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SIRT5 desuccinylates and activates SOD1 to eliminate ROS

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ABSTRACT

Cu/Zn superoxide dismutase (SOD1) is a key antioxidant enzyme. Deficiency of SOD1 is associated with various human diseases, including cancer. Here, we report that SOD1 is succinylated and that succinylation decreases its activity. SIRT5 binds to, desuccinylates and activates SOD1. SOD1-mediated ROS reduction is increased when SIRT5 is co-expressed. Furthermore, mutation of the SOD1 succinylation site inhibits the growth of lung tumor cells. These results reveal a novel post-translational regulation of SOD1 by means of succinylation and SIRT5-dependent desuccinylation, which is important for the growth of lung tumor cells.

1. Introduction

Reactive oxygen species (ROS) are generated as a result of normal intracellular metabolism in mitochondria and peroxisomes and from a variety of cytosolic enzyme systems [1]. In addition, a number of external agents can trigger ROS production [2]. A sophisticated intracellular enzymatic and non-enzymatic antioxidant defense system including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) counteracts and regulates overall ROS levels to maintain physiological homeostasis [3]. Low levels of ROS contribute to cell signaling and cell proliferation. Because of their highly reactive nature, however, excess ROS cause various damage to a range of cellular constituents, including proteins, lipids and, in particular, DNA, which may lead to cell death or to an acceleration in aging and age-related diseases [2]. In addition to these effects, a rise in ROS levels may also constitute a stress signal that activates specific redox-sensitive signaling pathways [3]. Once activated, these diverse signaling pathways may have either damaging or antioxidative functions.

Superoxide dismutases (SODs) are a class of enzymes that catalyze the detoxification of superoxide into oxygen and hydrogen peroxide, which is then converted to oxygen and water by Catalase [4]. SOD is believed to be present in all organisms that metabolize oxygen and is well conserved throughout evolution [4]. The physiological role of SOD is to balance the level of intracellular ROS. Most eukaryotes express two intracellular SODs, a Mn-containing SOD2 in the mitochondrial matrix [5] and a highly abundant Cu/Zn SOD1, which is largely cytosolic but is also found in the mitochondrial intermembrane space (IMS) [6,7]. SOD1-deficient mice ($SOD1^{-/-}$) have higher incidences of liver cancer compared with wild-type mice [8], which indicates an important role for SOD1 in tumor initiation and progression.

There have been several papers reporting that acetylation regulates antioxidative reactions [9–11]. In this study, we report that SOD1 is succinylated and that succinylation decreases its activity. We also found that SIRT5 binds to, desuccinylates and activates SOD1. SOD1-mediated ROS reduction is increased when SIRT5 is co-expressed. Furthermore, mutation of the SOD1 succinylation site inhibits the growth of lung tumor cells.

2. Materials and methods

2.1. Cell lysis and immunological procedures

Cells were lysed in an NP40 buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na_3VO_4 and 1 mM PMSF for 30 min and cell lysate was centrifuged at 13,000 rpm for 15 min. The supernatant was subjected to immunoprecipitation with Flag beads and then western analysis. Western blot was performed according to standard methods. Antibodies specific to Flag (Sigma), HA (Santa Cruz), actin (Sigma), SOD1 (Cell Signaling), SIRT5 (Abcam), pan anti-succinyllysine (Hangzhou Jingjie biotechnology

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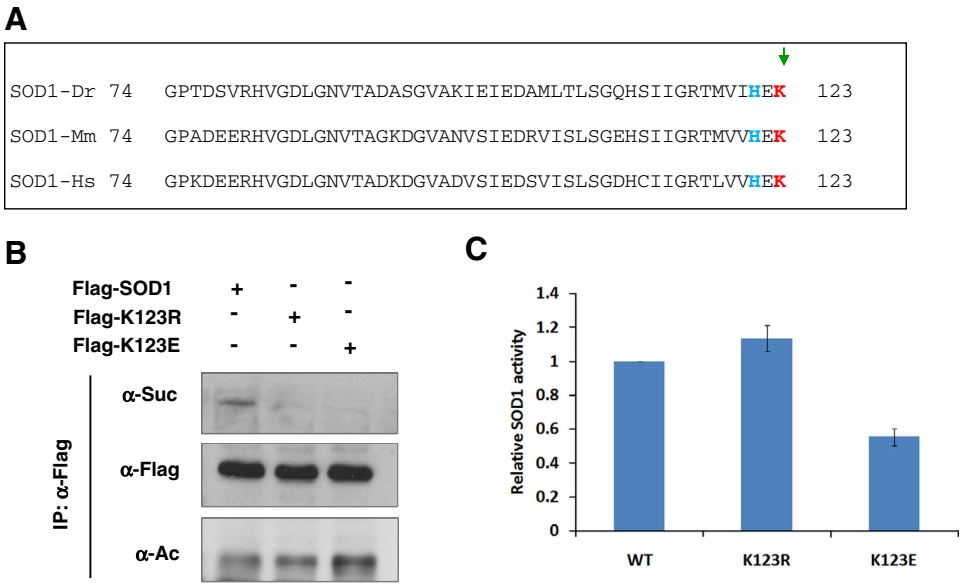

Fig. 1. Succinylation of K123 reduces SOD1 activity. (A) Amino acid sequence alignment of SOD1. 50 Amino acids (74–123) from human (Hs; *Homo sapiens*), two representative vertebrates: mouse (Mm; *Mus musculus*) and zebrafish (Dr; *Danio rerio*), are aligned. (B) K123 is the succinylation site of SOD1. Wild-type SOD1 and K123 mutants were expressed in HEK293T cells, affinity purified and analyzed by Western blot. (C) The succinylation mimetic mutation at K123 decreases SOD1 activity. Wild-type SOD1 and K123 mutants were expressed in HEK293T cells, affinity purified and subjected to the enzyme assay. Error bars represent ±SD for quadruplicate experiments.

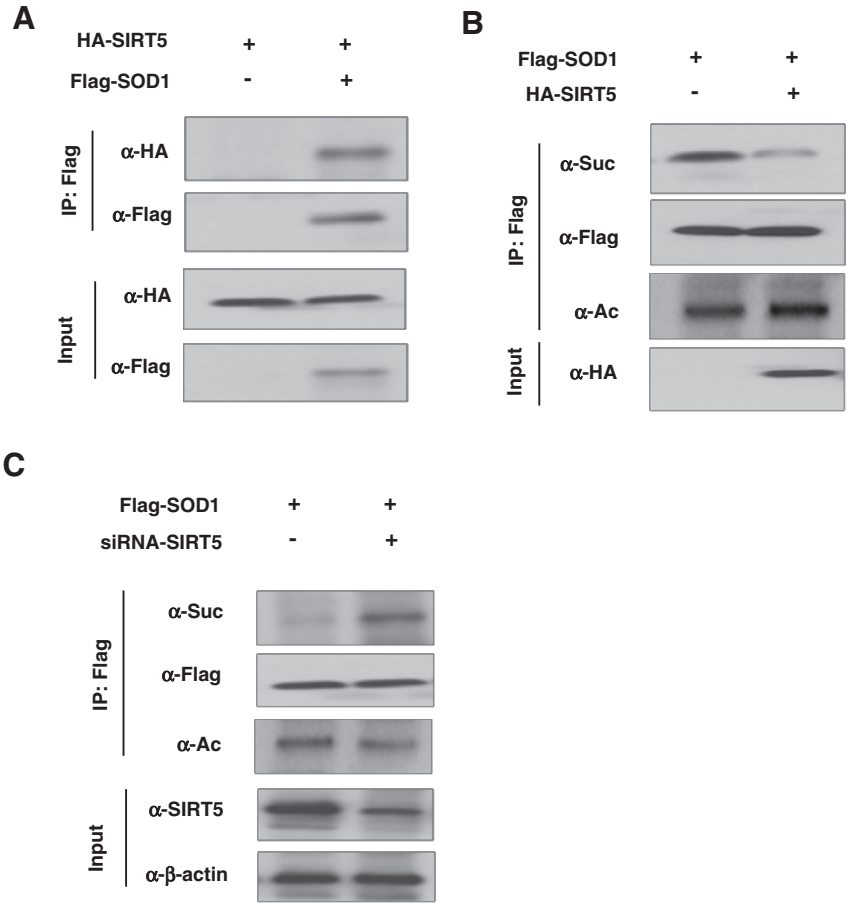

Fig. 2. SIRT5 binds to and desuccinylates SOD1. (A) SIRT5 interacts with SOD1. Flag-SOD1 was co-expressed in HEK293T cells with HA-SIRT5, immunoprecipitated and analyzed by Western blot. (B) Overexpression of SIRT5 desuccinylates SOD1. Flag-SOD1 was co-expressed in HEK293T cells with HA-SIRT5 as indicated. SOD1 proteins were purified by Flag beads and analyzed by Western blot. (C) Knocking down SIRT5 increases the level of SOD1 succinylation. Succinylation levels of overexpressed SOD1 in HEK293T cells with or without SIRT5 knockdown were analyzed by immunopurification and immunoblot with succinylation antibody.

limited company), acetylated-lysine (Cell Signaling) and SIRT5 siRNA (Santa Cruz) were purchased commercially.

2.2. SOD1 activity assay

The SOD1 activity assay was performed using the SOD Assay Kit (Dojindo Molecular Technology Inc.) according to the manufacturer's protocol with water-soluble tetrazolium salt (WST-1) as a substrate which produces a water-soluble formazan dye upon reduction with a superoxide anion. The assay procedure is as follow: First, add 20 μl sample solution or H_2O to each well, each assay was carried out in quadruplicate. Second, add WST working solution, dilution buffer, and enzyme working solution to each well. Third, incubate the plate at 37 °C for 20 min. At last, read the O.D. of the sample at 450 nm.

2.3. Measurement of intracellular ROS level

293T cells were transfected with indicated plasmids for 24–48 h. ROS production was determined by incubating HEK293T cells in PBS containing 20 μM fluorescent dye 2′,7′-dichlorofluorescein diacetate (Sigma) at 37 °C for 30 min, followed by flow cytometric analysis. Each assay was carried out in triplicate.

2.4. SOD1 knockdown and over expression

Flag-tagged human SOD1 and SOD1 K123R mutant containing two silent nucleotide substitutions in the sequence corresponding to the shRNA-targeted region were cloned into the retroviral vector pQCXIH, and retroviruses were produced as described below. Plasmids were co-transfected into HEK293T cells along with Gag and VSVG with the ratio of 5:3:3, respectively. Retroviruses were harvested 48 h after transfection. H1299 cells were infected with the retrovirus and selected in hygromycin (400 μg/ml) for 2 weeks. A shRNA targeting SOD1 was cloned into the pMKO vector. Viruses were produced and used to infect H1299 cells in the presence of polybrene (5 μg/ml) to increase the infection efficiency. Infected cells were selected in 2 μg/ml puromycin for 2 weeks. Whole cell lysates from stable cell lines expressing SOD1 and SOD1 K123R were prepared and knockdown and ever expression efficiency was analyzed by Western blot.

2.5. Cell proliferation analysis

5×10^4 H1299 cells either express wild type or K123R mutant of SOD1 stablely were seeded in triplicate in 6-well plates, and cell numbers were counted every 24 h over a four-day period. Each count was carried out in triplicate.

3. Results and discussion

3.1. Succinylation of K123 inhibits SOD1 catalytic activity

Protein function is often regulated by diverse posttranslational modifications, such as phosphorylation, ubiquitination and acetylation. It has been reported that many metabolic enzymes are modified by acetylation [12,13]. A recent paper reported a systematic study of the mammalian succinylome, which identified 2565 succinylation sites on 779 proteins [14], most of them do not overlap with acetylation sites, therefore suggesting different regulation of succinylation and acetylation. Among these proteins, SOD1 was found to be succinylated at K123 in the mass spectrometry analysis of SIRT5 knock out mouse liver tissue. Importantly, SOD1 K123 is conserved among all vertebrates (Fig. 1A). To confirm this finding, we generated a K123R succinylation resistant mutant and a K123E succinylation mimetic mutant and compared their succinylation level to wild-type SOD1 with pan anti-succinyllysine antibody (abbreviate for -Suc in the figures) and acetylated-lysine antibody (abbreviate for -Ac in the figures) as a control. We found that the succinylation levels of both mutants were significantly lower than wild type (Fig. 1B), confirming that SOD1 is succinylated and that K123 is the main succinylation site.

Notably, the amino acid H121, which is located near K123 and conserved in vertebrates (Fig. 1A), interacts with metallic cofactors essential for the activity and folding of SOD1 [15]. It is therefore probable that succinylation of K123 regulates SOD1 activity by affecting its binding to metallic cofactors. Analysis of SOD1 activity supports the idea that succinylation is inhibitory, as a 45% decrease in activity was observed in the mimetic mutant SOD1 K123E when compared with wild-type SOD1. Conversely, SOD1 K123R had a slight, but insignificant effect on the enzymatic activity of SOD1 (Fig. 1C).

3.2. SIRT5 binds to and desuccinylates SOD1

Given that the succinylation of SOD1 K123 was identified in SIRT5 knock out mouse liver tissue and that SIRT5 is the only known enzyme to date catalyzing Lys desuccinylation [16–18], we studied the interaction between Flag-SOD1 and HA-SIRT5. Immunoprecipitation and Western blot analysis demonstrate that SIRT5 can bind to SOD1 (Fig. 2A), supporting the notion that

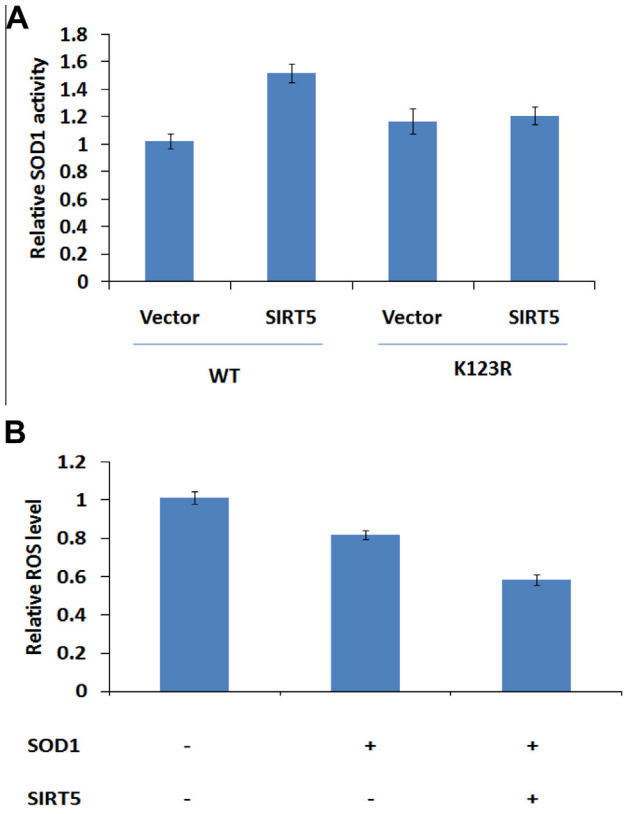

Fig. 3. SIRT5 activates SOD1 activity to eliminate ROS. (A) SIRT5 activates SOD1 activity via K123 desuccinylation. Wild-type and SOD1 K123R mutant were co-expressed in HEK293T cells with or without SIRT5, purified by Flag beads and subjected to the enzyme assay. Error bars represent ±SD for quadruplicate experiments. (B) SIRT5 promotes the SOD1-mediated reduction of ROS. Plasmids expressing SOD1 were expressed with or without SIRT5 in HEK293T cells and ROS levels were analyzed.

SIRT5 may be the desuccinylase of SOD1. To confirm this hypothesis, we overexpressed and knocked down SIRT5 in HEK293T cells and examined the succinylation level of SOD1. We found that co-expression of SIRT5 resulted in decreased wild-type SOD1 succinylation but nearly had no effect on the succinylation level of SOD1 K123R (Fig. 2B). Consistent with the overexpression data, knocking down SIRT5 significantly increased wild-type SOD1 succinylation (Fig. 2C). Taken together, SIRT5 can bind to and desuccinylate SOD1 on K123.

3.3. SIRT5 activates SOD1 activity and reduces ROS

To determine the functional significance of SIRT5 regulation of SOD1, we overexpressed SIRT5 in HEK293T cells and determined the enzyme activity of ectopically expressed SOD1. Overexpression of SIRT5 increased SOD1 activity by 50% but had no effect on the SOD1 K123R mutant (Fig. 3A), therefore providing additional evidence that succinylation of K123 negatively regulates the activity of SOD1. The main biological function of SODs is to remove ROS in cells [19]. To determine the effect of SOD1 succinylation on its physiological function, we first examined how changes in the succinylation levels of SOD1 affect ROS levels. We measured ROS levels in HEK293T cells with either SOD1 ectopically expressed alone or in combination with SIRT5. We found that the ROS level was reduced by almost 19% by the overexpression of SOD1 alone and by 43% by the co-expression of both SIRT5 and SOD1, indicating that SIRT5 activates SOD1 activity and reduces ROS.

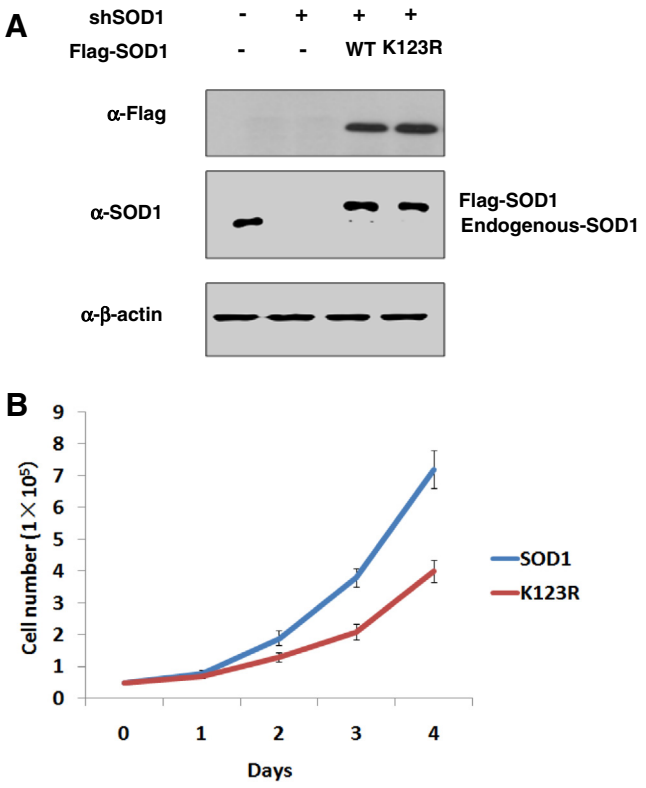

Fig. 4. Mutation of the SOD1 succinylation site inhibits the growth of lung tumor cells. (A) Expression of SOD1 and the SOD1 K123R mutant in H1299 cells. Whole cell lysates from stable cell lines expressing SOD1 and SOD1 K123R were prepared and analyzed by Western blot. (B) Mutation of SOD1 K123R inhibits the growth of lung tumor cells. 5×10^4 Wild-type SOD1 and SOD1 K123R mutant cells were seeded in each well. Cell numbers were counted every 24 h. Error bars represent ±SD for triplicate experiments.

3.4. Mutation of the SOD1 succinylation site inhibits the growth of lung tumor cells

To further determine whether SIRT5-mediated desuccinylation of SOD1 K123 is important for lung tumor cell growth, we first verified the knockdown of endogenous SOD1 and the stable ectopic expression of both wild-type SOD1 and the K123R mutant (Fig. 4A). We found that H1299/SOD1 wild-type cells proliferated twice as fast as the H1299/SOD1 K123R mutant cells (Fig. 4B), demonstrating a growth advantage conferred by the regulation of SOD1 via succinylation, which further indicates that the succinylation of SOD1 is critical for tumor cell growth.

SOD1 is highly expressed in various organisms [20,21]. Corson et al. found that less than 1% of total SOD1 is required to protect the aforementioned amino acid biosynthetic pathways and to prevent toxicity from superoxide [22]. The reason for producing such high levels of SOD1 is not well understood, and it is possible that the enzyme may have as yet unidentified functions in cell physiology.

Interestingly, a recent study found that SOD1 not only functions as an antioxidant enzyme, but it can also bind and protect key casein kinases from degradation in an O_2- and glucose-dependent manner. SOD1 is a vital component of nutrient sensing pathways and is essential for repressing respiration [23]. Our data may contribute to these findings by supporting the hypothesis that SIRT5 regulates nutrient sensing and respiration via the desuccinylation of SOD1, which we found to be very important for tumor cell growth, as mutation of the SOD1 succinylation site inhibits tumor cell growth significantly.

The function of SIRT5 is not completely understood. In the present study, we found that SIRT5 binds to, desuccinylates and activates SOD1 to eliminate ROS. Mutation of the SOD1 succinylation site inhibits the growth of lung tumor cells. These findings demonstrate that the regulation of SOD1 via succinylation is vital to lung tumor cell growth and provide a potential target for clinical cancer research and treatment.

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