Published in final edited form as:

Free Radic Biol Med. 2015 February ; 79: 176–185. doi:10.1016/j.freeradbiomed.2014.11.011.

Posttranslational Modification of Sirt6 Activity by Peroxynitrite

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Abstract

The mammalian sirtuin 6 (Sirt6) is a site-specific histone deacetylase that regulates chromatin structure and many fundamental biological processes. It inhibits endothelial cell senescence and inflammation, prevents development of cardiac hypertrophy and heart failure, modulates glucose metabolism, and represses tumor growth. The basic molecular mechanisms underlying regulation of Sirt6 enzymatic function are largely unknown. Here we hypothesized that Sirt6 function can be regulated via posttranslational modification, focusing on the role of peroxynitrite, one of the major reactive nitrogen species formed by excessive nitric oxide and superoxide generated during disease processes. We found that incubation of purified recombinant Sirt6 protein with 3-morpholinosydnonimine (SIN-1, a peroxynitrite donor that generates nitric oxide and superoxide simultaneously) increased Sirt6 tyrosine nitration and decreased its intrinsic catalytic activity. Similar results were observed in SIN-1-treated Sirt6 which was overexpressed in HEK293 cells,

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and on endogenous Sirt6 when human retinal microvascular endothelial cells were treated with SIN-1. To further investigate whether Sirt6 nitration occurs under pathological conditions, we determined Sirt6 nitration and activity in retina using a model of endotoxin-induced retinal inflammation. Our data showed that Sirt6 nitration was increased while its activity was decreased in this model. With mass spectrometry, we identified that tyrosine 257 in Sirt6 was nitrated after SIN-1 treatment. Mutation of tyrosine 257 to phenylalanine caused loss of Sirt6 activity, and abolished SIN-1-induced nitration and decrease in its activity. Mass spectrometry analysis also revealed oxidation of methionine and tryptophan in Sirt6 after SIN-1 treatment. Our results demonstrate a novel regulatory mechanism controlling Sirt6 activity through reactive nitrogen species-mediated post-translational modification under oxidative and nitrosative stress.

Keywords

Nitrosative stress; Oxidative stress; Inflammation; Reactive nitrogen species; Sirtuin; Sirt6; Nitration; Oxidation; Catalytic activity

INTRODUCTION

Mammalian sirtuins (Sirts) are the homologues of the yeast silent information regulator (Sir) 2, a nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase regulating life span of yeast [1]. Among the seven mammalian Sirts, Sirt6 most closely resembles yeast Sir2 regarding its intracellular location and function, and the animal phenotype caused by loss of Sirt6 [1, 2]. It is a site-specific histone deacetylase that regulates chromatin structure, prevents genomic instability, and represses the transcription activities of several transcription factors such as NF- κ B, c-JUN, and hypoxia-inducible factor (HIF)-1 α that are involved in fundamental biological processes [3–7]. Loss of Sirt6 results in premature aging, metabolic defects, cardiac hypertrophy, massive inflammation in several organs, and tumor genesis [1, 4, 6, 8].

Despite these important functions, the basic molecular determinants that regulate Sirt6 enzymatic function are not well understood. Recently, Sirt6 activity was shown to be positively regulated by binding to long-chain free fatty acids [9]. Phosphorylation of Sirt6 at evolutionarily conserved S338 modulates selected Sirt6 interactions [10], and ubiquitination of Sirt6 results in its protein degradation, likely through the proteasome [11]. Nevertheless, our knowledge is still limited regarding whether there are other factors that regulate Sirt6 biological activity at the post-translational level.

As a free radical, nitric oxide (NO) is an endogenous cell signaling molecule involved in the regulation of many physiological and pathological processes. NO and NO-related compounds exert both protective and cytotoxic effects, depending on the cellular context, the nature of the NO group and its concentration [12]. During oxidative stress, NO rapidly reacts with superoxide generated by NADPH oxidase, nitric oxide synthase uncoupling, or the mitochondrial respiratory complexes to form peroxynitrite, a highly reactive inflammatory and toxic molecule [13]. Peroxynitrite modifies proteins and alters the catalytic activity of enzymes, the organization of cytoskeleton, and the cell signal transduction events [14, 15]. Increases in peroxynitrite formation have been reported in

many physiological and pathological settings, including cardiovascular diseases, diabetes, inflammatory diseases, and aging. [13, 14, 16–18]. However, the downstream targets mediating the potential biological activities of peroxynitrite remain to be further elucidated. In this study, we determined whether Sirt6 is susceptible to nitrative modification and investigated the functional consequence of Sirt6 nitration.

MATERIAL AND METHODS

Antibody and reagents

Rabbit anti-Sirt6 antibody and rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA). Anti-3-nitrotyrosine, 3-morpholinosydnonimine (SIN-1) and His-tagged Sirt6 were obtained from Cayman Chemical Company (Ann Arbor, MI), and anti-Sirt6 (used for immunoprecipitation of samples for the detection of activity) was acquired from EMD Millipore (Billerica, MA). Anti-histone H3 was purchased from Abcam (Cambridge, MA) and anti-H3K9Ac antibody was purchased from Epitomics (Burlingame, CA). Anti-FLAG antibody was sourced from Sigma-Aldrich (St. Louis, MO). The secondary anti-mouse IgG or anti-rabbit IgG, ECLTM Western Blotting Detection Reagents, Protein G SepharoseTM 4 Fast Flow were purchased from GE Healthcare Bio-Sciences Corp (Piscataway, NJ). PVDF membrane was acquired from Bio-Rad Laboratories (Hercules, CA). CycLex Sirt6 Deacetylase Fluorometric Assay Kit was purchased from MBL International (Woburn, MA). Primers used to amplify mutant Sirt6 plasmids were synthesized by Integrated DNA Technologies (Coralville, IA).

Site-directed mutagenesis of Sirt6

pCDNA3.1-Sirt6-Flag construct for expressing full-length human Sirt6 was obtained from Addgene (Cambridge, MA). Mutant Sirt6 plasmids were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Each tyrosine (Y) residue of Sirt6 was mutated to phenylalanine (F). The following sets of forward and reverse primers were used to introduce mutations at tyrosine -5, -12, -148, -257: Y5F, GTC GGT GAA TTT CGC GGC GGG GCT GTC G (forward) and CGA CAG CCC CGC CGC GAA ATT CAC CGA C (reverse); Y12F, GGC TGT CGC CGT TCG CGG ACA AGG G (forward) and CCC TTG TCC GCG AAC GGC GAC AGC C (reverse); Y148F, GTA AGA CGC AGT TCG TCC GAG ACA CAT GCG (forward) and CGA CTG TGT CTC GGA CGA ACT GCG TCT TAC (reverse); Y257F, CCG CAT CCA TGG CTT CGT TGA CGA GG TCA TG (forward) and CAT GAC CTC GTC AAC GAA GCC ATG GAT GCG G (reverse). Mutagenesis was confirmed by automated nucleotide sequencing.

Cell Culture and Plasmid Transfection Analysis

HEK293 cells were grown in Dulbecco's modified Eagles medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum in humidified 5% CO₂ at 37°C. Wild-type (WT) or mutant pCDNA3.1-Sirt6-Flag plasmids were transfected into HEK293 cells using lipofectamine 2000 and Opti-MEM I reagents (Life Technologies) according to the manufacturer's instructions. 48 hours after transfection, cells were collected and homogenized in ice-cold RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Rockford, IL). The homogenates were centrifuged at 13800 g for 10

minutes at 4°C. Supernatants were collected, and protein concentrations were determined by a bicinchoninic acid assay (Thermo Fisher Scientific). Samples were stored at -80°C until used.

Primary human retinal microvascular endothelial cells (HRMECs) were purchased from Cell Systems (Kirkland, WA) and cultured in medium constituted of 50% CSC Serum-Containing Medium (Cell Systems) and 50% EGM™ Endothelial Cell Medium (Lonza Walkersville Inc., Walkersville, MD). HRMECs were permeabilized with 40 µg/ml digitonin (Sigma-Aldrich) for 5 minutes, then treated with 5 mM SIN-1 for 2 hours in Endothelial Basal Medium (Lonza Walkersville Inc.) at 37°C with 5% CO₂ before cells were collected and homogenized in ice-cold RIPA Lysis and Extraction Buffer [19].

Mouse model of endotoxin-induced retinal inflammation

All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the UTMB Institutional Animal Care and Use Committee (Assurance Number A3314-01). C57BL/6J mice received a single intraperitoneal injection of lipopolysaccharide (LPS, 4 mg/kg, Sigma-Aldrich). Control mice received an equal volume of PBS. At 3 hours after injection, retinas were collected and kept in liquid nitrogen for the analyses of Sirt6 nitration and its activity.

In Vitro nitration of Sirt6

Recombinant human Sirt6 from CycLex Sirt6 Deacetylase Fluorometric Assay Kit, His-tagged Sirt6, or immunoprecipitated Sirt6 from HEK293 in which wild-type and mutant Sirt6 were overexpressed were incubated with SIN-1 at 37°C for 30–60 minutes. Unreacted SIN-1 was removed by ultrafiltration membranes with a 3-kDa cutoff (Millipore). Nitration was detected by immunoblotting with anti-3-nitrotyrosine (anti-NT) antibody, and the intrinsic catalytic activity was measured with CycLex Sirt6 Deacetylase Fluorometric Assay Kit [20].

Sirt6 activity assay

The deacetylase activity of Sirt6 was determined by CycLex SIRT6 Deacetylase Fluorometric Assay Kit according to manufacturer's instructions [20]. Briefly, SIN-1 treated-recombinant Sirt6 or immunoprecipitated Sirt6 from cell lysates (15 µl) were mixed with a Sirt6 reaction buffer to form a 50 µl reaction mixture containing 50 mM Tris-HCl (pH 8.8), 0.5 mM DTT, 2.5 mAU/mL lysyl-endopeptidase, 1 µM trichostatin A, 800 µM NAD, 10 µM fluorosubstrate peptide, followed by incubation for 1 hour at 37°C. The fluorescence intensity was measured using a microtiter plate fluorometer with excitation at 490±10 nm and emission at 530±10 nm, and normalized to the protein concentration. All tests were performed in triplicate.

In vitro histone deacetylation assays

In vitro histone deacetylation assay was performed to detect Sirt6 deacetylase activity as described with minor modifications [21]. In brief, SIN-1-treated recombinant human Sirt6 (Sirt 6 from CycLex Sirt6 Deacetylase Fluorometric Assay Kit) was incubated with histone H3 (immunoprecipitated from 800 µg HEK293 cells with antibody for histone H3) in

deacetylation buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM NAD⁺, 3.3 mM DTT) at 37°C for 2 hours. Histone deacetylation was then determined by Western blot with H3K9Ac-specific antibody.

Immunoprecipitation and immunoblotting

Cell or tissue homogenates were diluted to same concentration with RIPA Lysis and Extraction Buffer. Samples were precleared by incubation with protein G beads for 2 hours, followed by incubation with 1–2 µg of primary antibodies overnight at 4°C. Protein G beads was added to the tube for a further 2-hour incubation. Samples were then centrifuged at 10,000 g for 1 minute at 4°C and the pellets were washed three times with immunoprecipitation buffer. For Sirt6 activity measurement, beads were additionally washed with PBS three times and subjected to activity measurement. For immunoblotting, bound proteins were eluted by boiling at 100°C for 10 minutes in SDS-PAGE loading buffer, and then isolated by centrifugation. The supernatants were separated on SDS-PAGE gels and then electrotransferred to a PVDF membrane. After blocking, membranes were incubated with primary antibodies overnight at 4°C and incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature, followed by development with ECLTM Western Blotting Detection Reagents.

Sample preparation and analysis by Mass Spectrometry

His-tagged human Sirt6 was treated with SIN-1, boiled in SDS sample buffer containing 125 mM DTT, and resolved on SDS-PAGE gel. Gel was stained with Coomassie Blue, and the gel band corresponding to Sirt6 was manually excised with a razor, destained, washed, cut and placed into 0.5 ml polypropylene tube. 100 µl of 50 mM ammonium bicarbonate buffer (pH 8.0) was added to each tube and the samples were then incubated at 37°C for 30 minutes. After incubation, the buffer was removed and 100 µl water was added to each tube followed by incubation at 37°C for 30 minutes. The water was then removed and 100 µl acetonitrile was added to each tube to dehydrate the gel pieces. Samples were placed in a speedvac for 45 minutes to remove excess solvent. The dried gel samples were digested with 10 ng/µl sequencing grade modified trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate buffer (pH 8.0) at 37°C for 15 hours.

The resulting tryptic peptides were analyzed by Nano-LC-MS/MS using a LTQ Orbitrap Velos from Thermo Finnigan, coupled with an Eksigent NanoLC 1D Plus. A 3 µl sample was injected onto a nano trap column (75 µm i.d. × 1 cm) for clean up, followed by a C18 reversed-phase column (75 µm i.d. × 10 cm, Agilent SB- C18, 5 µm). Flow rate was 400 nl/min with 60 minute LC gradient, where mobile phase is A (5% acetonitrile, 0.1% formic acid in water) and B (100% acetonitrile, 0.1% formic acid). Parameters included the following: tip voltage at +2.0 kV; FTMS mode for MS acquisition of precursor ions (resolution was set to be 60,000); ITMS mode for subsequent MS/MS of top 6 precursors selected; fragmentation accomplished via CID.

XCalibur raw data was analyzed using Thermo Proteome Discoverer 1.2.0.208 for protein identification and modifications. Precursor ion tolerance was set at 0.02 Da; MS/MS

fragment tolerance was set at 0.3 Da. The default significance of peptide matching threshold was $P < 0.05$. Manual analysis also was applied to the modified peptides.

Data analysis and statistics

Values were expressed as means $\pm$ SE and were obtained from at least three independent experiments. Statistical analyses of the results were carried out by one-way analysis of variance (ANOVA), followed by the Duncan's new multiple range method or the Newman-Keuls test. P values of < 0.05 were considered significant.

RESULTS

SIN-1 treatment increases Sirt6 nitration and inhibits its activity

Protein nitration may occur at the tyrosine residues when proteins are exposed to peroxynitrite generated during oxidative and nitrative stress. This is a selective posttranslational modification process and not all tyrosine-containing proteins are susceptible to nitration [16, 22]. Human Sirt6 protein contains four tyrosine residues and these residues are evolutionarily conserved across species. Additional non-conserved tyrosine residues were identified in mouse, rat, frog and chicken (Figure 1A). To determine whether human Sirt6 is susceptible to nitrative modification, recombinant human Sirt6 from the CycLex SIRT6 Deacetylase Fluorometric Assay Kit was incubated with SIN-1, a molecule that simultaneously generates nitric oxide and superoxide and functions as a peroxynitrite donor. Sirt6 nitration was determined by Western blot using a monoclonal antibody against 3-nitrotyrosine (3-NT). As shown in Figure 2A, SIN-1 treatment resulted in dose-dependent increases in Sirt6 nitration, which was very prominent at 5 mM SIN-1, a concentration within the range known to induce protein nitration [23–25]. Based on a study showing that within 30 or 60 minutes after the addition of SIN-1 the average peroxynitrite flow rate is about 0.1% of the added SIN-1 per minute under a similar experimental condition [26], it is estimated that 5 mM SIN-1 resulted in the flux of peroxynitrite at about 5 μ M/min during the treatment.

To determine whether the activity of Sirt6 is altered by this posttranslational modification, Sirt6 activity was measured with the CycLex SIRT6 Deacetylase Fluorometric Assay Kit. In this kit, fluorophore and quencher are coupled to amino terminus and carboxyl terminus of substrate peptide, respectively. This peptide is acetylated, preventing its cleavage by a protease. When incubated with Sirt6, a deacetylase, the substrate peptide is deacetylated and consequently cleaved by a protease to separate quencher from fluorophore so that fluorescence will be emitted. The deacetylase enzyme activity of Sirt6 is positively correlated with the increase in the fluorescence intensity. As shown in Figure 2B, in the presence of Sirt6, the fluorescence intensity was increased in a time-dependent manner, reflecting accumulation of cleaved peptide due to the deacetylase activity of Sirt6. However, the increase in fluorescence intensity was significantly reduced after Sirt6 was treated with 5 mM SIN-1, indicating significant loss of Sirt6 deacetylase activity. The deacetylase activity of Sirt6 was also slightly reduced by exposure to 0.5 mM SIN-1. As Sirt6 is a NAD^+ -dependent histone H3K9 deacetylase, we further determined its activity by incubating Sirt6 with histone H3 immunoprecipitated from HEK293 cells (Figure 2C). Significant reduction

of acetylated H3K9 (H3K9Ac) was observed when Sirt6 was incubated with histone H3 in the presence of NAD⁺, indicating the specificity of the reaction for Sirt6 H3K9Ac deacetylase activity. Consistent with the finding using the CycLex SIRT6 Deacetylase Fluorometric Assay Kit, SIN-1 treatment reduced Sirt6 deacetylase activity because SIN-1-treated Sirt6 did not remove H3K9Ac as efficiently as vehicle-treated Sirt6 (Figure 2C). These results indicate that recombinant human Sirt6 is susceptible to nitration, which is associated with SIN-1-induced reduction of Sirt6 deacetylase activity.

It has been demonstrated that Sirt6 catalytic efficiency increases after long fatty acid binding [9]. Therefore we extended the study to check whether SIN-1 treatment affects fatty acid-enhanced Sirt6 deacetylase activity. Our data show a 3.5 fold increase of Sirt6 deacetylase activity when myristic acid was added to the reaction (Figure 2D). However, myristic acid only boosted activity of SIN-treated Sirt6 to 2.1 fold of control, suggesting that peroxynitrite may inactivate Sirt6 under different settings.

Because it is possible that there are some differences in posttranslational modification (e.g. glycosylation) and molecular structure between recombinant Sirt6 expressed in prokaryotic cells and that expressed in eukaryotic cells, we further assessed Sirt6 nitration and its activity in mammalian cells. A Flag-tagged human Sirt6 was overexpressed in HEK293 cells, immunoprecipitated with anti-Flag antibody, and incubated with SIN-1 at different concentrations for various periods of time. Significant Sirt6 nitration was detected after immunoprecipitated Sirt6 was incubated with 5 mM SIN-1 for 1 hour (Figure 3A & 3B). Associated with increased Sirt6 nitration, the deacetylase activity of Sirt6 was reduced 32–70 % after SIN-1 treatment (Figure 3C).

Having demonstrated that nitrative modification of Sirt6 was associated with its inactivation using purified protein, we further investigated whether nitration of Sirt6 protein also occurs endogenously. We treated human retinal microvascular endothelial cells (HRMECs) with SIN-1 and found that significant Sirt6 nitration was detected at 1 hour and 2 hours after cells were treated with 5 mM SIN-1 (Figure 4A & 4B). To measure Sirt6 activity, Sirt6 was immunoprecipitated with a rabbit antibody against human Sirt6 from cell lysate. In order to confirm the specificity of the antibody, cell lysates were incubated with either anti-Sirt6 antibody or control IgG to immunoprecipitate Sirt6. Compared with control IgG, immunoprecipitant by the anti-Sirt6 antibody showed significant increase in the deacetylase activity, indicating the specificity of the measurement for endogenous Sirt6 activity (Figure 4C). Associated with increases in Sirt6 nitration, Sirt6 activity was dramatically reduced after cells were treated with SIN-1 (Figure 4D). In summary, these results demonstrate that Sirt6 is subject to nitration by peroxynitrite, a modification which may decrease Sirt6 activity.

Sirt6 is nitrated and inactivated during endotoxin-induced retinal inflammation

Oxidative stress and nitrative stress are involved in the pathogenesis of many diseases. To investigate whether Sirt6 nitration occurs in disease-related context, we determined Sirt6 nitration and activity in a mouse model of endotoxin-induced retinal inflammation. This is a model for uveitis which is a damaging ocular condition that can lead to severe vision loss and blindness [27]. The model is induced by injecting endotoxin (lipopolysaccharide, LPS)

into mice, resulting in an ocular inflammation similar to human acute anterior uveitis such as the breakdown of blood-ocular barrier, increases in retinal inflammatory cytokine production, leukocyte attachment to retinal vessels, and leukocyte infiltration into anterior segments [28–30]. After LPS injection, analysis of 3-nitratyrosine levels demonstrated that endotoxin induced significant nitration of retinal proteins (Figure 5A). Associated with increases in total protein nitration, Sirt6 nitration was significantly increased and its deacetylase activity was significantly reduced (Figures 5B & 5C). We conclude that Sirt6 nitration is associated with retinal inflammation.

LC-MS/MS identifies tyrosine257 as a nitration site in Sirt6

To determine which tyrosine residues in Sirt6 were nitrated, SIN-1-treated Sirt6 was analyzed by LC-MS/MS. A commercially available His-tagged human Sirt6 was used to provide sufficient protein for LC-MS/MS analysis. After SIN-1 treatment, nitration of Sirt6 was confirmed by Western blot with a monoclonal antibody against nitrotyrosine (Figure 6A). The amino acid sequence coverage obtained by LC-MS/MS is indicated in red (Figure 6B). Two tryptic peptides containing tyrosine were found by mass spectrometry analysis with good expectation values, which included peptide SVNIAAGLSPYADK (Y5 and Y12, E-value 1.38E-5) and IHGYVDEVMTR (Y257, E-value 7.94E-4). It was also found that peptide IHGYVDEVMTR had an oxidized form on methionine with an E-value 4.68E-4. Peptide containing Y148 (TQYVR) was not identified, perhaps due to its low molecular weight (666.36 Da). Assignments of nitration sites were verified by manual inspection of the tandem mass spectra. Our results indicated that Y257 was nitrated in SIN-1-treated Sirt6 (Figure 6C). As expected, the same original peptides were found in vehicle-treated Sirt6, but nitrated peptides were not identified.

Tyrosine 257 is critical for Sirt6 activity and nitration

To validate the results of LC-MS/MS and to determine whether Y148 is nitrated, we performed mutagenesis by changing tyrosine residue to phenylalanine residue, and then examined Sirt6 nitration in relation to its deacetylase activity in Sirt6-overexpressed mammalian cells. Significant tyrosine nitration was observed in SIN-1-treated wild-type (WT) Sirt6 and Sirt6 mutants including Y5F, Y12F and Y148F. In contrast, the level of tyrosine nitration in SIN-1-treated Y257F mutant was dramatically reduced compared with that of wild-type Sirt6 (Figure 7A). Sirt6 Y5F and Y12F mutants exhibited similar deacetylase activity as wild-type Sirt6 (Figure 7B). However, mutation of Y148F or Y257F reduced Sirt6 activity by 57% and 73%, respectively. SIN-1 treatment markedly inhibited the activity of wild-type Sirt6 and Y5F, Y12F and Y148F mutants (Figure 7B). Nevertheless, SIN-1 treatment did not further decrease the activity of Y257F mutant, suggesting the effect of SIN-1 on Sirt6 activity was mainly mediated by its induction of Y257 nitration. These results indicate that tyrosine 257 is critical for Sirt6 activity, is the major target of nitration, and is involved in the inactivation of Sirt6 by peroxynitrite.

Sirt6 is oxidized after SIN-1 treatment

In addition to inducing protein tyrosine nitration, peroxynitrite is a strong oxidant and has been shown to induce cystine oxidation to form sulfenic, sulfinic and sulfonic acids; induce methionine oxidation to form methionine sulfoxide; induce tryptophan oxidation to form

hydroxy-tryptophan, N-formylkynurenine and kynurenine; and induce histidine oxidation to form 2-Oxo-histidine [31–33]. To determine whether Sirt6 is oxidized by SIN-1 treatment, we further analyzed the LC-MS/MS data to search the above potential modifications. Among peptides identified by LC-MS/MS (Figure 6B), 3 peptides were found to contain methionine oxidation and 3 peptides were found to contain tryptophan oxidation (Table I). However, histidine oxidation was not identified. Since the covered amino acid sequence obtained by LC-MS/MS does not contain cystine residues, it is unknown whether cysteine oxidation occurred when Sirt6 was treated with SIN-1. Nevertheless, cysteine oxidation is a likely reaction induced by peroxynitrite [33].

DISCUSSION

Sirt6 is a H3K9 and H3K56 deacetylase that represses activities of several transcription factors involved in aging and inflammation, including NF- κ B, c-JUN, and HIF-1 α [3, 4, 6, 7]. Here, we measured nitration of Sirt6 in the retina in an animal model of endotoxin-induced retinal inflammation. Our results demonstrated that Sirt6 nitration was significantly increased while its intrinsic deacetylase activity was decreased. Together with studies showing that increased peroxynitrite is associated with H3K9 acetylation and production of inflammatory molecules in diabetic retinopathy and LPS-induced leukocyte activation [34–37], our findings suggest that nitration-induced loss of Sirt6 activity may represent a novel mechanism by which inflammatory stimuli (e.g. endotoxin and diabetes) induce retinal inflammation. Since increase in protein nitration has been causally linked to many pathologies associated with inflammation, aging, cancer, neurodegenerative, and cardiovascular diseases [38–41], and since Sirt6 is a key player in these processes [1, 4, 6, 8], results of this study warrant further investigation to determine whether nitration-induced loss of Sirt6 activity has a role in these conditions. Of note, under normal physiological conditions protein tyrosine nitration can occur and is a process involved in cellular signal transduction [42–46]. Because of exposure to light, visual cycle, and highly active neuronal activity and metabolism, retina produces significant amount of superoxide and nitric oxide. Therefore remarkable protein nitration has been observed in the retinas of control animals without inflammation (Figure 5A) [37, 47–49], although its role in retinal function under physiological conditions remains to be elucidated.

At this time, Sirt6 has received increased attention because of its pleiotropic functions in fundamental biological processes that include regulation of chromatin structure, maintenance of genomic stability, and control of glucose/lipid homeostasis [50]. However, mechanisms by which Sirt6 activity is regulated are largely unknown. Here, we demonstrate that Sirt6 is nitrated in the presence of a peroxynitrite donor or during retinal inflammation, and that Sirt6 nitration results in inhibition of its deacetylase activity. To our knowledge, this is the first evidence that suggests Sirt6 activity could be regulated by a posttranslational process widely used as a common mechanism for activation or inhibition of enzyme activity. Although our studies suggest that inactivation of Sirt6 by peroxynitrite is likely mediated by nitration of Sirt6 at tyrosine 257, our study also identifies that several residues in Sirt6 are oxidized after treated with SIN-1. Therefore it is possible that other post-translational modifications (e.g. oxidation) also contribute to inactivate Sirt6 during nitrate and oxidative stress, either by working alone or together with nitration of tyrosine 257. Further

studies are required to fully address the mechanism of peroxynitrite-induced inactivation of Sirt6 and to elucidate the specific contributions of these modifications in this process. Our study, together with a recent report that phosphorylation of Sirt6 affects its interaction with a subset of specific partners [10], indicates that Sirt6 activity and its selectivity of downstream targets can be modulated via posttranslational modification. Therefore, Sirt6 function may be altered by a pathophysiological process, even though its protein expression is not reduced. This is supported by data showing that Sirt6 protein level is increased during angiotensin II-induced cardiomyocyte hypertrophy but its activity is reduced [20].

Prior studies suggest that Sirt6 is involved in protecting cells against oxidative stress by mono-ADP-ribosylating poly [ADP-ribose] polymerase 1 (PARP-1), stimulating PARP1 poly-ADP-ribosylase activity, and enhancing repair of double-strand breaks that occur during oxidative stress [51]. We do not know whether nitration of Sirt6 also changes its ribosylation activity. However, our results suggest that Sirt6 function could be compromised when oxidative stress and nitrative stress occur together, as seen in inflammatory and chronic diseases. Therefore, agents that can reduce the level of peroxynitrite will be useful in protecting Sirt6 from nitration-induced loss of the deacetylase activity so that Sirt6 can fully execute its anti-aging and anti-inflammatory properties under diseases conditions.

Sirtuin proteins consist of a conserved central sirtuin domain, which is an enzymatic core flanked by variable N- and C-terminal extensions [5]. A functional analysis of Sirt6 mutants revealed that the N-terminal extension of Sirt6 and the core sirtuin domain are critical for its chromatin association and intrinsic catalytic activity [5]. Deleting the first 34 amino acid of Sirt6 or mutating histidine to tyrosine at residue 133 in the core sirtuin domain result in severe loss of its deacetylase activity. In contrast, the C-terminal extension of Sirt6 is critical for proper sub-cellular targeting but is dispensable for enzymatic activity [5]. Since the tyrosine at residue 5, 12 and 148 are located at these domains critical for Sirt6 activity, it is plausible that loss of Sirt6 activity may be due to nitration of one or more of these tyrosine residues. Unexpectedly, with both LC-MS/MS and mutagenesis analyses, we found Sirt6 is nitrated at tyrosine 257. Although tyrosine 257 is close to the C-terminal extension of Sirt6, mutation of tyrosine 257 to phenylalanine dramatically abolished Sirt6 deacetylase activity. This finding is consistent with the structural analysis of Sirt6 which shows tyrosine 257 as a key residue located in the large Rossmann fold domain required for binding to co-factors, such as NAD⁺ or ADP-ribose [52]. Sirt6 contains two globular domains composed of a large Rossmann fold for NAD⁺ binding and a smaller domain containing a zinc-binding motif. The large Rossmann fold domain is formed by a six-stranded parallel β -sheets sandwiched between two α helices on one side and four α helices on the other side [52]. In this domain, tyrosine 257 together with its adjacent glycine 256 and valine 258, link β 9 and α 8 and form a site that interacts with co-factors. In contrast to tyrosine 257, tyrosines at residue 5 and 12 are dispensable for Sirt6 activity and mutation of these two residues neither reduces Sirt6 nitration nor prevents nitration-induced loss of Sirt6 activity. These studies highlight the importance of tyrosine 257 in Sirt6 activity and suggest that nitration of tyrosine 257 is a novel posttranslational modification that inhibits Sirt6 function. Our study also identify tyrosine 148 is a critical residue for Sirt6 activity. In Sirt6, the zinc-binding motif and the large Rossmann fold domain are pulled together via the hydrogen bond between arginine 126 and glutamine 147 [52]. It is possible that tyrosine 148, which is adjacent to glutamine

147, plays an important role in maintaining the interaction between arginine 126 and glutamine 147 and therefore keeps Sirt6 in an active structure. Interestingly, although tyrosine 148 is critical for Sirt6 activity, it does not serve as a major target for nitration-mediated regulation of Sirt6 activity, additionally supporting the notion that nitration is a selective posttranslational modification process [16, 22]. As tyrosine residue can also be posttranslationally modulated by phosphorylation and sulfation, further studies are needed to investigate whether tyrosine 148 and/or 257 are subject to other modifications and therefore regulates Sirt6 activity.

In summary, our data unravel a novel regulatory mechanism by which Sirt6 activity is negatively regulated likely through reactive nitrogen species-mediated tyrosine nitration during oxidative and nitrative stress. Using proteomic and molecular biology approaches, we further show that Sirt6 nitration occurs at the site of tyrosine 257 and this residue is indispensable for Sirt6 activity. Our study highlights the important role of posttranslational modification in regulation of Sirt6 function in different physiological and pathological settings. Insight into mechanisms that regulate Sirt6 function via posttranslational modification, including phosphorylation, acetylation, nitrosylation, sulfation, oxidation, methylation and S-nitrosylation, may open important avenues for developing novel agents to regulate Sirt6 function and to treat diseases involving dysregulated Sirt6 activity.

Acknowledgments

This work was supported by National Institutes of Health grants EY022694, EY012231, EY018659, EY019309, and GM104934, AHA 11SDG4960005, the John Sealy Memorial Endowment Fund for Biomedical Research, a grant from Oklahoma Center for the Advancement of Science & Technology (OCAST), a grant from International Retinal Research Foundation, a grant from Retina Research Foundation, and an unrestricted grant from Research to Prevent Blindness.

Abbreviations

HIF-1α	hypoxia-inducible factor-1 α
HRMECs	human retinal microvascular endothelial cells
Sirt	Sirtuin
LPS	lipopolysaccharide
NAD	nicotinamide adenine dinucleotide
NO	nitric oxide
NT	nitrotyrosine
PARP-1	mono-ADP-ribosylating poly [ADP-ribose] polymerase 1
SIN-1	3-morpholiniosydnonimine
WT	Wild-type

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Human Sirt6	1	MSVNYAAGLS [*] PYADKGKCGLPEIFDPPEELERKWWELARLWQSSSVVFH
Mouse Sirt6	1	MSVNYAAGLS [*] PYADKGKCGLPEIFDPPEELERKWWELARLWQSSSVVFH
Rat Sirt6	1	MSVNYAAGLS [*] PYADKGKCGLPEIFDPPEELERKWWELARLWQSSSVVFH
Frog Sirt6	1	MSVNYAAGLS [*] PYADKGRCGLPEQFDSDELQKVEELAEMIRESSYVVFH
Chicken Sirt6	1	MSVNYAAGLS [*] PYADKGRCGLPEQFDSDELQKVEELAEMIRESSYVVFH
Human Sirt6	51	TGAGISTASGIPDFRGPHGWWTMEERGLAPKFDITTFESARPTQTHMALVQ
Mouse Sirt6	51	TGAGISTASGIPDFRGPHGWWTMEERGLAPKFDITTFENARPSKTHMALVQ
Rat Sirt6	51	TGAGISTASGIPDFRGPHGWWTMEERGLAPKFDITTFENARPSKTHMALVQ
Frog Sirt6	51	TGAGISTSCGIPDFRGPNGWWTLEEKGLDPKFDITTFETACPSPTHMALLK
Chicken Sirt6	51	TGAGISTASGIPDFRGPNGWWTMEERGLSPKFDITTFENARPSKTHMALLG
Human Sirt6	101	LERVGLLRFLVSQNVVDGLHVRSGFPRDKLAELHGNMFVEECAKCKTQYVR [*]
Mouse Sirt6	101	LERMGFLSFLVSQNVVDGLHVRSGFPRDKLAELHGNMFVEECAKCKTQYVR
Rat Sirt6	101	LERMGFLSFLVSQNVVDGLHVRSGFPRDKLAELHGNMFVEECAKCKTQYVR
Frog Sirt6	101	LQRVG ILKFLVSQNVVDGLHVRSGFPREQLAELHGNMFVEECSCAKQYVR
Chicken Sirt6	101	LQRVG ILKFLVSQNVVDGLHVRSGFPRDKLAELHGNMFVEECMKCGKQYVR
Human Sirt6	151	DTVVGTMGLKATGRLCTVAKARGLRACRGELRDTILDWEDSLPDRDLALA
Mouse Sirt6	151	DTVVGTMGLKATGRLCTVAKTRGLRACRGELRDTILDWEDSLPDRDLMLA
Rat Sirt6	151	DTVVGTMGLKATGRLCTVAKARGLRACRGELRDTILDWEDALPDRDLTLA
Frog Sirt6	151	DQVVGTMGLKPTGRFCVDPKVRGLRACRGKLDITLOWEDSLPDRDLNLA
Chicken Sirt6	151	DAVVGSMGLKPTGRCLCSVTKARGLRACRGKLDITLOWEDSLPDRDLTLA
Human Sirt6	201	DEASRNADLSITLGTSLQIRPSGNLPLATKRGGRLVIVNLQPTKHDRHA
Mouse Sirt6	201	DEASRTADLSVTLGTSLQIRPSGNLPLATKRGGRLVIVNLQPTKHDRQA
Rat Sirt6	201	DEASRTADLSVTLGTSLQIRPSGNLPLATKRGGRLVIVNLQPTKHDRQA
Frog Sirt6	201	DEACRKADLS ITLGTSLQIRPSGNLPL LTKRGGKLVIVNLQPTKHDKHA
Chicken Sirt6	201	DEACRKADLSVTLGTSLQIKPSGNLPL ITKKRGGKLV IVNLQATKHDRQA
Human Sirt6	251	DLRIHGYVDEVMTRLMKHLGLEIPAWDGPVRLERLALPPLPRPPTPKLEPK [*]
Mouse Sirt6	251	DLRIHGYVDEVMCRLMKHLGLEIPAWDGPVLDKALPPLPRPVAKAEP-
Rat Sirt6	251	DLRIHGYVDEVMCKLMKHLGLEIPTWDGPVLEKALPPLPRPVAPKAEP-
Frog Sirt6	251	DLRIHGYVDEVM IQLME LLGHKIPVWTG IHT-----KTEPT
Chicken Sirt6	251	DLRIHAYDDVMTKLLKHLGLEVPEWTGPVVVESA-----DSAKPEQL
Human Sirt6	301	EESPTRI NGS I PAGPKQEPCAQHNGSEPA [*] SPKRERPTSPAPHR-----
Mouse Sirt6	300	-----PVHLNGAVHVSYSK-----SKPNSP I LHR-----
Rat Sirt6	300	-----PVHLNG-----SYK-----PKPDSVPVPHR-----
Frog Sirt6	287	YEN-----HKEEDNSYHNSVLDANLDQKREGCKEESN-----
Chicken Sirt6	294	YTFKPEAHG LL-----KEEFP [*] SOHNGTAGQCP-----DLGTT LVEHRDSLKQEC
Human Sirt6	344	-----PPKRVKAKAVPS-----
Mouse Sirt6	323	-----PPKRVKTEAAPS-----
Rat Sirt6	319	-----PPKRVKTEAAS-----
Frog Sirt6	319	-----LELK-----KAKVEPACV
Chicken Sirt6	338	PSPDTGPPLTKMKMKVEP LLT

Figure 1. Sirt6 protein contains conserved tyrosine residues

Alignment of human, mouse, rat, frog and chicken Sirt6 protein sequences revealed four conserved tyrosine residues (*) among these species. Non-conserved tyrosine residues, (underlined) were identified in species other than human Sirt6.

Figure 2. SIN-1 nitrates purified Sirt6 and attenuates its deacetylase activity

(A) Recombinant human Sirt6 from the CycLex SIRT6 Deacetylase Fluorometric Assay Kit were incubated with 0.5 mM or 5 mM SIN-1 for 30 minutes at 37° C. Sirt6 tyrosine nitration was detected with anti-3-nitrotyrosine (anti-NT) by immunoblotting. (B) The fluorescence intensity, indicating the deacetylase activity of Sirt6, was measured at different times after SIN-1 or vehicle-treated Sirt6 was incubated with the Fluoro-Substrate Peptide from CycLex SIRT6 Deacetylase Fluorometric Assay Kit. Data are presented as means±SE. # $P < 0.05$ versus control. (C) After recombinant human Sirt6 was treated with vehicle or 5 mM SIN-1, it was incubated with histone H3 immunoprecipitated from HEK293 cells in the absence or presence of 10 mM NAD⁺ for 2 hours at 37° C. Acetylated H3K9 and total H3 was determined by Western blot. (D) After Sirt6 was treated with vehicle or 5 mM SIN-1, the deacetylase activity of Sirt6 was measured by CycLex SIRT6 Deacetylase Fluorometric Assay Kit in the absence or presence of 400 μM myristic acid (MA). Activity of Sirt6 treated with vehicle (PBS, control) in the absence of myristic acid was used as reference. Data are presented as means±SE. # $P < 0.05$ versus Sirt6 treated with vehicle. * $P < 0.05$ versus Sirt6 treated with vehicle in the presence of MA.

Figure 3. Sirt6 expressed in HEK293 cells is nitrated, and its deacetylase activity is reduced after SIN-1 treatment

Flag-tagged human Sirt6 was overexpressed in HEK293 cells and immunoprecipitated by anti-Flag antibody. Immunoprecipitated Sirt6 was incubated with 5 mM SIN-1 for 0.5 or 1 hour at 37° C and Sirt6 nitration was analyzed by Western blotting (**A**) Immunoprecipitated Sirt6 were incubated with 0.5 or 5 mM SIN-1 for 1 hour at 37° C, followed by immunoblotting for nitrated Sirt6 (**B**) or measuring deacetylase activity (**C**). Activity of Sirt6 treated with vehicle (PBS, control) was used as reference. Data are presented as mean $\pm$ SE. # $P < 0.05$ versus control.

Figure 4. SIN-1 treatment induces Sirt6 tyrosine nitration and reduces its activity in cultured human retinal microvascular endothelial cells (HRMECs)

HRMECs were incubated with 5 mM SIN-1 for 1 or 2 hours (A), or 0.5 or 5 mM of SIN-1 for 2 hours at 37° C (B, D). Nitration of Sirt6 was determined by immunoprecipitation using an anti-Sirt6 antibody and immunoblotting with an anti-nitrotyrosine antibody (A, B). The deacetylase activity of Sirt6 was measured by CycLex SIRT6 Deacetylase Fluorometric Assay Kit (C, D). The specificity of the measurement of endogenous Sirt6 activity was confirmed by comparing immunoprecipitant by anti-Sirt6 antibody vs. control IgG (C). (D) After SIN-1 treatment, Sirt6 was immunoprecipitated with the anti-Sirt6 antibody and its activity was measured. Activity of Sirt6 in cells treated with vehicle (PBS, control) was used as reference. Data are presented as means±SE. # $P < 0.05$ versus control.

Figure 5. LPS treatment results in the increase in Sirt6 nitration and the reduction of its activity in retinas

Mice were injected with LPS (4 mg/kg) or PBS (control) and retinas were collected 3 hours after injection. **(A)** Total tyrosine nitration in retina lysates was examined by immunoblotting. Tubulin was used as loading control. **(B, C)** Retina lysates (400 μ g) were immunoprecipitated with anti-Sirt6 antibody overnight. Sirt6 tyrosine nitration was detected by immunoblotting and its activity was measured with kit. [#] $P < 0.05$ versus control.

Figure 6. Identification of tyrosine nitration sites in SIN-1-treated Sirt6

Purified recombinant human Sirt6 was exposed to 5 mM SIN-1 for 1 hour at 37° C. (A) Sirt6 nitration was determined by Western blotting with an antibody against nitrotyrosine. Upper gel shows nitrated Sirt6 and lower gel shows total Sirt6. (B) After Sirt6 was nitrated with SIN-1, it was subjected to trypsin digestion, and peptides were separated on a reverse phase HPLC column on-line with nano spray ionization, orbi trap mass spectrometer. The amino acid sequence coverage obtained by LC-MS/MS is indicated in red. The verified nitrated peptide regions are underlined and asterisks designate nitrotyrosine residues. (C) Annotated mass spectrum of peptides containing nitrotyrosine was observed after the reaction of Sirt6 with SIN-1. Collision induced fragmentation MS/MS spectrum of precursor ion at m/z 460.88 (MH³⁺) corresponds to the amino acid sequence. Type b ions contain the N-terminal portion of the peptide and type y ions contain the C-terminal portion.

Figure 7. Mutation of tyrosine 257 on Sirt6 (Y257F) prevents nitration by SIN-1

HEK293 cells were transfected with either Flag-tagged wild-type (WT) or mutant Sirt6 plasmids. At 48 hours after transfection, cells were lysed and Sirt6 from cell lysates were immunoprecipitated with an anti-Flag antibody, followed by exposure to 5 mM SIN-1 for 1 hour at 37° C. Sirt6 nitration (**A**) and its deacetylase activity (**B**) were detected. Activity of WT Sirt6 treated with vehicle (PBS, control) was used as reference. Data are presented as means±SE. * $P < 0.05$ versus WT Sirt6 treated with vehicle. # $P < 0.05$ versus its relevant vehicle-treated control.