibrium in the cell. In this mechanism of regulation some deacetylases proteins participate such as sirtuins in the specific case of lysine residues[8].

The sirtuin family includes seven (SIRT1–SIRT7) deacetylase/ADP ribosyltransferase proteins that vary in cellular localization, tissue specificity, enzymatic activity and protein substrates. They are involved in post-translational modifications by deacetylation (SIRT1, 2, 3, 5 and 6) or ADP ribosylation (SIRT4, 5 and 6) and play an important regulatory role in many biological processes[9]. Sirtuins catalyse the deacetylation of lysine residues of target proteins using nicotinamide adenine dinucleotide (NAD^+) as a cofactor and liberating nicotinamide which, in high concentrations, is able to bind to sirtuin in a non-competitive form, and by a feedback-loop mechanism, inhibit its activity[10].

Due to widespread participation of sirtuins in many physiological processes such as metabolism, stress and ageing[11], it has been suggested that NAT1 and NAT2 could be affected by sirtuin-regulated deacetylation. Positive modulation of SIRT1 mRNA expression was able to prevent the hepatotoxicity induced by INH and rifampicin in mice[12]. Since NAT is the main metabolizing enzyme of INH, it is not surprising to suggest that NAT could be a target of SIRT1 causing, therefore, a decrease in NAT activity due to an increase of the deacetylation process generated by SIRT1 overexpression. To date it is unknown whether sirtuins are able to regulate NAT activity and the consequences of this regulation on immune cells. Therefore, this field needs to be explored.

Given the fact of NATs and SIRT1 and 6, are present in the same subcellular compartment, in this study we investigated the expression and the effects of sirtuins on the in situ enzymatic activity of NAT1 and NAT2 in peripheral blood mononuclear cells (PBMC). We found higher expression and activity of NAT2 compared to NAT1 in PBMC. The results from the current study provide the first evidence of possible modulation for NAT2, but not for NAT1, by the inhibition of sirtuin activity, which might have important implications for the cellular functions of NAT2.

2. Materials and methods

2.1. Population

A total of 17 healthy subjects, ranging between 20 and 32 years of age, were recruited for this study. Nine of them were males and eight females. For this group, biochemical parameters, such as glucose and triglycerides were measured. Subjects with infectious and/or autoimmune diseases and with antibiotic therapy, alcohol consumption, tobacco or illicit drugs were excluded. The Bioethics Committee of the Autonomous University of San Luis Potosi approved this work and all participants signed a written informed consent form.

2.2. Isolation of peripheral blood mononuclear cells

Blood samples were collected in 8 mL EDTA Vacutainer tubes (BD Biosciences, CA, USA) for expression and enzymatic activity analysis. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation. Blood was diluted with an equal volume of phosphate-buffered saline (PBS) pH 7.3, overlaid on layered Ficoll-Histopaque (Sigma, St. Louis, MO, USA) and centrifuged at 2500 rpm ($500 \times g$) for 20 min at 25 °C. The PBMC layer was removed and washed twice with PBS and resuspended in Dulbecco's modified Eagle's medium (DMEM) culture medium at 2×10^6 cells/mL. Media were supplemented with 10% fetal calf serum, 50 U/mL penicillin and 50 μg/mL streptomycin (Sigma). Cell viability was assessed by trypan blue exclusion assay.

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