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SIRT1 contributes to aldose reductase expression through modulating NFAT5 under osmotic stress: *In vitro* and *in silico* insights

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ABSTRACT

So far, a myriad of molecules were characterized to modulate NFAT5 and its downstream targets. Among these NFAT5 modifiers, SIRT1 was proposed to have a promising role in NFAT5 dependent events, yet the exact underlying mechanism still remains obscure. Hence, the link between SIRT1 and NFAT5-aldose reductase (AR) axis under osmotic stress, was aimed to be delineated in this study. A unique osmotic stress model was generated and its mechanistic components were deciphered in U937 monocytes. In this model, AR expression and nuclear NFAT5 stabilization were revealed to be positively regulated by SIRT1 through utilization of pharmacological modulators. Overexpression and co-transfection studies of NFAT5 and SIRT1 further validated the contribution of SIRT1 to AR and NFAT5. The involvement of SIRT1 activity in these events was mediated via modification of DNA binding of NFAT5 to AR ORE region. Besides, NFAT5 and SIRT1 were also shown to co-immunoprecipitate under isosmotic conditions and this interaction was disrupted by osmotic stress. Further *in silico* experiments were conducted to investigate if SIRT1 directly targets NFAT5. In this regard, certain lysine residues of NFAT5, when kept deacetylated, were found to contribute to its DNA binding and SIRT1 was shown to directly bind K282 of NFAT5. Based on these *in vitro* and *in silico* findings, SIRT1 was identified, for the first time, as a novel positive regulator of NFAT5 dependent AR expression under osmotic stress in U937 monocytes.

1. Introduction

NFAT5, a member of Rel family proteins, is a transcription factor activated upon osmotic stimuli [1]. When cells are exposed to osmotic stress, NFAT5 activates transcription of several osmoadaptive factors that are responsible for the accumulation of compatible osmolytes including betaine via BGT1, myo-inositol by SMIT and sorbitol by AR [1,2]. Osmotic stress dependent activation of NFAT5 was suggested to be mediated by a number of different mechanisms. Osmotic stress increases expression of NFAT5 protein and mRNA levels [3,4], causes nuclear translocation of NFAT5 [3,4], activates transactivation domain of NFAT5 [5] and increases phosphorylation of NFAT5 [6]. Other than phosphorylation, NFAT5 was proposed to be palmitoylated in which depalmitoylation was shown to accelerate its nuclear translocation [7]. In addition, sumoylation of NFAT5 was revealed to inhibit its transactivation [8]. NFAT5 was also documented to be part of a bulky complex, which consists of several other partners, such as catalytic subunit of PKA [9], ATM [10], RNA Helicase A [11], TAZ [12], FSP27 [13], β-catenin [14], AP-1 [15], HSP-90 [16] and PARP1 [16]. Among these interacting partners, PARP1, an inhibitor of transcriptional activity of NFAT5 [16], catalyzes poly (ADP-ribosyl)ation of proteins, as well as plays a role in DNA repair mechanism using NAD^+ as cofactor [17]. In this regard, PARP1 has also been shown to influence several intracellular pathways, reciprocally with a deacetylase, SIRT1 due to utilization of common cofactor NAD^+ [18–20]. Considering these evidences, a possible SIRT1 driven regulation of NFAT5 remains to be elucidated.

SIRT1 is a NAD^+ dependent histone/protein deacetylase which has been implicated in a wide array of cellular events including starvation, inflammation, oxidative stress and senescence [21]. SIRT1 mediates these cellular events in part through directly deacetylating several transcription factors such as p53 [22], FOXOs [23], Egr-1 [24], p65 [25], and NFATc3 [26]. Among these transcription factors, Rel family members p65 and NFATc3 were identified to be negatively regulated by direct SIRT1 dependent deacetylation [25,26]. Yet, presence of such mechanism and its consequence in another Rel family member, NFAT5, remain unsettled. Likewise, NFAT5 was proposed to be a candidate enzymatic target of SIRT1 in a stable isotope labeling with amino acids in cell culture (SILAC) method based study [27]. Nonetheless, the intracellular machinery of such targeting has also not been considered until now.

Abbreviations: AK, acetyllysine; AP-1, activator protein 1; AR, aldose reductase; BGT1, betaine/gamma-aminobutyric acid transporter; Egr-1, early growth response protein 1; FSP27, fat-specific protein 27; HSP90, heat shock protein 90; NAD^+, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NFAT5, nuclear factor of activated T-cells 5; ORE, osmotic response element; p38, mitogen-activated protein kinase p38; p53, cellular tumor antigen p53; p65, transcription factor p65; PARP1, poly (ADP-ribose) polymerase 1; PKA, protein kinase A; SIRT1, sirtuin 1; SMIT, sodium-myo-inositol transporter; TAZ, transcriptional coactivator with PDZ-binding motif.

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More recently, NFAT5 and SIRT1 have been anticipated to work synergistically under osmotic stress towards downregulation of prorenin receptor expression, supporting an influence of SIRT1 activity towards NFAT5 [28]. Based on these previous findings, the possibility of SIRT1 based regulation NFAT5 has warranted further studies.

As one of the canonical targets of NFAT5, AR (E.C: 1.1.1.21), the rate limiting enzyme of polyol pathway, catalyzes reduction of the glucose to sorbitol, along with the reduction of atherogenic aldehydes, steroids, phospholipid lipid aldehydes and their glutathione conjugates [29–31]. Reduction of glucose to sorbitol by AR, has been established to be osmoprotective in the cells of inner medulla of kidney [32–34]. Excess glucose flux through AR was also linked to secondary complications of diabetes such as atherosclerosis, nephropathy, neuropathy and retinopathy [35–38]. These complications have been reported to be mediated in part, by excess AR activity dependent depletion of NADPH, which is utilized in cellular protection against oxidative stress by glutathione reductase/glutathione peroxidase system [30,31,39]. Besides these well-recognized consequences of AR activity in progression of diabetic complications, SIRT1 has also been suggested to act as a protective element against these complications [40–43]. In a recent study on diabetic human AR overexpressing mice, excess AR flux was proposed to diminish SIRT1 activity, providing presence of a metabolic link between these proteins [44]. Regardless of these previous studies linking SIRT1 and AR, involvement of SIRT1 in regulation of AR expression in cells under osmotic stress remains ambiguous, which also prompted this study.

To the best of our knowledge, this is the first report investigating the contribution of SIRT1 on NFAT5 dependent AR expression in monocytes under osmotic stress. An osmotic stress model with concurrent expression of NFAT5 and SIRT1, was generated in U937 monocytes. Essential mechanistic details of the model were established by analyzing intracellular localization of NFAT5 and SIRT1, AR expression and oxidative stress. In this model, contribution of SIRT1 activity on AR expression, as well as stabilization of nuclear NFAT5, were explored using pharmacological modulators. By utilizing overexpression and co-transfection of NFAT5 and SIRT1 in HeLa cells, impact of SIRT1 activity on AR and NFAT5 was validated, while its role on DNA binding activity of NFAT5 was also demonstrated. Using co-immunoprecipitation method, the influence of osmotic stress on NFAT5–SIRT1 interaction was deciphered to comprehend the model more thoroughly. Possibility of direct interaction between NFAT5 and SIRT1 was also investigated via *in silico* analysis. In this regard, deacetylation favored lysine residues of NFAT5 and their binding to SIRT1 substrate binding site was evaluated. Among these lysines, K282 was proposed as the most plausible candidate for SIRT1 activity. In summary, here, for the first time, evidence on identification of novel intracellular SIRT1 target, NFAT5 dependent AR expression, was described.

2. Materials & methods

2.1. Cell culture and treatments

U937, human histiocytic lymphoma cell line, was obtained from Professor Giuseppe Poli, cultured in RPMI-1640 with 5 mM glucose, 10% FBS, 2 mM glutamine and 100 IU/ml penicillin/streptomycin. Cultures were maintained at 37 °C in a humidified incubator at an atmosphere of 5% CO_2. Before each experiment, cells were collected by centrifugation at 300 *g* for 5 min, resuspended in serum free medium (SFM) and seeded (1,000,000 cells/ml) in 96-well, 6-well, 12-well, 60 mm or 100 mm culture plates depending on the experiment. In all U937 experiments, osmotic agents were applied to cells from main stocks prepared in SFM and an equal amount of SFM was added to the control group. Actual rise in osmolality of the medium was validated using an osmometer (Osmomat 030, Gonatech, Berlin, Germany). Pretreatment with activators and inhibitors was done 1 h prior to applying osmotic stress agent. For each pretreatment experiment, DMSO (max 0.5%, v/v) was added to all controls. HeLa cells were cultured in DMEM supplemented with 5 mM glucose, 10% FBS and 100 IU/ml penicillin/streptomycin, maintained in a humidified incubator similar to U937 cells. In all HeLa experiments, osmotic agent was applied to cells from main stock prepared in culture medium.

2.2. Antibodies, kits and reagents

RPMI-1640, DMEM, FBS and antibiotics were purchased from Pan Biotech GmbH (Aidenbach, Germany). MTT Cell Proliferation Kit I, X-tremeGENE 9 DNA transfection reagent, positively charged nylon membranes, protease and phosphatase inhibitor cocktails were purchased from Roche (F. Hoffman-La Roche Ltd., Basel, Switzerland). FITC tagged Annexin V was purchased from Alexis Biochemicals (Enzo Life Sciences Inc., Farmingdale, NY, USA). NFAT5 (H-300), SIRT1 (H-300) and AR (G-1) antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, Texas, USA). SIRT1 (1 F3), PARP1, myc, Lamin A/C, Beta-Actin, acetyl-lysine, HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Dynabeads Protein G and chemiluminescent nucleic acid detection module kit were purchased from Life Technologies (Thermo Fisher Scientific Inc., Waltham, MA USA). AR ORE probe was purchased from Integrated DNA Technologies (Coralville, Iowa, USA). NaCl, glucose, mannitol, tris, glycine, and Tween-20 were purchased from Molekula Ltd. (Newcastle Upon Tyne, UK). All other antibodies and chemicals were obtained from Sigma (Darmstadt, Germany), unless otherwise stated.

2.3. Metabolic activity, cell death and oxidative stress assays

For metabolic activity assay, U937 cells were seeded in 96-well plates, treated as indicated and analyzed by MTT Cell Proliferation Kit I according to the manufacturer's instructions. For cell death assay, U937 cells were seeded in 12-well plates, treated as indicated and FITC tagged Annexin V staining was performed according to the manufacturer's protocol. Cells were then quantified by FACS (FACSCanto, Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed by Flowjo software. For oxidative stress assay, U937 cells were seeded in 12 well plates, treated as indicated and stained with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min at 37 °C. Cells were then collected into flow cytometry tubes, pelleted, washed and resuspended in PBS, analyzed by flow cytometry similar to the analysis of cell death assay.

2.4. Protein extraction and immunoblotting

For total protein extraction, cells were treated as indicated in 6-well, 60 mm or 100 mm culture plates and harvested by centrifugation at 300 *g* for 5 min. Cells were then resuspended in 1 ml of ice-cold PBS and transferred into 1.5 ml microcentrifuge tubes and spun at 13,200 rpm for 30 s. Pellet was lysed by incubation in total cell lysis buffer containing 50 mM Tris–HCl (pH: 8.0), 150 mM NaCl, 1% Nonidet P-40 (v/v), 1 mM phenylmethylsulfonyl fluoride (PMSF), protease and phosphatase inhibitors for 30 min, followed by centrifugation at 13,200 rpm for 10 min. Supernatant was collected as total protein extract and stored in −80 °C for immunoblotting analysis. For cytoplasmic–nuclear extraction, cells were treated as indicated in 6-well, 60 mm or 100 mm culture plates and harvested by centrifugation at 300 *g* for 5 min. Cells were then resuspended in 1 ml of ice-cold PBS and transferred into 1.5 ml microcentrifuge tubes and spun at 13,200 rpm for 30 s. For cytoplasmic extraction, pellet was first lysed by incubation in T1 buffer containing 10 mM Hepes-KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1% Nonidet P-40 (v/v), 1 mM DTT, 0.5 mM PMSF, protease and phosphatase inhibitors for 10 min, followed by centrifugation at 13,200 rpm for 10 min. Supernatant was collected as cytoplasmic extract and stored in −80 °C for immunoblotting analysis. For nuclear extraction, remaining pellet was resuspended in T2 buffer containing 50 mM Hepes-KOH, 2 mM $MgCl_2$,