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KEAP1 MODIFICATION AND NUCLEAR ACCUMULATION IN RESPONSE TO S-NITROSOCYSTEINE

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Abstract

Keap1 is a key regulator of the Nrf2 transcription factor which transactivates the Antioxidant Response Element (ARE) and upregulates numerous proteins involved in antioxidant defense. Under basal conditions, Keap1 targets Nrf2 for ubiquitination and proteolytic degradation and as such is responsible for the rapid turnover of Nrf2. In response to oxidants and electrophiles, Nrf2 is stabilized and accumulates in the nucleus. The mechanism for this effect has been proposed to involve thiol-dependent modulation of Keap1 leading to loss of its ability to negatively regulate Nrf2. We have previously shown that nitric oxide and S-nitrosothiols cause nuclear accumulation of Nrf2 and upregulation of the ARE-regulated gene HO-1. Here we show that nitric oxide and S-nitrosocysteine (CSNO) cause time and dose-dependent Keap1 thiol modification. These studies were carried out in HEK293 and in HEK293 cells overexpressing hemagglutinin-tagged Keap1. Furthermore we demonstrate that in response to CSNO Keap1 accumulates in the nucleus with a time course similar to that of Nrf2.

Keywords

Nitric Oxide; S-Nitrosocysteine; S-Nitrosylation; Oxidation; Keap1; Nrf2; Nuclear localization

INTRODUCTION

We and others have previously demonstrated that nitric oxide (NO) and related species activate the Nrf2/ARE pathway with subsequent induction of cytoprotective and antioxidant genes [1–6]. Several of these studies point to a role for protein kinases in mediating this response [1,5,6]. However reports from other labs suggest that modification of Keap1 is responsible for activation of the Nrf2/ARE pathway.

Keap1, or Kelch-like ECH-associated protein, is a member of the BTB-Kelch family of proteins [7,8]. In mammalian cells, Keap1 was first identified in 2 hybrid screens as a binding partner for NF-E2-related factor 2 (Nrf2), a basic leucine zipper (bZIP) transcription factor of the cap'n collar family [9] [10] [7] [8]. Nuclear Nrf2 forms heterodimers with other bZIP transcription factors, then binds to and transactivates the antioxidant response element (ARE) found in the

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promoter regions of numerous genes involved in cytoprotection and antioxidant defense [11] [12]. Importantly, Keap1 acts as a negative regulator of Nrf2 [13] [7] which modulates Nrf2 activity through a variety of proposed mechanisms.

Initially Keap1 was thought to be a cytosolic protein which binds to both Nrf2 and actin [7] [14]. In this way, Keap1 could sequester Nrf2 in the cytosol and prevent its nuclear localization and activation of ARE-regulated genes [7]. More recently, an alternative picture has emerged in which Keap1 targets Nrf2 for rapid turnover by proteolytic degradation [15] [16] [17]. Studies have shown that Keap1 acts as an adaptor protein for CUL3 which complexes with enzymes involved in ubiquitination [18]. Through the action of E3 ubiquitin ligase, ubiquitin groups are transferred to lysine residues in the Neh2 domain of Nrf2 and rapid degradation by the 26 S proteasome follows [18,19].

Interestingly, recent studies show that nuclear accumulation of Keap1 or truncated versions of Keap1 occurred in response to activators of the Nrf2/ARE pathway [20] [21,22]. In addition, Keap1 accumulated in the nucleus following the inhibition of the CRM1-dependent nuclear export by leptomycin B [20] [21,22]. This suggests that Keap1 contains a nuclear export signal (NES) sequence and, in fact, at least one putative NES sequence has recently been identified [21] [22] [20].

Numerous investigations have demonstrated that Keap1's functions are thiol-dependent [23]. Keap1 is a cysteine-rich protein with a high degree of sequence homology between species. Several of the cysteine thiols have been identified as very reactive *in vitro* [23] [24] [25]. Site-directed mutagenesis of Cys 273 and Cys 288 resulted in the loss of Keap1's ability to repress Nrf2 activity while Cys 151 was found to be essential for inducer-mediated activation of Nrf2 [24] [26] [17]. Similar effects were observed with oxidants and electrophiles which are known to be thiol-reactive [17] [26] [24].

Studies with purified Keap1 have shown oxidative modification in response to oxidants and the formation of Keap1 adducts in response to electrophiles [23–25,27–29]. Very few studies have measured Keap1 thiol modification in cellular systems. In one case, treatment with 3 different electrophiles led to the formation of disulfide-linked Keap1 dimers [24]. More recently, adducts of Keap1 thiols were identified following treatment of cells with 15-deoxy-prostaglandin J₂ [30] and iodoacetyl-N-biotinylhexylenediamine (IAB) [28]. The time course or dynamics of Keap1 thiol modification have not been reported in any cellular system.

S-nitrosylation has emerged as an important mechanism by which nitric oxide (NO) regulates protein function [31,32]. NO oxidizes to N₂O₃ which may react with thiols by S-nitrosylation [31,32]. In addition, S-nitrosothiols react directly with thiols through transfer of nitroso groups and by oxidation [33]. Both S-nitrosylation and thiol oxidation may mediate functional changes in proteins. We hypothesized that Keap1 might be sensitive to NO and related species by these mechanisms. In this study we investigated the modification of Keap1 thiols in response to spermine NONOate and CSNO. These experiments were carried out in HEK293 cells and in HEK293 cells overexpressing hemagglutinin (HA) - tagged Keap1. Furthermore we determined the nuclear localization of Keap1 under conditions which led to its modification. The time course of both of these responses was determined.

MATERIALS AND METHODS

Materials

Fetal bovine serum was obtained from HyClone (Logan, UT) and Minimal Essential Medium was from Sigma. All other cell culture reagents were from GIBCO BRL (Grand Island, NY). Tissue culture plasticware was obtained from Nunclon (Fisher Scientific, Raleigh, NC).

Spermine NONOate was from Cayman (Ann Arbor, MI). Tris-glycine SDS-PAGE gels, lipofectamine reagent, N⁵-(3-maleimidylpropionyl)biocytin (MPB) and NeutrAvidin-HRP were from Invitrogen (Carlsbad, CA). PD10 columns and enhanced chemiluminescence reagents were obtained from Amersham (Piscataway, NJ). Antibodies were from Santa Cruz Biotechnology (SCBT, Santa Cruz, CA) and Cell Signaling Technology (CST, Danvers, MA). BCA reagents were from Pierce (Rockford, IL). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Cell isolation and culture

HEK293H cells from Invitrogen were obtained from the Duke Cell Culture facility. They were grown on polylysine-coated dishes and flasks in Minimal Essential Medium containing 10% fetal bovine serum and 1% antibiotic-antimycotic and maintained at 37°C in a 5% CO₂ environment. Cells were passaged using trypsin.

Expression of Keap1

Keap1 cDNA was obtained from human umbilical endothelial cells by RT-PCR using the primer pair, 5'-GGA TCC ATG CAG CCA GAT CCC AGG-3' and 5'-TCT AGA TCA ACA GGT ACA GTT CTG CTG GTC-3'. A hemagglutinin (HA) tag was added by PCR and the construct was cloned into a mammalian expression vector, pcDNA3, under control of the CMV promoter for the expression of N-terminally tagged HA-Keap1. The HA-Keap1 construct was confirmed by DNA sequencing.

Transfection

At 50% confluence, HEK293H cells in wellplates (9 cm²) were transfected using 1.5 µg/well of HA-Keap1 expression vector DNA and 6 µg/well lipofectamine for 40 hours.

Experimental conditions

Confluent monolayers of cells in flasks or wellplates were rinsed 2 times with Hanks Balanced Salt Solution containing 25 mM HEPES (HHBSS) and incubated in the same for 15 minutes at 37°C in air prior to the start of the experiment. Immediately prior to the experiments, spermine NONOate was dissolved in 0.01 N NaOH. S-nitrosocysteine (CSNO) was freshly prepared immediately before use as a mol/mol solution of cysteine and sodium nitrite and its concentration was verified by UV/vis spectrophotometry. No changes to HHBSS pH were observed following addition of spermine NONOate or CSNO.

MPB Assay

This assay was carried out as previously described [34]. Cell monolayers adhered to tissue culture plasticware were rinsed twice with ice-cold PBS and lysed in 1 mL lysis Buffer containing 50 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, 1% Triton x-100, 0.5% sodium deoxycholate, 0.2 g SDS, 20 mM NEM and protease inhibitors. Solutions containing NEM were prepared immediately before use. Cell monolayers were placed on ice and cells were dislodged by scraping. Samples were subjected to centrifugation for 10 minutes at 16,000 × g at 4 °C. To remove unreacted NEM, the resulting supernatant was applied to PD-10 columns which had been preequilibrated with Tris-NaCl-EDTA buffer pH 8. Eluate was collected from the 3.5–4.5 mL fraction. Samples were incubated for 30 minutes on ice with 20 mM DTT and the unreacted DTT removed using the PD10 columns. Samples were incubated on ice for 30 minutes with 50 µM MPB and the unreacted MPB removed using the PD10 columns. Samples were divided into aliquots for protein assay, immunoblotting and immunoprecipitation. Samples for immunoprecipitation were treated with 50 µM glutathione and incubated on a rocking platform with 1 µg of anti-Keap or anti-HA antibody overnight at 4 °C. Protein A or Protein G Plus Agarose was added and the samples were further incubated for 1 hour. Samples

were subjected to centrifugation at $1000 \times g$ to obtain pellets which were then washed 4x with RIPA buffer consisting of a solution of 9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate and 150 mM NaCl, pH 7.4, to which was added 1% (vol/vol) Igepal CA-630, 0.5% (wt/vol) sodium deoxycholate and 0.1% (wt/vol) SDS. Samples were boiled 5 minutes at 100°C in 10 μL 2X Laemmli Sample buffer consisting of 0.125 M Tris HCl, pH 6.8, 20% glycerol, 4% SDS, 0.004% bromphenol blue and 10% β -mercaptoethanol. Samples were immunoblotted as described below using a 1:1000 dilution of NeutrAvidin-HRP.

Preparation of cell lysates

Cell lysates were prepared by rinsing flasks twice with ice-cold phosphate buffered saline and incubating on ice for 10 minutes with 1mL RIPA buffer containing protease inhibitors. Cells were collected by scraping, sonicated and subjected to centrifugation at $16,000 \times g$ for 10 minutes at 4°C . A 50 μL aliquot of the supernatant was removed for protein analysis. Supernatants were diluted with 4X sample buffer to a final concentration of 62.5 mM Tris-HCl pH 6.8, 50 mM dithiothreitol, 2% (wt/vol) SDS, 10% (vol/vol) glycerol and 0.004% (wt/vol) bromphenol blue. Samples were treated for 5 minutes at 100°C and stored at -20°C until analysis.

Preparation of cytosolic and nuclear extracts

Briefly, confluent monolayers of cells were rinsed twice with ice-cold phosphate buffered saline and incubated at 4°C for 10 minutes on a rocking platform in 1 mL cytoplasmic extraction buffer consisting of 10 mM Tris-HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT with protease inhibitors. The detergent Igepal CA-630 was added to a final concentration of 0.1% and cells were dislodged with a cell scraper. Nuclei were pelleted by centrifugation at $7200 \times g$ for 10 minutes while the supernatant served as the cytosolic extract. Nuclei were then incubated in 0.25 mL nuclear extraction buffer consisting of 20 mM Tris-HCl, pH 8, 400 mM NaCl, 1.5 mM MgCl_2 , 1.5 mM EDTA, 1 mM DTT, 25% glycerol, and protease inhibitors. Following sonication, an aliquot was removed for protein analysis. Both cytosolic and nuclear extracts were diluted with 4X sample buffer to a final concentration of 62.5 mM Tris-HCl pH 6.8, 50 mM dithiothreitol, 2% (wt/vol) SDS, 10% (vol/vol) glycerol and 0.004% (wt/vol) bromphenol blue. Samples were treated for 5 minutes at 100°C and stored at -20°C until analysis.

Immunoblotting

Immunoblotting was carried out in cell lysates, cytosolic extracts and nuclear extracts following determination of protein concentrations by the Micro-BCA Assay (Pierce). Primary antibodies included rabbit polyclonal anti-Nrf2 (H-300) SCBT #sc-13032, goat polyclonal anti-Keap1 (E-20) SCBT #sc-15246, mouse monoclonal anti-HA-probe (F-7) SCBT #sc-7392, rabbit polyclonal anti-Lamin A (H-102) SCBT sc#20680 and mouse monoclonal anti- α -Tubulin (TU-02) SCBT sc#8035. Blocking peptide was Keap1 (E-20) SCBT #sc-15246P. HRP-conjugated secondary antibodies included donkey anti-goat IgG-HRP SCBT #sc-2020, anti-mouse IgG-HRP CST #7076 and anti-rabbit IgG-HRP CST #7074. Immunoreactivity was visualized by enhanced chemiluminescence (ECL and ECL Plus). Estimation of protein molecular weights was based on prestained molecular weight markers transferred to the PVDF.

RESULTS

Keap1 Modification by Spermine NONOate and CSNO

These experiments investigated Keap1 modification by NO-related species using an assay developed by our laboratory to measure modification of specific proteins [34]. Following exposure to the agents, cells were lysed in the presence of NEM to block reduced thiols. After

removal of unreacted NEM, lysates were treated with DTT to reduce disulfide bridges, S-nitrosothiols, sulfenic acid derivatives and other reversible thiol modifications. Subsequently the unreacted DTT was removed and lysates were treated with MPB, a biotinylated, irreversible thiol reactive reagent. After removal of unreacted MPB, lysates were immunoprecipitated for the Keap1 protein and subjected to SDS-PAGE electrophoresis and immunoblotting. NeutrAvidin-HRP was used to identify MPB-labeled proteins. In this way, only DTT-sensitive modifications were detected. This included S-nitrosylated and glutathionylated species, disulfide bridges and sulfenic acid derivatives. Any disulfide bridges (or other DTT-sensitive modifications) found endogenously in Keap1 were visible in control cells as well as treated cells.

The first experiments involved HEK293H cells which were transfected with HA-tagged Keap1. Cells were treated for 40 hours with lipofectamine and HA-Keap1 plasmid or lipofectamine alone. Subsequently some cells were treated for 10 minutes with 500 μ M of the NO donor spermine NONOate or with 500 μ M of the S-nitrosothiol CSNO. NO and CSNO are reactive nitrogen species with different chemical reactivities. As shown in Figure 1A, Keap1 was overexpressed in cells treated with our HA-Keap1 construct (lanes 1–3 and lanes 6–8) compared with sham controls (lanes 4 and 9). Both immunoprecipitation with anti-HA (lanes 1–3) and immunoprecipitation with anti-Keap1 (lanes 6–8) resulted in an immunoreactive band at about 70 kDa, corresponding to Keap1, following immunoblotting with anti-HA. Sham controls lacked this immunoreactivity (lanes 4 and 9). A nonspecific band of higher molecular weight was noted in anti-HA immunoprecipitates (lanes 1–4). Treatment with spermine NONOate (lanes 2 and 7) and CSNO (lanes 3 and 8) had no effect on our ability to immunoprecipitate Keap1 protein using either the anti-HA or anti-Keap1 antibodies. As a control, lysate was preincubated with Keap1 blocking peptide prior to immunoprecipitation with anti-Keap1 and immunoblotting with anti-HA. This resulted in loss of immunoreactivity of the 70 kDa band as well as that of the other bands (Figure 1B, lane 1). These findings validate this method for evaluation of Keap1 oxidation.

We treated HEK293H cells, which had been transfected with HA-Keap1, for 0–15 minutes with 500 μ M spermine NONOate. Lysates were prepared by the MPB protocol and HA-Keap1 was immunoprecipitated using anti-HA. As shown in Figure 2A, increased Keap1 thiol modification was observed at 10 and 15 minutes in the case of spermine NONOate (lanes 1–4) and at 5 to 15 minutes in the case of CSNO (lanes 5–8). Both reactive nitrogen species were effective in modifying Keap1 thiols, although the response was much more pronounced using CSNO. We next tested the concentration-dependence of CSNO on Keap1 thiol modification in HA-transfected HEK293H cells. As shown in Figure 2B, increased modification was observed following 10 minutes of 100–500 μ M CSNO.

While the above experiments looked at Keap1 thiol modification under conditions of overexpression, the next experiments determined the modification of endogenous Keap1 in HEK293H cells which were not transfected. Following a 10 minute exposure to 0–500 μ M CSNO, cells lysates were prepared according to the MPB protocol and immunoprecipitated with anti-Keap1. Immunoblotting was conducted first with anti-Keap1 and then with neutrAvidin-HRP. Keap1 immunoreactivity is shown as a loading control. Importantly, treatment of cells with CSNO led to a concentration-dependent modification of endogenous Keap1 (Figure 3A). Next, a time course study was carried out in HEK293H cells treated with 500 μ M CSNO for 0–60 minutes. Keap1 immunoreactivity is shown as a loading control. Increased immunoreactivity of MPB-labeled proteins was observed by 15 minutes and maintained for 60 minutes (Figure 3B).

Nuclear Accumulation of Nrf2 and Keap1 in Response to CSNO

To confirm that CSNO treatment resulted in nuclear accumulation of Nrf2 as we have previously demonstrated in endothelial cells, HEK293H cells were treated with 500 μ M CSNO for 0–120 minutes. Nuclear extracts were prepared and immunoblotted with anti-Nrf2. A prominent immunoreactive band at about 90 kDa, corresponding to Nrf2, was observed in nuclear extracts, increasing in density over 120 minutes (Figure 4). The 70 kDa nuclear marker protein Lamin A is shown as a loading control. These results demonstrate that Nrf2 accumulated in the nucleus within 30 min following CSNO and continued over at least 2 hours.

Keap1 localization was also determined in cytosolic and nuclear extracts from HEK293H cells which were transfected with HA-Keap1 and treated with 500 μ M CSNO for 0, 60 and 120 min. Using anti-Keap1, we observed decreased Keap1 immunoreactivity in the cytosolic compartment and increased Keap1 immunoreactivity in the nuclear compartment at 60 and 120 minutes of CSNO treatment (Figure 5). Marker proteins for cytosolic and nuclear compartments, α -tubulin (55 kDa) and lamin A (70 kDa) respectively, are shown as loading controls. Additional experiments demonstrated nuclear accumulation of Keap1 as early as 30 minutes after CSNO (data not shown). Similar results were obtained using anti-HA (data not shown). These experiments show that Keap1 modification was accompanied by the nuclear localization of both Keap1 and Nrf2.

Similar studies were conducted in HEK293H cells which were not transfected with HA-Keap1. Disappearance of Keap1 from the cytosol and appearance of Keap1 in the nucleus was seen at 60 and 120 minutes but not at 30 minutes following CSNO (data not shown). Figure 6 shows the nuclear accumulation of both Keap1 and Nrf2 at 90 minutes following treatment of cells with 500 μ M CSNO. In this experiment, half of the samples were pretreated with leptomycin B (5 ng/ml for 2 hours) to block nuclear export. Through this mechanism, leptomycin B would be expected to cause the nuclear accumulation of proteins containing nuclear export signals (NES) in their sequences [21]. As shown in Figure 6, leptomycin B did not increase nuclear levels of either Keap1 or Nrf2 in control or CSNO-treated cells. The marker protein Lamin A is shown as a loading control. These results are interesting since both Nrf2 and Keap1 may contain at least one NES.

DISCUSSION

Keap1 Modification

Results from this study clearly demonstrate that Keap1 thiols were modified in response to NO and CSNO. This is the first report of Keap1 thiol modification by reactive nitrogen species in an intact cell model. Similar responses were observed in HEK293H cells overexpressing Keap1 and in HEK293H cells containing only endogenous Keap1. It should be noted that the response to NONOate was much less pronounced than the response to CSNO. There are several possible explanations for this observation. First the dose of NO released from the donor compound spermine NONOate, which has a half-life of 39 minutes, was not likely to be comparable to the dose of CSNO. Secondly, NO and CSNO are known to have different chemical reactivities. Although NO can slowly oxidize to N_2O_3 and as such participate in S-nitrosylation reactions, it can be metabolized to other forms as well. Thirdly, the known active transport of CSNO by LAT transporters may have led to rapid accumulation of a reactive nitrogen species [35]. In contrast, NO derived from spermine NONOate may have had to diffuse across cellular membranes and be converted to N_2O_3 before reacting with intracellular thiols [31,32].

Our findings add to only a handful of other studies showing Keap1 thiol modification *in vivo*. In contrast to these other studies where only one time point was studied, i.e. 1 hour for 15-deoxy-prostaglandin J_2 , 2 hour for IAB and 6 hour for the 3 electrophiles sulfurophane,

1,2,-dithiole-3-thione and bis(2-hydroxybenzylidene) acetone [24,28,30], we determined Keap1 modification over an hour. We found that both NONOate and CSNO-mediated responses were rapid in onset, occurring within 5–10 minutes, and were sustained over 15–60 minutes.

Our method for determining thiol modification does not distinguish between several different products which could have been formed initially. S-nitrosylation and oxidation to glutathionylated species, disulfide bridges and sulfenic acid derivatives are all possibilities [33]. Hence the MPB assay is a good measure of the initial products formed by reaction of S-nitrosothiols with thiols. Furthermore, in cellular systems, some newly formed S-nitrosothiols are metabolized to oxidized forms by enzymatic and nonenzymatic reactions while others are protected from degradation due to their subcellular localization or by the location of the SNO bond in the protein tertiary structure [32,33,36]. These oxidized species formed secondarily can also be detected by the MPB assay.

In addition, this approach does not determine which cysteines of Keap1 were modified in response to NO and CSNO. However two likely candidates are the highly reactive Cys 273 and Cys 288 found in the central linker domain. These have been found to be essential for Keap1's repression of Nrf2 activity [17,24,26,30]. Modification of these cysteines by oxidants and electrophiles *in vitro* has been shown in many cases to correlate with activation of the Nrf2/ARE pathway *in vivo* [28]. For example, adducts of Keap1 involving Cys 241, Cys 257 and Cys 288 were observed after treatment of cells with IAB, which led to significant Nrf2/ARE activation [28]. In one case where Keap1 adducts were observed without concomitant induction of Nrf2-mediated gene expression, the modified thiols were located outside of the central linker domain [28]. Another candidate for thiol modification is the Cys 151 found in the BTB domain, which plays a key role in Keap1 homodimerization [26]. Cys 151 was found to be essential for inducer-mediated Nrf2 activation [26]. Although no modification of this cysteine has yet been shown in a cellular model, formation of Keap1 adducts by sulfurophane was found to occur primarily in the BTB domain and was correlated with significant activation of the Nrf2 pathway [27].

Keap1 Nuclear Accumulation

Our study clearly shows that Keap1 disappeared from the cytosol and accumulated in the nuclear fraction over 30–120 minutes following CSNO treatment. Similar results were obtained with endogenous and overexpressed Keap1. Importantly, we found that Keap1 thiol modification preceded its nuclear accumulation. Furthermore, nuclear accumulation of Keap1 and Nrf2 was temporally associated suggesting that they may have localized to the nucleus in tandem.

Earlier studies proposed that the cytosolic Nrf2-Keap1 complex dissociated in response to an oxidant or electrophile allowing Nrf2 to translocate to the nucleus while Keap1 remained in the cytosol tethered to the cytoskeleton [23,24]. Additional studies led to the proposal that the complex does not dissociate immediately following thiol modification but that a conformational change in Keap1 occurs leading to decreased CUL3-dependent Nrf2 ubiquitination [17,19,25]. It has been proposed that Keap1 conformational changes lead to a disorientation of the ubiquitination sites found in the Neh2 domain of Nrf2, resulting in decreased ubiquitination of Nrf2 [19]. In one study, decreased Nrf2 ubiquitination was accompanied by increased Keap1 ubiquitination [15]. We can not at this point rule out the possibility that the decrease in cytosolic Keap1 observed in the present study was due to Keap1 ubiquitination. However this scenario could not explain the accumulation of Keap1 in the nucleus which we also observed.

Since Keap1 appears to lack a NLS sequence, it may accumulate in the nucleus through its association with Nrf2 which contains a single localization sequence [19,21]. Once in the nucleus Nrf2 and Keap1 may both associate with binding partners. Nrf2 is known to form heterodimers with MafG proteins and to bind the ARE [11]. Studies *in vitro* suggest a role for the nuclear protein prothymosin α in binding Keap1 by displacing Nrf2 [22].

Alternatively, Keap1 or Nrf2 may contain a NES sequence which is redox sensitive. Oxidants and electrophiles could block the function of this NES, leading to nuclear accumulation. While there is no evidence of redox sensitivity in the putative NES sequences identified in Keap1 [20–22], a redox sensitive NES sequence in Nrf2 has been reported [37]. Results from the present study show that leptomycin B, an inhibitor of the CRM1-dependent nuclear export pathway, had no effect on levels of Keap1 and Nrf2 in the nucleus in control cells and in cells treated with CSNO. Similar results have been reported for Keap1 [19]. These contradictory results from our lab and others leave open the role of NES sequences in the nuclear accumulation of Nrf2 and Keap1.

In summary, results from this study were the first to show rapid Keap1 thiol modification in cells treated with reactive nitrogen species. We found that modification led to redistribution of Keap1 and Nrf2 to the nucleus. This mechanism, presented in schematic form in Figure 7, likely mediates NO-mediated upregulation of the Nrf2/ARE pathway. In this manner, thiol modification of a key protein signals the upregulation of cytoprotective and antioxidant genes.

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Figure 1. Overexpression of Keap1 in HEK293H cells

HEK293H cells were transfected with HA-tagged Keap1 or with lipofectamine alone (sham) as described in Methods. **(A)** Treatment was with 500 μ M spermine NONOate or CSNO for 10 minutes in HHBSS. Cellular lysates were immunoprecipitated with either anti-HA or anti-Keap1 and were immunoblotted with anti-HA as described in Methods. Keap1 has an apparent molecular weight of about 70 kDa. **(B)** Lysate was pre-incubated with Keap1 blocking peptide prior to immunoprecipitation and immunoblotting (lane 1) and compared with control lysates (lane 2).

Figure 2. Thiol Modification of Overexpressed Keap1 in response to Spermine NONOate and CSNO

HEK293H cells were transfected with HA-tagged Keap1 and treated with spermine NONOate or CSNO in HHBSS. Lysates were prepared according to the MPB protocol, as described in Methods, followed by immunoprecipitation using anti-HA and immunoblotting with NeutrAvidin-HRP. **(A)** Time-response to 500 μ M spermine NONOate or CSNO for 0–15 min. **(B)** Dose-response to 0–500 μ M CSNO for 10 min.

Figure 3. Thiol Modification of Endogenous Keap1 in Response to CSNO

HEK293H cells were treated with CSNO. Lysates were prepared according to the MPB protocol, followed by immunoprecipitation using anti-Keap1 and immunoblotting with NeutrAvidin-HRP or anti-Keap1. **(A)** Dose response to 0–500 μ M CSNO for 10 min. **(B)** Time response to 500 μ M CSNO for 0–60 min.

Figure 4. Nuclear Accumulation of Nrf2 in response to CSNO

HEK293H cells which were not transfected were treated with 500 μ M CSNO for 0–120 minutes. Extracts were prepared from isolated nuclei obtained from lysates as described in Methods and immunoblotted using anti-Nrf2 and anti-Lamin A. Nrf2 has an apparent molecular weight of 90 kDa. The nuclear protein Lamin A which has a molecular weight of 70 kDa was used as a loading control.

Figure 5. Cytosolic disappearance and nuclear accumulation of Keap1 in response to CSNO HEK293H cells which were transfected with HA-tagged Keap1 were treated with 500 μ M CSNO for 0–120 minutes. Cytosolic and nuclear extracts were prepared, as described in Methods, and immunoblotted using anti-Keap1, anti- α -tubulin and anti-Lamin A. The cytosolic protein α -tubulin and the nuclear protein Lamin A were used as loading controls. The molecular weight of α -tubulin is 55 kDa.

Figure 6. Nuclear accumulation of Keap1 and Nrf2 in response to CSNO and leptomycin B
HEK293H cells which were not transfected were treated with 0 or 500 μ M CSNO. Half of the cells were pre-treated with 5 ng/ml leptomycin B for 2 hours prior to the addition CSNO. After 90 minutes, lysates were prepared and immunoblotted using anti-Keap1, anti-Nrf2 and anti-Lamin A. The nuclear protein Lamin A was used as a loading control.

Figure 7. A proposed scheme for the activation of the ARE by nitrosative stress

Key thiols in Keap1 are modified by nitrosative stress as well as by oxidative stress and electrophiles. This alters the interaction of the Keap1-Nrf2-Cul3 complex in the cytosol and results in decreased degradation of Nrf2 and possibly of Keap1. Nrf2 accumulates in the nucleus where it forms a heterodimer with MafG and activates the ARE leading to transcriptional upregulation of cytoprotective and antioxidant genes.