ELECTROPHILIC NITRO-FATTY ACIDS ACTIVATE NRF2 BY A KEAP1 CYSTEINE 151-INDEPENDENT MECHANISM

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Nitro-fatty acids (NO₂-FAs) are electrophilic signaling mediators formed in vivo via nitric oxide (NO)- and nitrite (NO₂)-dependent reactions. NO₂-FAs modulate signaling cascades via reversible covalent post-translational modification of nucleophilic amino acids in regulatory proteins and enzymes, thus altering downstream signaling events such as Keap1-Nrf2-antioxidant response element (ARE) regulated gene expression. In this study, we investigate the molecular mechanisms by which 9- and 10-nitro-octadec-9-enoic acid (OA-NO₂) activate the transcription factor Nrf2, focusing on the post-translational modifications of cysteines in the Nrf2 inhibitor Keap1 by nitroalkylation and its downstream responses. Of the two regioisomers, 9-nitro-octadec-9-enoic acid was a more potent ARE inducer than 10-nitro-octadec-9-enoic acid. The most OA-NO₂-reactive Cys residues in Keap1 were Cys38, Cys226, Cys257, Cys273, Cys288 and Cys489. Of these, Cys273 and Cys288 accounted for approximately 50% of OA-NO₂ reaction in a cellular milieu. Notably, Cys151 was among the least OA-NO₂-reactive of Keap1 Cys residues, with mutation of Cys151 having no effect on net OA-NO₂ reaction with Keap1 or on ARE activation. Unlike many other Nrf2-activating electrophiles, OA-NO₂ enhanced, rather than diminished, the binding between Keap1 and the Cul3 subunit of the E3 ligase for Nrf2. OA-NO₂ can therefore be categorized as a Cys151-independent Nrf2 activator, which in turn can influence the pattern of gene expression and therapeutic actions of nitroalkenes.

Nitroalkene derivatives of unsaturated fatty acids (NO₂-FAs) are electrophilic signaling mediators formed in vivo via nitration of unsaturated fatty acids by NO- or NO₂-derived species. NO₂-FAs trigger signaling cascades via covalent and reversible post-translational modifications (S-nitroalkylation) of susceptible nucleophilic amino acids in transcriptional regulatory proteins and enzymes, altering their function and downstream signaling events (1). Molecular targets of NO₂-FAs include the p65 subunit of nuclear factor κB (NF-κB) (2), the enzyme xanthine oxidoreductase (3), and the transcription factor peroxisome proliferator-activated receptor γ (PPARγ) (4). Moreover, NO₂-FAs activate heat shock (5) and antioxidant response pathways (5;6), via mechanisms that remain to be defined.

Antioxidant response element (ARE)-regulated genes play an essential role in the protection against endogenous and exogenous stresses (7). The transcription factor nuclear factor E2-related factor-2 (Nrf2) can activate these genes via binding to AREs as a heterodimer with small Maf proteins (7). Under basal conditions, Nrf2 is bound to its inhibitor Kelch-like ECH-associated protein 1 (Keap1), which functions as an adaptor molecule in the Cul3-based E3 ligase complex. Nrf2 is then rapidly ubiquitinated and degraded (8;9). During periods when cellular concentrations of oxidative or electrophilic species are elevated, the interaction of Nrf2 with the ubiquitin ligase...
complex is disrupted, enabling the escape of Nrf2 from degradation, its nuclear translocation and transactivation of target genes.

Keap1 is a Cys-rich protein with 27 Cys residues in the human and 25 Cys residues in the murine protein. Keap1 has four functional domains: the Bric-a-Brac, tramtrack, broad complex (BTB) domain, the intervening region (IVR), the Kelch domain (also known as the double glycine repeat) and the C-terminal region. Alkylation or oxidation of Keap1 Cys residues, predominantly within the IVR, leads to the inactivation of Keap1 and is the central mechanism for the activation of Nrf2 (10-12). A number of studies utilizing mass spectrometry (MS) analysis show that electrophilic inducers of Nrf2 modify several different Cys residues in recombinant Keap1. These data indicate that there is no single Cys modified by electrophiles in Keap1, and reveal that in addition to Cys151 in the BTB domain, the most reactive residues are within the IVR (13).

Functional assays performed with Keap1 mutants lacking specific Cys residues support that Cys273 and Cys288 of the IVR and Cys151 within the BTB domain have critical but very different regulatory roles. Cys273 and Cys288 are important for the repression of Nrf2 in basal conditions (11;14-17). In contrast, mutation of Cys151 significantly reduces ARE activation in response to electrophile exposure (17-19). This suggests that Cys151 is critical for electrophile sensing of Keap1. Adduction of Cys151 causes dissociation of Keap1 from Cul3, facilitating Nrf2 escape from proteosomal degradation and subsequent activation of its target genes via binding to AREs (18;20;21). However, there are Nrf2-activating electrophiles which act independently of Cys151. Recent analysis of different electrophile actions on the antioxidant response in a zebrafish model revealed that the cyclopentenone prostaglandins 15-deoxy-

EXPERIMENTAL PROCEDURES

Materials. OA-NO₂ (an equimolar mixture of 9- and 10-nitro-octadeca-9,12-dienoic acid) (6) and OA-NO₂ (9- or 10-nitro-octadec-9-enoic acid) (5) activate the Keap1-Nrf2-ARE system. Inasmuch as NO₂-FAs are endogenous electrophilic signaling mediators, we assessed whether Keap1 is covalently adducted by NO₂-FAs and identified the specific Keap1 Cys residues that are targeted for reaction. Herein, we identify Cys273 and Cys288 as the functionally important residues modified by OA-NO₂, and that NO₂-FA are Cys151-independent Nrf2 activators.
lysozyme, and Complete EDTA-free protease inhibitor (Roche)]. Soluble protein was then purified by Ni-NTA agarose (Qiagen) and HiLoad Superdex 200 column (GE Healthcare). The isolated mouse Keap1 protein at 0.2 mg/ml was exchanged into protein buffer containing 20 mM Tris–HCl, pH 8.4, 10% glycerol, 2 mM Tris(2-carboxyethyl)phosphine, and 0.5 mM dithiothreitol.

**Plasmids.** The following plasmids were used for this study: pGL3-SV40-2xGCLM-ARE-luc (26), pCl-Nrf2 (27), p3xFLAG-CMV-10 (Sigma-Aldrich), p3xFLAG-mKeap1-wt (28), p3xFLAG-mKeap1-C257S, p3xFLAG-mKeap1-C273S and p3xFLAG-mKeap1-288S (14). Mutagenesis of Cys38, Cys151, Cys226, and Cys489 in p3xFLAG-mKeap1 was performed with the Stratagene XL site-directed mutagenesis kit using the following primers: C38S, 5'-GCCTCCACGGGAGCAAGGCAAGGCAGAGG-3'; C151S, 5'-GTGGGCGAGAAGAGTGTCCTGCACGTG-3'; C226, 5'-CAACCTGTCACACAGCCAGCTGGCCAC-3'; C489, 5'-GGCTTAACTCCGCAGAAAGTTACTATCCAGAGAGG-3'. The correct mutations were verified by sequencing. For cloning of HA-Cul3, Cul3 was PCR amplified from the full-length cDNA clone IRATp970E06107D (RZPD, Germany) using the primers 5'-ATTCCCGGGATGTCGAATCTGAGCAAAGGC-3' and 5'-ATTCTCGAGTGAGTTCCCTTTCAACCACC-3'. SmaI-XhoI digested PCR-product was first cloned into EcoRI-blunt-XhoI digested pGem7Z (Promega), and the product was then further digested with SmaI-XhoI and cloned into XmnI-XhoI site of pReceiver-M06a (Genecopoeia).

**LC/MS detection and analysis of Keap1 post-translational modifications.** Purified recombinant Keap1 (10 µg) was incubated in the presence or absence of different concentrations of OA-NO2 for 60 min in 50 mM phosphate buffer, pH 7.4. Keap1 was then immediately reduced with 2 mM TCEP for 10 min at room temperature and alkylated in the dark for 20 min using 5 mM of iodoacetamide. After alkylation, Keap1 was digested using MS grade modified trypsin (trypsin:Keap1 ratio of 1:50) for 16 h at 37 °C. The peptide digest was analyzed by microLC-MS/MS for post-translational modifications using an Agilent 1200 Series HPLC system (Agilent) coupled to a LTQ mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ionization source. Peptides (3 µg on column) were loaded onto a C18 Zorbax SB (150 mm length, 0.5-mm i.d., 0.5-µm particle size, Agilent) reverse-phase column resolved using a linear gradient of solvent A (0.1% formic acid in HPLC grade water) and solvent B (0.1% formic acid in acetonitrile) at a flow rate of 8 µl/min. Chromatographic conditions were as follows: 3% solvent B for 10 min, followed by a linear gradient to 65% solvent B for 160 min, to then move to 100% solvent B for 30 min and re-equilibration to return to the initial condition (3% solvent B) for 20 min. MS analysis was carried out in the positive ion mode with source parameters optimized for the detection of peptides containing nitro-alkylated Cys as follows: source voltage 5 kV, capillary temperature 220 °C, tube lens 70 V, capillary voltage 50 V and collision energy of 35 V. MS/MS spectra was acquired using data-dependent acquisition in which one full MS spectrum was followed by MS-MS spectra of top 5 ions. Peptide analysis was performed using Bioworks (Thermo Fisher Scientific). MS/MS spectra (b and y ions) of detected modified peptides (presenting a 327 amu mass shift corresponding to OA-NO2) were manually confirmed by comparing their fragmentation pattern to the native peptides (containing IAM alkylation in case of Cys modifications).

**Cell culture.** Human embryonic kidney (HEK)-293T cells were purchased from ATCC and maintained in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich), supplemented with 10% (v/v) fetal bovine serum (HyClone) and 1% penicillin/streptomycin (Invitrogen). Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords obtained from the maternity ward of the Kuopio University Hospital with the approval of its ethics committee. HUVECs were cultivated as previously described (12). A

**Reporter gene assay.** HEK-293T cells were seeded on 96-well plates and transfected the next day with the calcium phosphate transfection method using the following plasmids: 20 ng of
pGL3-SV40 as control or pGL3-SV40-2xGCLM-ARE-luciferase (26), 40 ng of empty pCI as control or pCI-Nrf2 (27), 80 ng of p3xFLAG-CMV as control or p3xFLAG-mKeap1-wt (28), p3xFLAG-mKeap1-C38S, p3xFLAG-mKeap1-C151S, p3xFLAG-mKeap1-C257S, p3xFLAG-mKeap1-C273S, p3xFLAG-mKeap1-C288S or p3xFLAG-mKeap1-C489S. For normalization, cells were also transfected with 20 ng of pCMV-β-gal vector. 24 h after transfection, cells were treated with indicated concentrations of OA-NO₂, 15d-PGJ₂ or SFN in 1% conditions. 16 h after treatment, luciferase activities were measured with Britelite Reporter Gene Assay (Perkin Elmer) according to the manufacturer’s instructions. Luciferase activities were normalized to β-galactosidase activities measured as previously described (26).

Chromatin immunoprecipitation (ChIP). ChIP was performed as in (29), with modifications. HUVECs were seeded on T75 flasks and treated with vehicle, 5 µM 9,10-OA-NO₂, 9-OA-NO₂ or 10-OA-NO₂ for 2 h. Nuclear proteins were cross-linked to DNA by adding formaldehyde directly to the medium to a final concentration of 1% and incubating for 10 min at room temperature on a rocking platform. Cross-linking was stopped by adding glycine to a final concentration of 0.125 M and incubating for 10 min at room temperature on a rocking platform. Medium was removed and the cells were washed twice with ice-cold PBS. The cells were collected and lysed with 1 ml lysis buffer (10 mM NaCl, 5 mM MgCl₂, 0.1% Nonidet P-40, 10 mM Tris-HCl pH 7.4) and incubated on ice for 10 min. The lysates were centrifugated (1500 x g, 5 min at 4 °C) to pellet the nuclei. The pellets were washed once with the lysis buffer and resuspended in 500 µl SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, protease inhibitors) and aliquoted for immunoprecipitation. 2.5 µl BSA (100 mg/ml) was added to each aliquot. Chromatin solutions were incubated overnight at 4 °C on a rocking platform with 3.6 µg of specific Nrf2 antibody (Santa Cruz Biotechnologies sc-722) or 1 µg of nonspecific IgG (anti-rabbit IgG, Upstate Biotechnology). The immuno-complexes were collected with 20 µl of Magna ChIP Protein A Magnetic Beads (Upstate Biotechnology) for 1 h at 4 °C with rotation. The beads were separated with a magnetic rack and washed sequentially for 3 min with 700 µl of the following buffers: Low Salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1), High Salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, pH 8.1) and LiCl wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Finally, the beads were washed twice with 700 µl TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.1). The immuno-complexes were then eluted by adding 300 µl of elution buffer (25 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% SDS) and incubating for 30 min at 64 °C. Proteins were digested from the eluate by adding 2.5 µl of proteinase K (934 U/ml, Fermentas) and incubating overnight at 64 °C. DNA was recovered by phenol/chloroform/isoamyl alcohol (25:24:1) extractions and precipitated with 1:10 volume of 3 M sodium acetate, pH 5.2, and 2 volumes of ethanol using glycogen as carrier. Immuno-precipitated chromatin DNA was then used as a template for real-time quantitative PCR.

PCR of chromatin templates. Real-time quantitative PCR of ChIP templates was performed using specific primers for the HMOX1 chromatin region (5'-TGAGTAAATCCTTTCCCGAGC-3’ and 5'-GTGACTCAGCGAAAACAGACA-3’) and Maxima™ SYBR Green/ROX qPCR Master Mix in a total volume of 10 µl in a LightCycler® 480 System (Roche).

Analysis and quantification of β-ME adducts. HEK-293T cells were seeded on 10 cm plates and transfected the next day using Lipofectamine (Invitrogen) with 3 µg of p3xFLAG-CMV, p3xFLAG-mKeap1-wt,
p3xFLAG-mKeap1-C257S-C288S or p3xFLAG-mKeap1-C151S-C273S-C288S. 24 h after transfection, cells were treated with indicated concentrations of OA-NO₂. After 2 h, cells were collected and 3 mg of total protein was immunoprecipitated using anti-FLAG-affinity gel (Sigma-Aldrich). The following day, the immunoprecipitates were washed and eluted using the FLAG-peptide. Supernatants, containing specific FLAG-tagged proteins, were assayed for OA-NO₂ content by LC-MS analysis following β-mercaptoethanol (β-ME) trans-nitroalkylation reaction as previously (30).

Keap1-Cul3 binding assay. HEK-293T cells were seeded on 6 cm plates and co-transfected the next day using the calcium phosphate transfection method with 1 µg of p3xFLAG-CMV, p3xFLAGmKeap1-wt or HA-Cul3. 48 h after transfection, cells were treated with indicated concentrations of OA-NO₂, 15d-PGJ₂ or SFN. After 4 h, the cells were collected and 0.5 mg of total protein was used for immunoprecipitation with anti-FLAG-affinity gel (Sigma-Aldrich) or HA-antibody (BioSite, Täby, Sweden). The following day the immunoprecipitates were washed and analyzed with Western blot as described before (5).

Statistical analysis. Statistical analysis was performed with GraphPad Prism and the data were analyzed by Student t-test or 1-way ANOVA with Bonferroni’s post hoc comparison. Data is expressed as mean ± SD and differences were considered significant at * p < 0.05, ** p < 0.01, *** p < 0.001.

RESULTS

Modification of recombinant Keap1 by OA-NO₂. Post-translational modification of nucleophilic Keap1 residues mediate electrophile-induced signaling actions via Nrf2- and ARE-regulated gene transcription. We used MS as the first approach to examine sites of recombinant Keap1 protein adduction by OA-NO₂. The coverage obtained after trypsinization and LC-MS-MS analysis was 75% (by total number of residues) that included 17 of 25 Keap1 Cys residues (68%). At the lowest OA-NO₂ concentration (50 µM) and a molar ratio of Keap1 to OA-NO₂ of 1:7, Cys38, Cys226, Cys257, Cys273, Cys288 and Cys489 were modified (Table 1 and Fig. S1). Greater OA-NO₂ concentrations gave additional modified Cys residues, including Cys151 at 250 µM OA-NO₂ and the adduction of all 17 Cys residues at 500 µM OA-NO₂ (Table 1).

Functional contributions of OA-NO₂-reactive Cys residues to Keap1-Nrf2 signaling. In order to test whether the OA-NO₂-reactive Keap1 Cys residues Cys38, Cys226, Cys257, Cys273, Cys288, and Cys489 are functionally important for the regulation of Nrf2-dependent genes, ARE activity was measured using luciferase reporter assays. HEK-293T cells were transfected with a reporter construct containing two AREs from the glutamate-cysteine ligase modifier subunit (GCLM) promoter (26), in combination with expression plasmids for Nrf2 and Keap1. Each OA-NO₂-reactive Cys of Keap1 was individually mutated to Ser and then the effect on basal and OA-NO₂-inducible conditions, indicating that these residues were crucial for repression of Nrf2. The level of ARE activity was higher in OA-NO₂ treated vs. non-treated cells due to the activation of endogenous Nrf2. The ability of the other mutated constructs to inhibit ARE activation was comparable to wild type Keap1, suggesting that the other Cys residues are not functionally important (Fig. 1A).

The Keap1 Cys151 is important for facilitating Nrf2 activation by the electrophiles tert-butyldihydroquinone (tBHQ) and sulforaphane (SFN) (16;18;22;31), with Cys151-independent Nrf2 activation also reported (22). For this reason, the role of Cys151 in Nrf2 activation was evaluated, despite a relatively low reactivity with OA-NO₂ (Table 1). The Cys151-dependent Nrf2 inducer SFN (16) and the electrophilic cyclopentenone prostaglandin 15d-PGJ₂ that activates Nrf2 independent of Keap1 Cys151 (22) were used as controls. SFN required Cys151 for full Nrf2-dependent ARE activation, while it was not required for Nrf2 activation by OA-NO₂, analogous to 15d-PGJ₂ (Fig. 1B).

Binding of OA-NO₂ with Keap1 in intact cells. The initial screening of OA-NO₂-reactive...
Cys residues was performed with recombinant Keap1 treated with OA-NO\(_2\). In order to confirm that the addition of Keap1 by OA-NO\(_2\) occurs in intact cells, where fatty acid metabolism and alternative competing reactions can occur, a MS-based method was utilized to measure adducted NO\(_2\)-FA levels (30;32). The method quantifies OA-NO\(_2\)-protein adducts after an exchange reaction with exogenously added β-ME (trans-nitroalkylation). FLAG-tagged Keap1 was transiently transfected to HEK-293 cells, which were exposed to increasing concentrations of OA-NO\(_2\). After FLAG-immunoprecipitation, Keap1 was detected on silver stained gels (Fig. 2A, lower panel). Keap1 was immunoprecipitated within treatment groups with similar efficiency and was not detected in empty vector controls. When immunoprecipitated Keap1 was subjected to transnitroalkylation with β-ME, an OA-NO\(_2\)-β-ME adducts was detected. Thus, OA-NO\(_2\) covalently reacts with ectopically expressed Keap1 in HEK-293T cells at low µM concentrations (Fig. 2B).

**Activation of ARE by different regioisomers of OA-NO\(_2\).** Both endogenously-generated OA-NO\(_2\) and the synthetic OA-NO\(_2\) used herein is an equimolar mixture of 9- and 10-nitro-octadec-9-enolic acids (33). Recently, isomer-specific binding of OA-NO\(_2\) to redox-sensitive thiols has been reported, with 10-nitro-octadec-9-enolic acid being more reactive than 9-nitro-octadec-9-enolic acid towards Cys285 in the ligand-binding domain of PPAR\(γ\) (34). In order to explore whether ARE activation is OA-NO\(_2\) regioisomer-selective, ARE activation was measured using luciferase reporter assays. In contrast to patterns of PPAR\(γ\) adduction, 9-nitro-octadec-9-enolic acid was more potent than both 10-nitro-octadec-9-enolic acid and an equimolar 1:1 mixture of both regioisomers in inducing ARE activity (Fig. 3A). After treatment with the two isomers of OA-NO\(_2\), ChiP was performed to study the binding of Nrf2 to the ARE-containing distal enhancer region of the HMOX1 gene. In accordance with reporter analysis-based observations, 10-nitro-octadec-9-enolic acid was less potent than 9-nitro-octadec-9-enolic acid and the 1:1 regioisomer mixture in enhancing Nrf2 binding to AREs. This supports the notion that 9-OA-NO\(_2\) is a more favorable inducer of ARE activation (Fig. 3B).

The effect of OA-NO\(_2\) on Cul3-Keap1 interaction. According to the current paradigm, Keap1 binding to Cul3 enables the complex to degrade Nrf2 by ubiquitination under basal conditions. Electrophile adduction of Keap1 Cys151 modulates the interaction of the protein with Cul3, leading to the precept that modification of Cys151 may dissociate Keap1 from Cul3 and promote the escape of Nrf2 from proteasomal degradation (20;21). The binding of Cul3 and Keap1 after treatment with the Cys151-dependent electrophile SFN (21) and the two Cys151-independent electrophiles, OA-NO\(_2\) and 15d-PGJ\(_2\), revealed that SFN diminished Keap1 and Cul3 binding, whereas both OA-NO\(_2\) and 15d-PGJ\(_2\) enhanced Keap1 and Cul3 interaction (Fig. 4).

**DISCUSSION**

Organisms are continuously exposed to electrophiles that are either endogenously produced via redox reactions or are derived from exogenous sources (35). It is now evident that multiple cell signaling mechanisms have evolved to sense and respond to electrophiles, thus linking gene expression and protein function with metabolic and inflammatory status. Electrophiles can react with nucleophilic amino acids of proteins via Michael addition, thereby altering protein structure and function. While such modifications have typically been viewed as toxic, recent data affirm that low concentrations or rates of generation of reversibly-reactive electrophiles can elicit a broad range of adaptive protein functional and gene expression responses in the absence of toxicity (23;35). One of the pathways activated by electrophiles, such as nitroalkene fatty acid derivatives, is the Keap1-Nrf2-ARE system (5). The reactive effector protein in this pathway, Keap1, is exemplary for revealing how post-
translational modifications by electrophiles can elicit specific biological responses. The modifications of Keap1 reported from the use of a variety of different electrophiles and methods show significant variations in specific thiol residues as Michael addition targets of different electrophiles (13). This has important implications for the mechanisms underlying activation of ARE-regulated genes, patterns of gene expression and ultimately, the phenotypic characteristics of differentiated cell responses. These issues motivated the investigation of Keap1 Cys modifications by the electrophilic fatty acid nitroalkene, OA-NO$_2$.

In this study, six Keap1 Cys residues were identified as susceptible to modification by nitroalkylation at the lowest OA-NO$_2$ concentrations used. While LC-MS determinations have revealed several Cys residues susceptible to adduction during Keap1 electrophile sensing, the interpretation of this data can be influenced by a number of issues. The efficiencies for the detection of peptides by MS are affected by a number of factors, resulting in up to 3 orders of magnitude differences in the sensitivity of detection of different tryptic peptides stemming from the same protein. Thus, the relative detection limits for different modified peptides can widely differ. Also, depending on the specific electrophile, sample preparation approaches and both ionization and fragmentation efficiencies of different mass spectrometers, there can be differences in the identification of reactive residues. Despite these limitations, of the six nitroalkene-reactive Cys residues identified in the present study, Keap1 Cys257, Cys273, and Cys288 have been frequently reported as targets for adduction (13;23).

Previous proteomic analyses and functional studies identified Keap1 Cys273, Cys288 and Cys151 as critical targets of electrophile-thiol interactions (23). The reactivity of Cys273 and Cys288 that are located in the IVR is reaffirmed in this study. Recently, the structure of Keap1 dimer was studied by single particle electron microscopy (36). Keap1 was identified as a forked dimer with two large globular domains constituting 86.5% of the total volume of the protein. This study also revealed a close proximity of IVR with DC domain (DGR [double glycine repeat] and the C-terminal region of Keap1) that forms a β-propeller structure interacting with the Neh2 domain of Nrf2. Neh2 has two evolutionarily conserved motifs (DLG and ETGE), that have different electrostatic potentials defining their binding affinities towards the Keap1-DC domain (37;38). According to the two-site recognition model of Keap1-Nrf2 interaction, Keap1-homodimer interacts with a single Nrf2 molecule. Under basal conditions, both ETGE and DLG motifs of Neh2 interact with Keap1-DC. However, during oxidative or electrophilic stress, the low affinity DLG motif that positions the lysines within the Neh2 domain for ubiquitination detaches from the Keap1-DC domain, resulting in disruption of polyubiquitination and degradation of Nrf2. The proximity of IVR with the DC-domain (36) supports the notion that covalent modification of Cys273 and Cys288 induces conformational changes in the IVR that in turn affect the structural integrity of adjacent Keap1-DC, eventually disrupting the interaction with the DLG motif of Nrf2.

In this study, the functionally important Cys273 and Cys288 residues were among the most reactive towards OA-NO$_2$ in the recombinant protein, but only accounted for approximately 50% of net OA-NO$_2$ reaction with intracellular Keap1. Mutation of either Cys alone had no impact on overall reaction. This is consistent with recent click chemistry-based detection of Keap1 adduct formation with sulfoxythiocarbamate (39). The adduction of single Cys273Ala and Cys288Ala Keap1 mutants with sulfoxythiocarbamate did not significantly differ from that of wild type Keap1. In contrast, double Cys273Ala/Cys288Ala and triple Cys151Ala/Cys273Ala/Cys288Ala mutants showed markedly-reduced sulfoxythiocarbamate adduction. The lack of reactivity of the single Cys mutants could be explained by the change of protein conformation by the mutation of one Cys to favor reaction with another. Alternatively, the two cysteines might act cooperatively. Dinkova-Kostova et al. proposed that Keap1 is a Zinc metalloprotein, and that C273 and C288 would coordinate binding of Zn$^{2+}$ (40). Upon inducer sensing, Zn$^{2+}$ could be released and the two cysteine residues as the more reactive thiolate anion can then react with electrophiles.
Herein, OA-NO$_2$ was not particularly reactive towards Cys151 in recombinant Keap1 and was not critical for ARE-dependent gene activation. Furthermore, ectopically expressed Keap1, with combined Cys151Ser, Cys273Ser and Cys288Ser mutations, was no less reactive in β-ME-based electrophile capture assays than the Cys273Ser and Cys288Ser double mutant. This also supports that Cys151 is not a major site of OA-NO$_2$ reaction in a cellular milieu. Cys151 has been reported to be sensitive to adduction (23) and critical in Nrf2 activation by electrophiles, including SFN, tBHQ (21; 31) and IAB (20). These data are based on luciferase reporter assays (16; 21; 31), zebrafish embryos overexpressing mouse Keap1 (22), and mouse embryonic fibroblasts derived from Keap1 Cys151 transgenic animals (17). The studies investigating Cys151-dependent Nrf2 activation have revealed that Cys151 is required for Keap1 and Cul3 interaction. Modification of Cys151 appears to decrease Cul3 interactions, leading to inhibition of Keap1-dependent ubiquitination of Nrf2 (21). Unfortunately, there are no structural data defining the Keap1-Cul3 interface. However, molecular contacts between the cullin proteins and their BTB domain-containing substrate adaptor proteins are highly conserved. Modeling of the Keap1-Cul3 interaction interface reveals that Keap1 residues 125–127 and 162–164 within the BTB domain are predicted to interact with Cul3 (21). While Cys151 is not located at the predicted Keap1-Cul3 interface (41), it is suggested that a bulky modification at this site would cause conformational changes that alter Cul3 binding, allowing Nrf2 to escape proteasomal degradation (18). Although several reports support a crucial role of Keap1 Cys151 for Nrf2 activation, the electrophilic prostaglandins PGA$_2$ and 15d-PGJ$_2$ (22) and the heavy metal arsenic (31) all activate Nrf2 independent of Cys151. Another class of Cys151-independent ARE activators, the cyclopentenone prostaglandins PGA$_2$ and 15d-PGJ$_2$, do not react with Cys151 as assessed by MS analysis of recombinant or ectopically expressed Keap1 (22; 42). We observed that both OA-NO$_2$ and 15d-PGJ$_2$ had a similar effect on Keap1-Cul3 interactions, i.e. both electrophiles increased Keap1-Cul3 binding, whereas treatment with SFN diminished the interaction of Keap1 and Cul3. It can therefore be envisioned that the class of Cys151-independent ARE activators do not disrupt the Keap1-Cul3 interaction, but nevertheless inhibit Nrf2 ubiquitination and proteasomal degradation, possibly via disruption of the dynamic assembly/disassembly of Keap1 with the Cul3–Rbx1 E3 ubiquitin ligase complex (21; 31). That Keap1 has multiple sensing mechanisms for activation is further supported by the report that Keap1 has separate sensors for nitric oxide, metals and alkenals, all acting independently (43).

Herein, we show Nrf2 is activated preferentially by 9-OA-NO$_2$. In redox signaling by electrophiles, the position of the electrophilic carbon in relation to the nucleophilic target thus appears critical in defining net reactivity. Importantly, key structural motifs of the target protein and critical structural elements of the fatty acid nitroalkene (the hydrophobic methyl end, the anionic nitro and carboxylic acid substituents and the cis double bond configuration) will influence rates of Michael addition. In this regard, OA-NO$_2$ shows regioisomer-selective reactivity towards the ligand binding domain cysteine residue (Cys285) in PPARγ, with 10-OA-NO$_2$ more reactive than 9-OA-NO$_2$ (4). Also, xanthine oxidoreductase activity is inhibited by 9-OA-NO$_2$ and the mixture of 9- and 10-OA-NO$_2$ but not by other structural variants of OA-NO$_2$ that differ in the nitroalkenyl and carboxylic acid moieties (3). Moreover, the extent of PPARγ activation is different by the 4 nitroalkenyl regioisomers of linoleic acid (9, 10, 12 or 13-nitro-octadeca-9, 12-dienoic acid) (44). These data underscore the notion that even small changes in nitroalkene fatty acid structure can impact on biological signaling actions.

In summary, Cys273 and Cys288 are significant electrophile reaction sites contributing to Keap1 function but are not the only fatty acid nitroalkene-reactive Cys residues of Keap1. Proteomic analyses with LC-MS/MS supported that reactions with His or other nucleophilic amino acids of Keap1 were not significant under these conditions. Furthermore, Cys151 is not notably reactive with OA-NO$_2$ nor necessary for ARE activation, as OA-NO$_2$ potently induced ARE-dependent gene expression at low µM concentrations (5). OA-NO$_2$ can therefore be categorized as a Cys151-independent Nrf2 activator. Inasmuch as nitroalkenenes represent...
endogenously-produced NO and nitrite-derived signaling mediators, this data adds to our understanding of their properties and therapeutic potential.

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FIGURE LEGENDS

FIGURE 1. Functional importance of OA-NO₂ reactive Cys residues. A, B. HEK-293T cells were co-transfected with ARE-luciferase reporter vector, Nrf2 overexpressing vector, and vector expressing wild type Keap1 or Cys to Ser mutations of indicated amino acids. 24 h after transfection, cells were treated with OA-NO₂, 15d-PGJ₂ or SFN for 16 h, and luciferase activity was measured. Results are normalized to β-galactosidase and represented as fold change vs. pGL3-SV40-control vector for each treatment. Values are represented as mean ± SD, *p < 0.05, **p < 0.01 when compared to ARE-luc+ Nrf2 + Keap1-wt.

FIGURE 2. OA-NO₂ reacts with Keap1 in a cellular milieu. HEK-293T cells were transfected with FLAG-CMV (empty vector) or FLAG-Keap1 overexpressing vector and treated with indicated concentrations of OA-NO₂. A. Keap1 adducted OA-NO₂ was exchanged to β-ME from immunoprecipitated Keap1 in the presence of [¹³C₁₈]OA-NO₂ internal standard and quantified by liquid chromatography-MS/MS as β-ME-OA-NO₂. Lower panels show transfection efficiency of FLAG-Keap1 constructs. C. β-ME-OA-NO₂ levels captured upon exchange to β-ME in immunoprecipitated wild type (WT) Keap1 and the following Cys to Ser mutant Keap1: C273S, C288S, C273S&C288S, or C151S&C273S&C288S. β-ME exchange reactions were conducted in the presence of [¹³C₁₈]OA-NO₂ and quantified by LC-MS/MS. Values are represented as mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001 when compared to respective control. ns = not significant.

FIGURE 3. Nitroalkene regioisomer-specific activation of ARE. A. HEK-293T cells were transfected with the ARE luciferase reporter and β-galactosidase control vector. 24 h after transfection, the cells were incubated with 9-nitro-octadec-9-enoic acid (9-OA-NO₂), 10-nitro-octadec-9-enoic acid (10-OA-NO₂), or 1:1 mix of both isomers for 16 h. The data is represented as fold change to control (vehicle) ± SD, n=4, *p < 0.05, **p < 0.01, ***p < 0.001 when compared to mix of both isomers, *p < 0.05, **p < 0.01, ***p < 0.001 when compared to 10-OA-NO₂. B. HUVECs treated with vehicle, 5 µM 1:1 mix of 9-OA-NO₂ and 10-OA-NO₂, 9-OA-NO₂ or 10-OA-NO₂ for 2 h. ChIP assays were performed with chromatin extracts using an anti-Nrf2 antibody. Real-time quantitative PCR was performed using primers specific for a distal enhancer region of the HMOX1 gene containing multiple ARE elements. Non-precipitated input chromatin served as reference and IgG-precipitated template as specificity control. Fold induction of Nrf2 association was calculated. Values are represented as mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001 when compared to vehicle

FIGURE 4. Exposure to OA-NO₂ or 15d-PGJ₂ does not cause dissociation of Keap1 and Cul3. HEK-293T cells were co-transfected with FLAG-Keap1 and HA-Cul3 and treated with 15d-PGJ₂, OA-NO₂ or sulforaphane (SFN). Cell lysates were immunoprecipitated with FLAG or HA antibodies (upper panel) and the amount of bound Keap1 or Cul3 was detected with Western blot using FLAG or HA antibodies. The lower panel shows the transfection efficiency of total cell lysates (input control). Western blots are representative of three independent experiments.
Table 1. Cysteines 38, 226, 257, 273, 288, and 489 are the most OA-NO$_2$ reactive Keap1 cysteines. Recombinant Keap1 protein was treated with different concentrations of OA-NO$_2$. After tryptic digestion, the modified cysteine residues were detected with mass spectrometry.

| PEPTIDE | Cys | Keap1 domain | MH+  | OANO$_2$ (µM) |
|---------|-----|--------------|------|---------------|
|         |     |              | 50   | 100 | 200 | 250 | 500 |
| C*PEGAGDAVMYASTEC@K | Cys38 | N-term | 2116.12 | +  | +  | +  | +  | +  |
| QEEFFNLSHC@QLATLISR  | Cys226 | IVR | 2463.45 | +  | +  | +  | +  | +  |
| YDC@PQR              | Cys257 | IVR | 1108.72 | +  | +  | +  | +  | +  |
| C@HALTPR             | Cys273 | IVR | 1124.8  | +  | +  | +  | +  | +  |
| C@EILQADAR           | Cys288 | IVR | 1345.89 | +  | +  | +  | +  | +  |
| LNSAEC@YYPER         | Cys489 | Kelch | 1671.98 | +  | +  | +  | +  | +  |
| SGAGVC@VLLHC*IAAAGGYDGQDQLNSVER | Cys513 | Kelch | 3380.78 | +  | +  | +  | +  | +  |
| SGVGVAVTMEPC@R       | Cys613 | C-term | 1633.02 | +  | +  | +  | +  | +  |
| PTQAVPC@R            | Cys319 | IVR | 1198.84 | +  | +  | +  | +  | +  |
| LSQQLC@DVTLSVK       | Cys77  | BTB | 1802.19 | +  | +  | +  | +  | +  |
| C*ESEVFHAC@IDWVK     | Cys249 | IVR | 2050.15 | +  | +  | +  | +  | +  |
| LADLQVPRSGLAGC@VVGGLLYAVGGR | Cys368 | Kelch | 2868.79 | +  | +  | +  | +  | +  |
| IGVGVIDLHGYAVGSHGC@IHHSSVER | Cys434 | Kelch | 3083.76 | +  | +  | +  | +  | +  |
| C@VLHVMNGAVMYQIDSVVR | Cys151 | BTB | 2461.46 | +  | +  | +  | +  | +  |
| C@ESEVFHACIDWVK      | Cys241 | IVR | 1993.13 | +  | +  | +  | +  | +  |
| C@KDYLVQIFQELTLHKPTAVPCR | Cys297 | IVR | 3157.87 | +  | +  | +  | +  | +  |
| DYLQIFQELTLHKPTAVPC@R | Cys319 | IVR | 2926.77 | +  | +  | +  | +  | +  |
| SGLAGC@VVGGLLAVGGR   | Cys368 | Kelch | 1976.28 | +  | +  | +  | +  | +  |
| C*PEGAGDAVMYASTEC*K  | Cys23  | N-term | 2116.12 | +  | +  | +  | +  | +  |

* IAM modified Cys; OA-NO$_2$ modified Cys
Figure 2, Kansanen et al 2011
Figure 3, Kansanen et al 2011
Figure 4. Kansanen et al 2011

| OA-NO₂ | - | - | + | - | - |
| 15d-PGJ₂ | - | - | - | + | - |
| SFN | - | - | - | + | - |
| FLAG-Keap1 | - | + | + | + | + |
| HA-Cul3 | + | + | + | + | + |

**FLAG IP**
- HA
  - FLAG
  - Keap1
  - Cul3

**HA IP**
- FLAG
  - HA
  - Keap1
  - Cul3

**Input**
- HA
  - FLAG
  - Keap1
  - Cul3
Electrophilic nitro-fatty acids activate Nrf2 by a Keap1 cysteine 151-independent mechanism
Emilia Kansanen, Gustavo Bonacci, Francisco J. Schopfer, Suvi Linna, Kit I. Tong, Hanna Leinonen, Steven R. Woodcock, Masayuki Yamamoto, Carsten Carlberg, Seppo Ylä-Herttuala, Bruce A Freeman and Anna-Liisa Levonen

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