Sirtuins (e.g. human Sirt1–7) catalyze the removal of acetyl groups from lysine residues in proteins in an NAD⁺-dependent manner, and loss of sirtuin deacetylase activity correlates with the development of aging-related diseases. Although multiple reports suggest that sirtuin activity is regulated by oxidative post-translational modifications of cysteines during inflammation and aging, no systematic comparative study of potential direct sirtuin cysteine oxidative modifications has been performed. Here, using IC₅₀ and kᵢ₅₀/Kₑ analyses, we quantified the ability of nitrosothiols (S-nitrosogluthathione and S-nitroso-N-acetyl-D,L-penicillamine), nitric oxide, oxidized GSH, and hydrogen peroxide to post-transcriptionally modify and inhibit the deacetylase activity of Sirt1, Sirt2, Sirt3, Sirt5, and Sirt6. The inhibition was correlated with cysteine modification and assessed with chemical-probe and blot-based assays for cysteine S-nitrosation, selenylation, and glutathionylation. We show that the primarily nuclear sirtuins Sirt1 and Sirt6, as well as the primarily cytosolic sirtuin Sirt2, are modified and inhibited by cysteine S-nitrosation in response to exposure to both free nitric oxide and nitrosothiols (kᵢ₅₀/Kₑ ≥ 5 M⁻¹ s⁻¹), which is the first report of Sirt2 and Sirt6 inhibition by S-nitrosation. Surprisingly, the mitochondrial sirtuins Sirt3 and Sirt5 were resistant to inhibition by cysteine oxidants. Collectively, these results suggest that nitric oxide–derived oxidants may causatively link nuclear and cytosolic sirtuin inhibition to aging-related inflammatory disease development.

Sirtuins catalyze the removal of acetyl and longer acyl-chains from protein lysine residues using NAD⁺ as a cosubstrate, producing O-acetyl-ADP-ribose and nicotinamide (1). Humans encode seven sirtuin isoforms (Sirt1–7), each with distinct subcellular localization, protein targets, and deacetylase activities (2, 3). Both Sirt1 and Sirt2 display nuclear-cytoplasmic shuttling (4, 5); however, Sirt1 localization is predominantly nuclear, whereas Sirt2 is primarily cytosolic (2). Sirt3, Sirt4, and Sirt5 primarily localize to the mitochondria (6), whereas Sirt6 and Sirt7 localize exclusively to the nucleus (7). In addition to diverse subcellular distribution, sirtuins possess unique protein targets and distinct deacetylase activities (2, 3). Sirt1 and Sirt2 harbor robust deacetylase activity and also remove longer acyl chains such as hexanoyl, decanoyl, and myristoyl chains (8). Sirt3 is the primary mitochondrial deacetylase (9), and Sirt5 demalonylates (10), desuccinylates (11–13), and deglutarylates (14) protein lysine residues (6). Compared with Sirt1–3, Sirt6 deacetylase activity is considered weak, although Sirt6 deacetylase activity is more robust against whole protein substrates compared with peptides (15) and is activated by long-chain fatty acids (16, 17). Moreover, Sirt6 harbors robust and preferential deacetylase activity against longer acyl chains (8). The deacetylase activities of Sirt4 and Sirt7 have only recently been discovered. Sirt4 can deacetylate lipoyl- (18), methylglutaryl-, hydroxymethylglutaryl-, and 3-methylglutacoyl-lysine (6, 19), and Sirt7 can deacetylate, desuccinylate, and demyristoylate nuclear protein targets (20–22); however, Sirt7 deacetylase activity is highly dependent on the presence of nucleic acid polymers (21, 22).

Despite the diversity of sirtuin activity in terms of localization, acyl group specificity, and protein targets, sirtuins are collectively considered cytoprotective proteins. Loss of sirtuin activity may contribute to aging-related inflammatory disease states including type II diabetes, neurodegeneration, and cardiovascular disease (23, 24). However, how sirtuin activity is inhibited in an aging or inflammatory disease context is largely unknown. The free radical theory of aging suggests that excessive production of oxidants and free radicals results in cumulative damage to proteins, lipids, and nucleic acids, thereby reducing lifespan and promoting related pathologies (25). More recent evidence has corroborated the importance of oxidants and inflammatory molecules in aging-related disease development (26). Indeed, multiple reports suggest that oxidation inhibits the deacetylase activity of several sirtuin isoforms. Previous work by us and others suggests that Sirt1 deacetylase activity can be inhibited by cysteine S-nitrosation (27–34) and glutathionylation (35–37). Additionally, previous work suggests that Sirt6 can be modified (38) and its deacetylase activity can be inhibited (39) by cysteine sulfinylation (38, 39), and Sirt3 deacetylase activity can be inhibited by cysteine glutathionylation (40). These data indicate that sirtuin oxidation may link decreased sirtuin activity to the development of aging-related disease states. However, much of this previous work was performed in a cellular context, making it difficult to decouple direct sirtuin inhibition from inhibition of upstream signaling pathways. Moreover, these previous studies have largely focused on inhibition of a single sirtuin by a single oxidant, often at a single concentration and/or a single time point, and a direct, comparative, concentration-dependent, and time-dependent study of sirtuin inhibition by oxidants was lacking.

Here, we tested the ability of the nitrosothiols S-nitrosoglutathione (GSNO) and S-nitroso-N-acetyl-D,L-penicillamine (SNAP), NO, H₂O₂, and GSSG to inhibit or activate the
deacetylase activity of Sirt1, Sirt2, Sirt3, Sirt5, and Sirt6 using IC<sub>50</sub> and k<sub>inact</sub>/K<sub>i</sub> analyses. Furthermore, we used biotin switch assays for S-nitrosation, dimedone analog-based assays for sulfonylation, and anti-GSH immunoblotting to determine the identity of the oxidative post-translational modifications leading to sirtuin inhibition. Despite the high degree of sequence conservation among sirtuins, differential sensitivity of sirtuins to cysteine oxidative inhibition was observed. In particular, we demonstrate that nuclear and cytosolic sirtuins (Sirt1, Sirt6, and Sirt2) are S-nitrosated at their Zn<sup>2+</sup>-tetrathiolate cysteines and selectively inhibited by both NO and nitrosothiols, whereas Sirt3 and Sirt5 are surprisingly resistant to inhibition by the oxidants tested. This study is the first direct, systematic comparison of sirtuin inhibition by oxidants, as well as the first report of Sirt2 and Sirt6 inhibition by S-nitrosation. This study provides the basis for future cellular studies of differential sirtuin inhibition by cysteine oxidants.

Results

Sirtuin isoforms are differentially inhibited by physiological cysteine oxidants

To directly compare the susceptibility of human sirtuins to inhibition by common cellular oxidants, Sirt1, Sirt2, Sirt3, Sirt5, and Sirt6 were treated for 1 h with 100 µM of the nitrosothiols GSNO and SNAP, NO (2 mol NO released/mol MAHMA-NONOate, t<sub>1/2</sub> = 2.7 min), H<sub>2</sub>O<sub>2</sub>, and GSSG. The deacetylase activity of treated sirtuins was measured at subsaturating concentrations near the K<sub>m</sub> values of both acylated peptide and NAD<sup>+</sup>, either by an enzyme-coupled continuous assay for nicotinamide formation (Sirt1 and Sirt3) (41) or an HPLC fixed time point assay monitoring acylated and deacylated peptide (Sirt2, Sirt5, and Sirt6) (8). Deacetylase activity of the primarily nuclear Sirt1 (Fig. 1A) was significantly inhibited (>50% inhibition, p < 0.05) by GSNO and SNAP. Demyristoylase activity of the nuclear Sirt6 (Fig. 1B) was significantly inhibited by GSNO and NO. Demyristoylase activity of the primarly cytosolic Sirt2 (Fig. 1C) was significantly inhibited by GSNO, SNAP, and NO. Interestingly, the mitochondrial sirtuins Sirt3 and Sirt5 were resistant to inhibition by cysteine oxidants with neither the deacetylase activity of Sirt3 (Fig. 1D) nor the desuccinylase activity of Sirt5 (Fig. 1E) significantly inhibited by greater than 23% by any oxidant tested. Together, these data suggest that nuclear and cytosolic sirtuins are inhibited by NO-derived oxidants, whereas mitochondrial sirtuins are highly resistant to oxidative inhibition, at least by the oxidants tested.

Sirt1 deacetylase activity is inhibited by cysteine S-nitrosation

The initial kinetic oxidant screen was performed at a single concentration of each oxidant (100 µM) generally considered to be above physiologically relevant oxidant concentrations that are estimated to be in the low-micromolar range (42–44). Thus, to compare relative potency and assess the potential for oxidants to inhibit in vivo, concentration-response titrations were performed for oxidants demonstrating significant inhibition (p < 0.05) greater than 50% in the initial 100 µM oxidant screen. To determine the relative ability of GSNO to inhibit Sirt1, purified Sirt1 was treated with varying concentrations (1–100 µM) of GSNO for 1 h at 37°C, and Sirt1 deacetylase activity was assessed via an enzyme-coupled continuous assay (41) (Fig. 2A). A concentration-dependent decrease in Sirt1 deacetylase activity was observed in response to GSNO (IC<sub>50</sub> = 43 ± 8 µM).

GSNO can both transnitrosate and glutathionylate proteins (42), and evidence for Sirt1 S-nitrosation and glutathionylation have been shown in response to GSNO treatment (27–29, 35, 36). To differentiate Sirt1 inhibition by S-nitrosation from inhibition by glutathionylation, Sirt1 was treated with varying concentrations (1–100 µM) of GSNO for 1 h at 37°C, and S-nitrosation and glutathionylation were comparatively assessed via the biotin switch assay (43) (Fig. 2B) and anti-GSH immunoblot (Fig. 2C), respectively. Sirt1 displayed concentration-dependent S-nitrosation (Fig. 2B), which was also dependent on ascorbate as a nitrosothiol-reducing agent. This also correlated with inhibition of Sirt1 deacetylase activity (Fig. 2A). However, Sirt1 glutathionylation above background was only observed at 100 µM GSNO, GSSG, or H<sub>2</sub>O<sub>2</sub>/GSH (Fig. 2C), and the anti-GSH signal for Sirt1 was weaker than that of the GST-GAPDH–positive control. Taken together, these results are consistent with GSNO inhibiting Sirt1 deacetylase activity primarily by S-nitrosation and not glutathionylation.

Although IC<sub>50</sub> analysis provides valuable information about the relative concentration dependence of a given inhibitor, our data suggest that Sirt1 (and other sirtuins as detailed below) is inhibited by oxidants via post-translational modification of
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Inhibition of Sirt1 inhibition by GSNO was examined via a single time point, as was done in all previous studies (27, 29, 35, 36, 44). The resulting concentrations of GSNO for varying time points, and the resulting deacetylase activity was assessed via a continuous enzyme-coupled assay (41) following incubation with varying concentrations of GSNO (μM), and the apparent second-order rate constant for GSNO inhibition of Sirt1 was 0.029 ± 0.004 min⁻¹, the apparent Kᵢ value was 40 ± 15 μM, and the apparent kᵢ/Kᵢ ratio was 12 ± 3 M⁻¹ s⁻¹ (Table 1). Taken together, these data suggest that Sirt1 is selectively modified and inactivated by transnitrosation donors such as GSNO and comparatively resistant to inhibition by other cysteine oxidants.

Sirt6 deacetylase activity is inhibited by cysteine S-nitrosation of the Zn²⁺-tetrathiolate

To compare the relative inhibition of Sirt6 by GSNO and NO, the two oxidants that significantly (>50% inhibition; p <

![Figure 2. Sirt1 deacetylase activity is inhibited by S-nitrosation.](image-url)

Table 1

| Oxidant | kᵢ | Kᵢ | kᵢ/Kᵢ |
|---------|----|----|-------|
| GSNO    | 0.029 ± 0.004 min⁻¹ | 40 ± 15 N.D. | 0.97 ± 0.11 |
| NO      | 0.0024 ± 0.0014 min⁻¹ | 0.010 ± 0.006 N.D. | 0.77 ± 0.05 |
| GSNO    | 0.0036 ± 0.0006 min⁻¹ | 0.011 ± 0.005 N.D. | 0.97 ± 15 N.D. |

*Note: All values are mean ± S.E.*
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Figure 3. Sirt6 demyristoylase activity is inhibited by S-nitrosation. A, comparison of Sirt6 inhibition by GSNO and NO. Sirt6 (0.5 μM) demyristoylase activity was assessed via an HPLC fixed time point assay (8) following incubation with varying concentrations of GSNO (red circles) or NO (2 mol NO released/mol MAHMA-NONOate) (gray triangles) for 1 h at 37 °C. The percentage of demyristoylase activity in the absence of oxidant was plotted versus log_{10} of the oxidant concentration (n = 3 ± S.E.). B, relative S-nitrosation of Sirt6 treated with varying concentrations of GSNO and DEA-NONOate for 1 h at 37 °C was assessed via the biotin switch assay (representative blot, n = 3). C, glutathionylation of Sirt6 treated with 100 μM GSNO, GSSG, or H2O2/GSH for 1 h at 37 °C was assessed via anti-GSH immunoblot (representative blot, n = 4). D, relative S-nitrosation of Sirt6 WT and Zn2+-tetrathiolate tetraalanine mutant (4C→A) treated with 100 μM GSNO for 1 h at 37 °C was assessed via the biotin switch assay (representative blot, n = 3). E, comparison of Sirt6 WT and 4C→A deamidase activity. Sirt6 WT and 4C→A (1 μM) demyristoylase activity was assessed via an HPLC fixed time point assay (8). The data are plotted as the percentage of Sirt6 WT demyristoylase activity (n = 3 ± S.D.). F, sulfenylation of Sirt6 or GST-tagged GAPDH treated with 100 μM H2O2 for 1 h at 37 °C was assessed via the DYn-2 assay (representative blot, n = 3). Sirt6 and GST-GAPDH samples were run on the same SDS-PAGE gel and imaged simultaneously. The image was subsequently cropped to bring Sirt6 and GST-GAPDH into proximity for comparison and to remove lanes irrelevant to the current study. G, comparison of Sirt6 inactivation kinetics by NO-derived oxidants. Sirt6 (0.5 μM) deacetylase activity was assessed via an HPLC fixed time point assay (8) following incubation with varying concentrations of GSNO (red circles) or NO (2 mol NO released/mol MAHMA-NONOate) (gray triangles) for 0–90 min at 37 °C. The rates of inactivation were plotted versus oxidant concentration (n = 3 ± S.E.).

0.05) inhibited Sirt6 in the initial 100 μM oxidant screen (Fig. 1B), purified Sirt6 was treated with varying concentrations (1–100 μM) of each oxidant, and the demyristoylase activity was assessed via an HPLC fixed time point assay (8) (Fig. 3A). A concentration-dependent decrease in Sirt6 demyristoylase activity was observed in response to both oxidants, with both GSNO (IC_{50} = 82 ± 8 μM) and NO (IC_{50} = 63 ± 12 μM) inhibiting Sirt6 to a similar extent (p = 0.32).

S-Nitrosation of Sirt6 was examined via the biotin switch assay (43) following treatment with varying concentrations (6–100 μM) of GSNO and NO (Fig. 3B), and glutathionylation was assessed via anti-GSH immunoblot following incubation with 100 μM GSNO, GSSG, and H2O2/GSH (Fig. 3C). Sirt6 displayed a concentration-dependent increase in biotin switch signal in response to treatment with GSNO and NO (Fig. 3B), correlating with the observed concentration dependence of demyristoylase inhibition (Fig. 3A). In contrast, anti-GSH reactivity was not observed in response to treatment with GSNO (Fig. 3C). Although mild Sirt6 glutathionylation was observed (compared with the GST-GAPDH positive control) in response to GSSG and H2O2/GSH treatment (Fig. 3C), Sirt6 demyristoylase activity was not inhibited by GSSG (Fig. 1B), suggesting that inhibition of Sirt6 demyristoylase activity by GSNO and NO is the direct result of Sirt6 S-nitrosation, not glutathionylation.

Because the Zn2+-tetrathiolate was previously demonstrated to be the kinetically relevant site of Sirt1 S-nitrosation (27, 28) and the Zn2+-tetrathiolate is conserved among sirtuins, we hypothesized that the Zn2+-tetrathiolate is also the site of Sirt6...
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S-nitrosation. Indeed, mutation of the four Sirt6 Zn^{2+}-tetra-thiolate cysteines to alanine (Cys^{141}, Cys^{144}, Cys^{166}, and Cys^{177}) ameliorated GSNO-dependent S-nitrosation (Fig. 3D). Additionally, no demyristoylase activity was observed for the Sirt6 tetraalanine mutant (Fig. 3E), consistent with S-nitrosation of the Zn^{2+}-tetra-thiolate resulting in Sirt6 inhibition. However, mutation of the Zn^{2+}-tetra-thiolate and disruption of Zn^{2+} binding may alter the Sirt6 structure such that the efficiency of S-nitrosation is diminished at Sirt6 cysteine residues distal to the Zn^{2+}-tetra-thiolate.

Inhibition of Sirt6 deacetylase activity by cysteine sulfenylation (39) has previously been demonstrated; however, Sirt6 demyristoylase activity was not inhibited by H_{2}O_{2} in our assays (Fig. 1B). To determine whether Sirt6 was sulfenylated by H_{2}O_{2}, the DYn-2 sulfenylation assay (51) (Fig. 3F) was performed following treatment with 100 μM H_{2}O_{2}. Sirt6 sulfenylation was not observed in the DYn-2 assay (Fig. 3F), despite observing robust sulfenylation of GST-GAPDH by 100 μM H_{2}O_{2} under the same conditions on the same SDS-PAGE gel. These results are consistent with the lack of inhibition of Sirt6 demyristoylase activity by H_{2}O_{2} observed in the initial kinetic oxidant screen (Fig. 1B). Taken together, these data suggest Sirt6 demyristoylase activity is selectively inhibited by S-nitrosation.

To examine the concentration and time dependence of Sirt6 inhibition by GSNO and NO, k_{inact}/K_{I} analyses were performed as detailed under “Experimental procedures” (Fig. 3G). The k_{inact} value of NO (0.010 ± 0.006 min^{-1}) was higher than that of GSNO (0.0024 ± 0.0014 min^{-1}), and the same was true of the apparent K_{I} values for NO (∼37 μM) and GSNO (∼2 μM) (Table 1). Comparisons of apparent k_{inact}/K_{I} ratios revealed greater inhibition by GSNO (∼23 m^{-1} s^{-1}) compared with NO (∼5 ± 4 m^{-1} s^{-1}) (Table 1). Taken together, these data suggest that low-micromolar concentrations of both GSNO and NO inhibit Sirt6 by S-nitrosation at the Zn^{2+}-tetra-thiolate.

Sirt2 demyristoylase activity is inhibited by S-nitrosation of the Zn^{2+-tetra-thiolate}

To compare the relative inhibitory potency of GSNO and NO, purified Sirt2 was treated with varying concentrations (1–100 μM) of each oxidant and the demyristoylase activity assessed via an HPLC fixed time point assay (8) (Fig. 4A). Sirt2 demyristoylase activity was inhibited in a concentration-dependent manner by both oxidants, with NO (50 ± 14 μM) and GSNO (IC_{50} = 52 ± 10 μM) inhibiting Sirt2 to a similar extent (p = 0.99).

To determine the identity of inhibitory post-translational modifications, Sirt2 S-nitrosation and glutathionylation were examined via the biotin switch assay (Fig. 4, B and C) and anti-GSH immunoblot (Fig. 4D), respectively. A concentration-dependent increase in Sirt2 S-nitrosation was observed in response to treatment with varying concentrations (1–100 μM) of GSNO (Fig. 4B) or NO (Fig. 4C), which depended on the presence of ascorbate as a nitrosothiol-reducing agent, consistent with trends in inhibition of demyristoylase activity (Fig. 4A).

As observed for Sirt6 (Fig. 3, D and E) and consistent with what has been previously demonstrated for Sirt1 (27, 28), mutation of the four Sirt2 Zn^{2+}-tetra-thiolate cysteines to alanine (Cys^{195}, Cys^{200}, Cys^{221}, and Cys^{224}) ameliorated GSNO-dependent S-nitrosation (Fig. 4E), and deacetylase activity of the Sirt2 tetraalanine mutant was not detected (Fig. 4F). These data are consistent with the Zn^{2+}-tetra-thiolate as the primary and kinetically relevant site of Sirt2 S-nitrosation. Moreover, despite the robust glutathionylation of the GST-GAPDH–positive control, Sirt2 did not exhibit glutathionylation in immunoblots when treated with 100 μM GSNO, GSSG, or H_{2}O_{2}/GSH (Fig. 4D), suggesting inhibition of Sirt2 by GSNO is mediated by S-nitrosation, not glutathionylation. Taken together, these data suggest that Sirt2 demyristoylase activity is selectively inhibited by S-nitrosation of the Zn^{2+}-tetra-thiolate. Again, we cannot rule out the possibility that mutation of the Zn^{2+}-tetra-thiolate and disruption of Zn^{2+} binding may alter the conformation of Sirt2 such that S-nitrosation is diminished at additional distal cysteine residues.

To examine the concentration and time dependence of Sirt2 inhibition by GSNO and NO, k_{inact}/K_{I} analyses were performed as detailed under “Experimental procedures” (Fig. 4G). Although the apparent K_{I} value for NO (∼26 μM) was higher than that of GSNO (0.77 ± 0.75 μM)), the maximal rate of Sirt2 inactivation by GSNO (k_{inact} = 0.0036 ± 0.0006 min^{-1}) was slower than that of NO (k_{inact} = 0.011 ± 0.005 min^{-1}). Comparison of apparent k_{inact}/K_{I} ratios revealed greater inhibition of Sirt2 by GSNO (78 ± 67 μM^{-1} s^{-1}) compared with NO (7 ± 6 μM^{-1} s^{-1}). Taken together, these data suggest that, similar to Sirt6, low-micromolar concentrations of both GSNO and NO inhibit Sirt2 by S-nitrosation at the Zn^{2+}-tetra-thiolate.

Mitochondrial sirtuin deacetylase activity is relatively insensitive to cysteine oxidation

Although Sirt3 and Sirt5 were not inhibited by greater than 23% by any cysteine oxidant tested, this lack of inhibition does not preclude modification at a site that does not result in inhibition of deacetylase activity. Therefore, we examined the ability of cysteine oxidants to modify Sirt3 and Sirt5. Although mild glutathionylation of Sirt3 was observed in response to 100 μM GSNO, GSSG, and H_{2}O_{2}/GSH (Fig. 5A), Sirt3 deacetylase activity was not inhibited by GSNO or GSSG (Fig. 1D), suggesting that Sirt3 glutathionylation by these oxidants is not inhibitory in a physiological context and that modification occurs on a kinetically irrelevant reactive cysteine or that the fraction of modification is insufficient to induce inhibition.

Although Sirt3 deacetylase activity and Sirt5 desuccinylase activity were not impacted by GSNO treatment (Fig. 1, D and E), significant biotin switch signal indicative of S-nitrosation was observed for both Sirt3 and Sirt5 treated with 100 μM GSNO but not NO (Fig. 5B). Surprisingly, mutation of the four Zn^{2+}-tetra-thiolate cysteines to alanine for both Sirt3 (Cys^{256}, Cys^{259}, Cys^{280}, and Cys^{283}) and Sirt5 (Cys^{166}, Cys^{169}, Cys^{207}, and Cys^{214}) ameliorated biotin switch signal for both sirtuins (Fig. 5C), consistent with the Zn^{2+}-tetra-thiolate as the primary site of mitochondrial sirtuin S-nitrosation. Additionally, deacetylase activity was not detected for either the Sirt3 or Sirt5 tetra-alanine mutants (Fig. 5D), demonstrating that, like other sirtuins, the Zn^{2+}-tetra-thiolate is critical for Sirt3 and Sirt5 deacetylase activity. However, because Sirt3 and Sirt5 were not significantly inhibited by the oxidants tested, these data...
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Discussion

Recent studies suggest sirtuin deacylase activity can be modulated by cysteine oxidative post-translational modifications, including S-nitrosation (Sirt1) (27–34), glutathionylation (Sirt1 and Sirt3) (35–37, 40), and sulfonylation (Sirt6) (38, 39). However, many of these studies were performed either in cellular contexts where the oxidant responsible for the modification is unclear or using high concentrations (>100 μM) of oxidants in vitro, rendering it difficult to determine physiological relevance or discriminate effects of direct sirtuin post-translational modification from indirect effects on upstream signaling pathways. To address these limitations, we performed a systematic, comparative analysis of sirtuin oxidative inhibition using a panel of physiological cysteine oxidants and human sirtuins. We also explored the concentration and time dependence of sirtuin inhibition to gain insight into the physiological relevance of each inhibitory oxidation. The results of this analysis both revealed novel insights, such as inhibition of Sirt2 (Fig. 4) and Sirt6 (Fig. 3) through S-nitrosation of their Zn²⁺-tetrathiolate cysteine(s), and also confirmed some observations of previous studies. However, several results also appeared contradictory to prior observations.

Previous studies demonstrated that Sirt1 can be glutathionylated by GSNO (35, 36). However, we and others have also shown that Sirt1 is S-nitrosated by GSNO (27–29). Other reports demonstrate Sirt1 S-nitrosation as a downstream response to cytokine treatment in cellular and whole-animal...
models (28, 31, 33). Although glutathionylation of Sirt1 was not observed in our previous study in response to GSNO or H₂O₂/GSH (27), the Sirt1 construct used in our previous study was truncated after one of the putative glutathionylation sites (Cys⁶⁷) (35, 36). Therefore, the physiological relevance of Sirt1 glutathionylation was not fully examined in our previous study (27), particularly at Cys⁶⁷. The full-length Sirt1 construct used herein was modified both by S-nitrosation and glutathionylation in response to high GSNO concentrations (100 μM), consistent with previous reports (27–30, 35, 36). However, glutathionylation of full-length Sirt1 was only observed at 100 μM GSNO, GSSG, and H₂O₂/GSH and not at lower oxidant concentrations (Fig. 2C), whereas S-nitrosation was concentration-dependent and directly correlated with increased deacetylase inhibition (Fig. 2B). Taken together, these data suggest that S-nitrosation, not glutathionylation, is the post-translational modification inducing Sirt1 inhibition.

We did not observe Sirt6 sulphenylation at 100 μM H₂O₂ (Fig. 3F). Previous studies observed Sirt6 sulphenylation at Cys¹⁸⁶ in response to 500 μM H₂O₂ treatment of either recombinant Sirt6 or RKO cells or at Cys¹⁴⁴ in response to LPS stimulation of THP-1 cells (38, 39). Therefore, H₂O₂ concentrations greater than 100 μM may be necessary to directly observe Sirt6 sulphenylation. Alternatively, because sulphenylation occurs via nucleophilic substitution, wherein a cysteine thiolate reacts with a two-electron oxidant such as a peroxide (52, 53), peroxides other than H₂O₂ can sulphenylate cysteine residues in cellular contexts (55, 56). For example, an organic peroxide, such as a lipid hydroperoxide (54), may be responsible for the Sirt6 sulphenylation observed in previous studies (38, 39).

Consistent with previous studies, we demonstrate that Sirt1 is S-nitrosated by GSNO (27–29). Interestingly, we also observed S-nitrosation and inhibition of Sirt2 and Sirt6, suggesting that all nuclear and cytosolic sirtuins may be S-nitrosated and inhibited under inflammatory conditions in cells. Physiological concentrations of NO lie in the nanomolar range under basal conditions and increase to low-micromolar concentrations under conditions of injury or chronic inflammation (55, 56). Nitrosothiols such as GSNO have been measured in the low (1–20 μM) range in cells (56). Previous work has shown that Sirt1 can be S-nitrosated in cellular and whole-animal systems in response to cytokine treatment (28, 31, 33), suggesting that Sirt1 is modified in vivo under inflammatory conditions. Moreover, our measured IC₅₀ (43–82 μM) and apparent Kᵢ (1–40 μM) values (Table 1) indicate that Sirt1 (Fig. 2), Sirt2 (Fig. 4), and Sirt6 (Fig. 3) can be S-nitrosated at low-micromolar concentrations of NO or GSNO, suggesting that, like Sirt1, Sirt2 and Sirt6 S-nitrosation is a physiologically relevant means to post-translationally inhibit Sirt2 and Sirt6 deacetylase activity.

In addition to IC₅₀ and apparent Kᵢ values, the kinetics of sirtuin inactivation are important to consider, because modification and inhibition may occur on a time scale too slow to be
physiologically relevant. The efficiency of enzyme inactivation (described by the apparent \( k_{\text{inact}}/K_I \)) is dependent both on the inherent rate constant for the reaction of a particular oxidant and cysteine thiol and on the rate constants of other molecular events (e.g. nitrosation binding, conformational change, loss of Zn\(^{2+}\) from the tetrahiolate, disulfide formation following S-nitrosation, etc.) required to inactivate the enzyme. Inactivation efficiencies (i.e. apparent \( k_{\text{inact}}/K_I \) values) faster than the bimolecular rate constant for the reaction of an oxidant with cysteine or other thiol outside the context of a protein suggest that modification occurs at a privileged cysteine residue. This could be due to the local chemical environment (e.g. surrounding residues, bound cofactors, specific binding, and/or positioning of an oxidant) promoting faster or more specific reaction between a given oxidant and a particular cysteine residue. Conversely, inactivation efficiencies slower than the bimolecular rate constant suggest additional steps beyond reaction of the oxidant with a given cysteine are required for inactivation.

The second order rate constant of transnitrosation varies between 1 and 300 M\(^{-1}\) s\(^{-1}\) (57), depending on the transnitrating agent and cysteine residue. The apparent \( k_{\text{inact}}/K_I \) values for inactivation of Sirt1 \((12 \pm 3\) M\(^{-1}\) s\(^{-1}\)) and Sirt6 \((\sim 23\) M\(^{-1}\) s\(^{-1}\)) are both the same \( \sim 2\) M\(^{-1}\) s\(^{-1}\). Subsequent reaction of Sirt2 with a cysteine thiol forms a nitrosothiol (rate constant \( \sim 0.06\) \( \times \) 8 \( \times \) 10\(^{-5}\) M\(^{-1}\) s\(^{-1}\)) (58). At the apparent \( K_I \) values for NO of Sirt2 and Sirt6 (average of \( \sim 30\) \( \mu \)M; Table 1), the second-order rate constant of NO oxidation to N\(_2\)O\(_3\) is \( \sim 60\) M\(^{-1}\) s\(^{-1}\) (30 \( \mu \)M NO times 2 \( \times \) 10\(^{10}\) M\(^{-2}\) s\(^{-1}\) = 60 M\(^{-1}\) s\(^{-1}\)). The inactivation efficiencies of Sirt2 \((5 \pm 4\) M\(^{-1}\) s\(^{-1}\)) and Sirt6 \((7 \pm 6\) M\(^{-1}\) s\(^{-1}\)) by NO are an order of magnitude slower than the rate constant describing N\(_2\)O\(_3\) formation, suggesting that N-nitrosation of Sirt2 is not rate-limiting, and subsequent steps may be rate-limiting for inactivation of Sirt2 and Sirt6 by NO (Figs. 3G and 4G and Table 1). Although MAHMA-NONOate was used as a NO donor, its \( t_{1/2} \) is relatively rapid at 2.7 min and therefore should not significantly impact the rate of sirtuin inactivation observed. Overall, the apparent \( k_{\text{inact}}/K_I \) values for Sirt1, Sirt2, and Sirt6 inactivation by GSNO- and NO-mediated S-nitrosation suggest that significant inhibition of nuclear and cytosolic sirtuins may only occur under conditions of chronic inflammation where NO and nitrosothiols are present at low-micromolar concentrations for prolonged periods. Alternatively, exposure of nuclear and cytosolic sirtuins to low concentrations of NO-derived oxidants may modify a small subset of each population, serving to fine-tune sirtuin activity under conditions of oxidative stress.

In conclusion, our findings suggest that nuclear and cytosolic sirtuins are inhibited by NO-derived oxidants including GSNO, whereas mitochondrial sirtuins are highly resistant to inhibition by all cysteine oxidants tested (Fig. 1). Studies by our laboratory and others suggest that oxidation of sirtuins may occur under inflammatory conditions (27-40) or potentially as a consequence of aging (28). Post-translational regulation of sirtuin activity by oxidation may regulate cellular dynamics and processes, including the subcellular localization equilibrium of sirtuins, as well as regulate cellular metabolism and oxidative stress responses. Overall, we add important insights supporting the hypothesis that chronic inflammation results in oxidative post-translational modification of nuclear and cytoplasmic (but not mitochondrial) sirtuins, thereby inhibiting cytoprotective sirtuin deacetylase activity and increasing susceptibility to aging-related inflammatory disease development.

**Experimental procedures**

**Materials**

Acetone, BSA, coumaric acid, copper sulfate, dichloromethane, DTT, EDTA, L-glutamic dehydrogenase (from bovine liver), hydrazine, hydrogen peroxide, imidazole, iodoacetamide, methanol, myristoyl chloride, NAD\(^{+}\), N-methyl-2-pyrrolidone (NMP), succinimido, triethanolamine, TFA, TCEP, and Tween 20 were purchased from Millipore–Sigma. GSNO, MAHMA-NONOate, and SNAP were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Acetic acid, Fmoc amino acids, \( \alpha \)-ketoglutaric acid, \( \alpha,\alpha \)-disopropylamidine (DIPEA), reduced GSH, hexafluoro phosphate benzotriazole tetramethyl uronium, hydroxybenzotriazole, luminol, NADH, GSSG, and triisopropylsilane were purchased from Chem-Impex (Wood

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**Inhibition of sirtuin activity by nitrosation**

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**Materials**

Acetone, BSA, coumaric acid, copper sulfate, dichloromethane, DTT, EDTA, L-glutamic dehydrogenase (from bovine liver), hydrazine, hydrogen peroxide, imidazole, iodoacetamide, methanol, myristoyl chloride, NAD\(^{+}\), N-methyl-2-pyrrolidone (NMP), succinimido, triethanolamine, TFA, TCEP, and Tween 20 were purchased from Millipore–Sigma. GSNO, MAHMA-NONOate, and SNAP were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Acetic acid, Fmoc amino acids, \( \alpha \)-ketoglutaric acid, \( \alpha,\alpha \)-disopropylamidine (DIPEA), reduced GSH, hexafluoro phosphate benzotriazole tetramethyl uronium, hydroxybenzotriazole, luminol, NADH, GSSG, and triisopropylsilane were purchased from Chem-Impex (Wood
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Dale, IL, USA). Acetonitrile, ampicillin, ascorbic acid, glycerol, β-mercaptoethanol, isopropyl-1-thio-β-D-galactopyranoside, kanamycin, sodium chloride, SDS, and 96-well polystyrene clear flat-bottomed plates were purchased from WVR (Radnor, PA, USA). Diethyl ether and N,N-dimethylformamide were purchased from Oakwood Chemical (Estill, SC, USA). HEPES, non-fat dry milk, 2× YT medium, and terrific broth medium were purchased from Research Products International (Mt. Prospect, IL, USA). Diethylenetriaminepentaacetic acid (DPTA), EZ-Link iodoacetyl-PEG2-Biotin, horseradish peroxidase–linked goat anti-mouse and anti-rabbit secondary antibodies and Wheaton 12 × 32-mm polypropylene HPLC vials were purchased from Thermo Fisher Scientific. Neocouproine was purchased from TCI chemicals (Tokyo, Japan). 5-Tetramethylrhodamine azide was purchased from AAT Bioquest (Sunnyvale, CA, USA). SYPRO Ruby was purchased from Molecular Probes. H3K14ac peptide was purchased from Peptide 2.0 (Chantilly, VA, USA). Anti-GSH antibody was purchased from Virogen (catalog no. 101-A, lot GS-D8/G2-70). Rink-amine 4-methylbenzhydrylamine resin was purchased from Novabiochem. Nickel–nitrilotriacetic acid superflow resin was purchased from 5-PRIME (Hilden, Germany) and GE (Marlborough, MA, USA). HiLoad Superdex 75-70 10 × 600-mm and HiLoad Superdex 200-20 10 × 1000-mm columns, TGX Stain-Free gels, and urea were purchased from Bio-Rad. GSNO stocks were made in HDN (100 mM HEPES, pH 7.7, 1 mM DPTA, 0.1 mM neocouproine), and the concentration was determined using the extinction coefficient 0.92 mm⁻¹ cm⁻¹.

Solid-phase peptide synthesis

A 5-mer acetyl-lysine p53-based peptide (p53W; acetyl-RHKK(acetyl)-NH2), 11-mer acetyl-lysine H3K14-based peptide (H3K14ac; H2N-KSTGGK(acetyl)APRKQ-NH2), 16-mer myristoyl-lysine H3K9-based peptide (H2N-KQTAQK(myristoyl)STGKKAPRW-W-NH2), 9-mer acetyl-lysine OTC-based peptide (H2N-IIINQK(acetyl)RFNDF-NH2), and 12-mer succinyl-lysine GDH-based peptide (H2N-SGASEK(succinyl)DIVHSG-NH2) were synthesized using standard tBu/Fmoc solid-phase peptide synthesis techniques (61). Acetyl derivatives were synthesized from corresponding commercially available Fmoc-Lys (Ac)-OH building block. Dde was used as a protecting group for myristoyl- and succinyl-lysine residues that were derivatized after amino acid coupling. The resin was swollen in DCM overnight at 4 °C and then Dde-lysine–deprotected with 2% (v/v) hydrazine in N,N-dimethylformamide for 10 min. Deprotection was repeated twice. The resin was then washed three times with 5 ml of DCM and three times with 5 ml of NMP. Deprotection of Dde-lysine was confirmed via positive Kaiser test (62). Myristoylation was performed by incubating resin with 5 equivalents of myristoyl chloride and 5% (v/v) DIPEA in NMP for 1 h. Succinylation was performed by incubating resin with 5 equivalents of succinic anhydride and 5% (v/v) DIPEA in NMP for 1 h. The resin was then washed three times with 5 ml of DCM and three times with 5 ml of NMP. Complete coupling was confirmed by negative Kaiser test. Following TFA cleavage and diethyl ether precipitation, crude peptides were redissolved in water with 0.1% (v/v) TFA and purified by semipreparative HPLC on a μBondapak C18 column (Waters, 3.9 × 300 mm) using an Agilent 1100 series HPLC, using a gradient of 0–80% (v/v) acetonitrile in water with 0.1% (v/v) TFA. Fractions were collected and lyophilized to yield final peptides as dry, white powders. Purified peptide masses were confirmed by direct injection ESI MS (QExactive, Thermo Scientific). The observed mass for each peptide matched the predicted mass. Peptide concentrations were determined from the mass of the peptide as a TFA salt of basic residues, assuming 1 equivalent TFA/cationic group or by A280 for Trp-containing peptides using an extinction coefficient of 5.6 mm⁻¹ cm⁻¹ for each tryptophan.

Expression and purification of nicotinamidase

Maltose-binding protein–tagged nicotinamidase (pTEV6) was purified from BL21(DE3) Escherichia coli via nickel-affinity chromatography as previously described (27, 41).

Expression and purification of sirtuins

Recombinant Sirt1 (pET24a aa 1–747), Sirt2 (pET28a aa 38–356 based on UniProtKB Q8IXJ6) or pQE80 aa 1–373 based on GenBank AAF67015.1), Sirt3 (pET28a aa 113–399), Sirt5 (pET28a aa 20–310), and Sirt6 (pQE80 aa 1–355) WT or Zn²⁺-tetrathiolate tetracysteine to tetraalanine mutants (4C→A) were purified from BL21(DE3) E. coli by nickel-affinity chromatography. The cells were transformed and grown at 37 °C in 2× YT medium supplemented with 50 mg/liter kanamycin (pET24 or pET28a) or ampicillin (pQE80) to an A₆₀₀ of ~0.7. Protein expression was induced for 20 h with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 18 °C. The cells were harvested via centrifugation at 5,000 × g, and cell pellets were frozen at −80°C until lysis. Frozen cells were thawed on ice, resuspended in lysis buffer (Table S1), and lysed via sonication. The lysates were cleared via centrifugation for 30 min at 30,000 × g. Cleared lysate was incubated with nickel–nitrilotriacetic acid resin (0.75 ml resin/liter culture) at 4 °C for a minimum of 1 h. Bound resin was pelleted via centrifugation at 4,200 × g, resuspended in 10 × resin volume of lysis buffer, and packed into a column. Packed resin was washed with 10 column volumes of wash buffer (Table S1) and purified enzymes eluted with 5 column volumes of elution buffer (Table S1). The enzymes were further purified and exchanged into storage buffer via size-exclusion chromatography using an Enrich SEC 650 10 × 300-mm (Sirt1) or Enrich SEC 70 10 × 300-mm column (Sirt2, Sirt3, Sirt5, and Sirt6). Concentrated purified enzymes were aliquoted and stored at −80°C.

Expression and purification of GST-GAPDH

GST-tagged GAPDH (pGEX 6P-3 vector) was purified from BL21(DE3) E. coli using GSH affinity chromatography, as previously described (27).

Continuous enzyme-coupled sirtuin deacetylase assays

Deacetylase activity of Sirt1, Sirt2, and Sirt3 WT and 4C→A mutants were monitored under initial rate conditions using a
continuous enzyme-coupled assay for nicotinamide-producing enzymes as previously described (41). In brief, the assay was performed at 25 °C in a reaction mixture containing 20 mM potassium phosphate, pH 7.5, NAD\(^+\), acetylated peptide, 2–2.5 \(\mu\)M maltose-binding protein–tagged pyrazinamidase, 3.3 mM \(\alpha\)-ketoglutarate, 200 \(\mu\)M NADH, 2.5 units of L-glutamic dehydrogenase, and 0.5 \(\mu\)M Sirt1, 2 \(\mu\)M Sirt2, or 0.5 \(\mu\)M Sirt3 (Sirt3 was desalted with a Micro BioSpin-6 column (Bio-Rad) to remove the 5 mM DTT in the storage buffer prior to oxidant treatment with 100 \(\mu\)M GSNO, SNAP, NO, GSSG, or \(\text{H}_2\text{O}_2\)). 100 \(\mu\)M p53ac (\(k_{\text{inact}}/K_I\) analyses) or 100 \(\mu\)M H3K14ac (oxidant screen and IC\(_{50}\) analyses) peptides were used to monitor Sirt1 deacetylase activity, 30 \(\mu\)M H3K14ac peptide was used to monitor Sirt2 deacetylase activity, and 100 \(\mu\)M OTCase peptide was used to monitor Sirt3 deacetylase activity. 500 \(\mu\)M NAD\(^–\) was used to monitor Sirt1 deacetylase activity, 100 \(\mu\)M NAD\(^+\) was used to monitor Sirt2 deacetylase activity, and 100 \(\mu\)M NAD\(^–\) was used to monitor Sirt3 deacetylase activity. Reactions were initiated by addition of acetylated peptide and NAD\(^+\). The rates were monitored continuously for 10 min in a clear flat-bottomed 96-well plate (Greiner Bio-One) on a Molecular Devices FlexStation multimode microplate reader (San Jose, CA, USA) and initial rates were determined using our Interactive Continuous Enzyme Kinetics Analysis Tool (ICEKAT) (64).

**HPLC sirtuin deacetylase assay**

Demyristoylation activity of Sirt2 and Sirt6 WT and 4C→A mutants and desuccinylase activity of Sirt5 WT and 4C→A mutants were assessed via an HPLC fixed time point assay as previously described (8). In brief, the assay was performed at 25 °C in a reaction mixture containing 20 mM potassium phosphate, pH 7.5, NAD\(^+\), acetylated peptide, and 0.5–1 \(\mu\)M Sirt2, 0.5–1 \(\mu\)M Sirt6, or 0.5 \(\mu\)M Sirt5. 10 \(\mu\)M H3K9myr peptide was used to monitor Sirt2 demyristoylation activity, 20 \(\mu\)M H3K9myr was used to monitor Sirt6 activity, and 12.5 \(\mu\)M GDHSucc was used to monitor Sirt5 activity. 10 \(\mu\)M NAD\(^+\) was used to monitor Sirt2 activity, 20 \(\mu\)M NAD\(^+\) was used to monitor Sirt6 demyristoylation activity, and 31 \(\mu\)M NAD\(^+\) was used to monitor Sirt5 desuccinylase activity. The reactions were initiated with the addition of acetylated peptide and NAD\(^+\). After 15 min (Sirt2), 45–60 min (Sirt6), or 2.5 min (Sirt5), the reactions were quenched with 1% (v/v) final concentration of TFA. The samples were centrifuged at 15,000 \(\times\) \(g\) prior to HPLC analysis to remove insoluble debris. Acetylated and deacetylated peptides were resolved on a C18 column (4.6 \(\times\) 250 mm, Thermo Scientific catalog no. 25005-254630) using an Agilent 1100 series HPLC. A gradient of 5–70% (v/v) acetonitrile in water with 0.1% (v/v) TFA was applied over 20 min to resolve H3K9 and H3K9myr peptides, where H3K9 eluted at 15.3 min and H3K9myr eluted at 21.5 min. This 5–70% acetonitrile gradient was applied over 40 min to resolve H3K9 and H3K9myr in reactions containing GSNO, where H3K9 eluted at 19.9 min, H3K9myr eluted at 32 min, and GSNO eluted at 18.7 min. A gradient of 5–50% (v/v) acetonitrile in water with 0.1% (v/v) TFA was applied over 20 min to resolve GDH and GDHSucc peptides, where GDH eluted at 13 min and GDHSucc eluted at 14.4 min. The area under the curve at 280 nm was integrated and used to calculate the percentage of conversion of the H3K9myr peptide. The area under the curve at 230 nm was integrated and used to calculate the percentage of conversion of the GDHSucc peptide.

**Oxidant IC\(_{50}\) analysis**

Concentration-dependent oxidant responses were determined by treating 2 \(\mu\)M purified sirtuins with varying concentrations of GSNO (Sirt1, Sirt2, and Sirt6) or NO (2 mol NO released/mol MAHMA-NONOate) (Sirt1, Sirt2, and Sirt6) for 1 h at 37 °C. Deacetylation rates of Sirt1–3 were determined at subsaturating concentrations of acetylated peptide and NAD\(^+\) using the continuous enzyme-coupled assay (41) as described above. Demyristoylation rates of Sirt2 and Sirt6, and desuccinylation rates of Sirt5 were determined at subsaturating concentrations of myristoylated or succinylated peptide and NAD\(^+\) via the HPLC fixed time point assay as described above. The data were plotted as percentages of deacylase activity in the absence of oxidant versus oxidant concentration and fit to Equation 1 using GraphPad Prism (La Jolla, CA, USA).

\[
\% \text{Deacylation activity} = 100 \left(1 - \frac{[\text{Oxidant}]}{\text{IC}_{50} + [\text{Oxidant}]} \right) \quad \text{(Eq. 1)}
\]

**Oxidant \(k_{\text{inact}}/K_I\) analysis**

Inactivation kinetics were examined using previously described methods (47, 48, 50). Each sirtuin (1 \(\mu\)M) was treated with varying concentrations of GSNO (Sirt1, Sirt2, and Sirt6) or NO (2 mol NO released/mol MAHMA-NONOate) (Sirt2 and Sirt6) for 0, 5, 10, 15, 30, 45, 60, and 90 min at 37 °C. Initial deacetylation rates were determined as detailed above at each oxidant concentration and time point. The fractional remaining activities at each time point and oxidant concentration were calculated relative to the untreated control at time 0. To determine the apparent first-order rate constants of inactivation (\(k_{\text{obs}}\)) at each oxidant concentration, the decrease in natural logarithm of the fraction remaining activity was plotted as a function of the time of oxidant incubation. The negative slopes of the resulting lines were calculated at each oxidant concentration, and the slope of the line in the absence of oxidant was subtracted to yield \(k_{\text{obs}}\) values with units of inverse time. The \(k_{\text{obs}}\) values were then plotted as a function of oxidant concentration, and the inactivation kinetic parameters were determined using Equation 2,

\[
k_{\text{obs}} = k_{\text{inact}} \left(\frac{[\text{Oxidant}]}{K_I + [\text{Oxidant}]} \right) \quad \text{(Eq. 2)}
\]

where [Oxidant] represents the oxidant concentration, \(k_{\text{inact}}\) is the limiting maximal inactivation rate constant at infinite oxidant concentration, and the apparent \(K_I\) is not a true binding constant but is the oxidant concentration that yields a \(k_{\text{obs}}\) value of half the \(k_{\text{inact}}\) value.
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Biotin switch assay for S-nitrosation

To fully prereduce all cysteine residues, purified Sir1, Sir2, Sir3, Sir5, and Sir6 (20 μM) were incubated with 1 mM TCEP for 15 min at room temperature and desalted with a Micro Bio-Spin-6 column (Bio-Rad). Desalted protein was incubated with 1–100 μM GSNO or the molar equivalent of NO released from MAHMA-NONOate (2 mol NO released/mol MAHMA-NONOate) for 60 min at 37 °C in PBS in a 50-μl total volume under low ambient light conditions. Free thiols were alkylated by incubation with an equal volume of 2X HDNSU-IA blocking buffer (200 mM HEPES, pH 7.7, 2 mM DPTA, 0.2 mM neocuproine, 2% (w/v) SDS, 6 mM urea, 200 mM iodoacetamide) for 1 h at 37 °C. The proteins were precipitated with 1 ml of cold (−20 °C) high purity acetone overnight at −80 °C. Precipitated protein was pelleted via centrifugation at 12,000 × g at 4 °C, and the supernatant was discarded. Pellets were washed once with 1 ml of cold (−20 °C) acetone and pelleted via centrifugation at 12,000 × g, and the supernatant was removed. The pellets were air-dried for 15 min at room temperature, resuspended into label buffer (PBS with 1% (w/v) SDS, 30 mM ascorbate, 100 μM EZ-link biotin iodoacetamide), and incubated for 1 h at 37 °C. Ascorbate was omitted as a control condition from the reduction and labeling step. The reactions were quenched with 6× Laemmli sample buffer containing 10% (v/v) β-mercaptoethanol, and each sample was loaded onto a Bio-Rad Any kDa TGX stain-free gel. Equal protein loading was verified by stain-free gel imaging under UV light using a ChemiDoc MP imager (Bio-Rad). The proteins were transferred to a nitrocellulose membrane using a TransBlot Turbo semi-dry transfer system (Bio-Rad), and the membrane was blocked for 1 h at room temperature in PBS containing 0.1% (v/v) Tween 20 (PBST) and 2.5% (w/v) dry milk. Vectastain solution was made up according to the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA), where reagents A and B were diluted into PBST and incubated for 20 min at room temperature prior to use. The membrane was then washed three times for 5 min with PBST and rocked for 1 h at room temperature with Vectastain solution. Following incubation, the membrane was again washed three times for 5 min in PBST and imaged after a 2-min incubation in chemiluminescence buffer (50 mM Na2HPO4, 50 mM NaHCO3, 150 mM NaCl, 10 mM NaBO3, 225 μM p-coumaric acid, 1.5 mM luminol, and 0.6% (v/v) H2O2).

Sulfenylation assays

The in vitro sulfenylation protocol was adapted from Truong and Carroll (51). Purified sirtuins and GST-GAPDH were diluted to 10 μM in Tris label buffer (50 mM Tris, pH 7.4, 150 mM NaCl), reduced with 1 mM TCEP for 30 min at room temperature, and desalted with Micro BioSpin-6 columns (Bio-Rad) pre-equilibrated with Tris labeling buffer. Desalted sirtuins and GST-GAPDH (10 μM) were treated with 0 or 100 μM H2O2, and 1 mM DYN-2 for 1 h at 37 °C in Tris labeling buffer. Treated sirtuins and GST-GAPDH were desalted a second time with Micro BioSpin-6 columns pre-equilibrated with click label buffer (50 mM triethanolamine, pH 7.4, 1% (w/v) SDS) and subsequently treated with 100 μM tetramethylrhodamine azide, 1 mM TCEP, 100 μM tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]-amine, and 1 mM CuSO4 for 1 h at room temperature, shielded from light and shaking continuously. The reactions were quenched with the addition of 1.3 ml of ice-cold MeOH and incubated for 18 h at −80 °C to precipitate proteins. Proteins were pelleted via centrifugation at 16,000 × g for 30 min at 4 °C. The supernatant was discarded, protein pellets were washed once with 1.3 ml of ice-cold MeOH, and pelleted again by centrifugation at 16,000 × g. The supernatant was discarded, and the resulting pellets were air-dried for 30 min at room temperature, shielded from light. The pellets were suspended into 1× Laemmli sample buffer with 10% (v/v) β-mercaptoethanol, incubated at 100 °C for 5 min, and centrifuged at 16,000 rpm for 2 min. Proteins were resolved on a 10% SDS-polyacrylamide gel. The gel was then incubated 2× 10 min in destain solution (40% (v/v) MeOH, 10% (v/v) acetic acid, 50% (v/v) water), then equilibrated in water for 10 min before imaging on a Typhoon Trio visible mode imager using an excitation wavelength of 532 nm and an emission wavelength of 580 nm for tetramethylrhodamine. Following in-gel fluorescence detection, the gel was again incubated in destain solution for 30 min at room temperature, followed by incubation in SYPRO Ruby stain for 18 h at room temperature. The gel was washed twice for 15 min in SYPRO Ruby wash solution (10% (v/v) MeOH, 7% (v/v) acetic acid, and 83% (H2O) water) and once for 10 min in water, and SYPRO Ruby signal was imaged on a Typhoon Trio visible mode imager using an excitation wavelength of 532 nm and an emission wavelength of 610 nm.

Anti-glutathionylation blots

Purified sirtuins (2 μM) were incubated with 100 μM (Sir1–3, Sir5, and Sir6) or 0–100 μM (Sir1) freshly dissolved GSNO, GSSG, and H2O2/GSH for 1 h at 37 °C. Purified GST-GAPDH was used as a positive control for glutathionylation. GST-GAPDH (2 μM) was incubated with 100 μM H2O2 and 100 μM GSH for 1 h at 37 °C as previously described (63). Glutathionylation reactions were quenched with the addition of nonreducing Laemmli sample buffer, and samples were resolved by nonreducing SDS-PAGE, transferred to nitrocellulose, and blocked with PBST with 3% (w/v) BSA for 1 h. The membranes were incubated with mouse anti-GSH antibody (Virogen catalog no. 101-A, lot GS-D8/G2-70) diluted 1:1000 in PBST with 1.5% (w/v) BSA for 16 h at 4 °C and subsequently washed three times for 10 min with PBST. The membranes were then incubated with anti-mouse horseradish peroxidase secondary antibody diluted 1:5000 in PBST with 1.5% (w/v) BSA for 1 h at room temperature. The membranes were again washed three times for 10 min with PBST and imaged after a 2-min incubation in chemiluminescence buffer (50 mM Na3HPO4, 50 mM NaHCO3, 150 mM NaCl, 10 mM NaBO3, 225 μM p-coumaric acid, 1.5 mM luminol, and 0.6% (v/v) H2O2). A stain-free gel image was used as a loading control.

Data availability

All of the data described in the article are contained within the article.

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HPLC assay data, Dr. Michael Olp for assistance with peptide synthesis and for development of an interactive fitting tool for processing continuous enzyme kinetic data, Trudy Holyst in the Protein Chemistry Core Laboratory at the BloodCenter of Wisconsin (Part of Versiti) for assistance with peptide synthesis, and Dr. Kirkwood Pritchard (Medical College of Wisconsin) for the gift of the ABI 433A peptide synthesizer.

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Abbreviations—The abbreviations used are: aa, amino acid(s); DCM, dichloromethane; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl; Fmoc, N-(9-fluorenyl)methoxycarbonyl; DIPEA, N,N-diisopropyl-ethylamine; DPTA, diethylenetriaminepentacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSNO, N-nitroso-N-acetylimino-

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