Electrophiles modulate glutathione reductase activity via alkylation and upregulation of glutathione biosynthesis

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**A B S T R A C T**

Cells evolved robust homeostatic mechanisms to protect against oxidation or alkylation by electrophilic species. Glutathione (GSH) is the most abundant intracellular thiol, protects cellular components from oxidation and is maintained in a reduced state by glutathione reductase (GR). Nitro oleic acid (NO2-OA) is an electrophilic fatty acid formed under digestive and inflammatory conditions that both reacts with GSH and induces its synthesis upon activation of Nrf2 signaling. The effects of NO2-OA on intracellular GSH homeostasis were evaluated. In addition to upregulation of GSH biosynthesis, we observed that NO2-OA increased intracellular GSSG in an oxidative stress-independent manner. NO2-OA directly inhibited GR in vitro by covalent modification of the catalytic Cys61, with $k_{\text{on}}$ of $(3.45 \pm 0.04) \times 10^{-5} \text{ M}^{-1} \text{s}^{-1}$, $k_{\text{off}}$ of $(4.4 \pm 0.4) \times 10^{-4} \text{ s}^{-1}$, and $K_{eq}$ of $(1.3 \pm 0.1) \times 10^{-7} \text{ M}$. Akin to NO2-OA, the electrophilic Nrf2 activators bardoxolone-imidazole (CDDO-Im), bardoxolone-methyl (CDDO-Me) and dimethyl fumarate (DMF) also upregulated GSH biosynthesis while promoting GSSG accumulation, but without directly inhibiting GR activity. In vitro assays in which GR was treated with increasing GSH concentrations and GSH depletion experiments in cells revealed that GR activity is finely regulated via product inhibition, an observation further supported by theoretical (kinetic modeling of cellular GSSG/GSH levels) approaches. Together, these results describe two independent mechanisms by which electrophiles modulate the GSH/GSSG couple, and provide a novel conceptual framework to interpret experimentally determined values of GSH and GSSG.

1. **Introduction**

Glutathione (GSH) is the most abundant intracellular thiol and is central to the detoxification of electrophiles and oxidants. Enzymatic or non-enzymatic oxidation to glutathione disulfide (GSSG) and subsequent NADPH-dependent reduction to GSH by glutathione reductase (GR) is key to maintaining thiol homeostasis. Although the pKa of the GSH Cys thiol is relatively high (8.94–9.42) \cite{1,2}, suggesting low reactivity under cellular conditions, its central role in reduction-oxidation biology stems from highly efficient synthesis, enzyme-catalyzed transfer and recycling pathways, as well as mass-action afforded by high intracellular concentrations.

The expression of enzymes responsible for biosynthesis of GSH is under the transcriptional control of Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) \cite{3,4}. Nrf2 also regulates expression of the import of the GSH substrate cysteine through the cystine/glutamate antiporter \cite{5}. Under basal conditions, Keap1, a BTB/E3 ubiquitin ligase, negatively regulates the transcription factor Nrf2 by targeting it for proteosomal degradation. Oxidation or alkylation of sentinel cysteine residues in Keap1 stabilize the Keap1/Nrf2 protein complex, sequestering Keap1 and allowing newly synthesized Nrf2 to accumulate and translocate to the nucleus to activate transcription of hundreds of cytoprotective enzymes involved in GSH biosynthesis.

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genes [4,6,7]. In addition to regulating GSH biosynthesis, canonical Nrf2 targets include enzymes responsible for detoxification such as heme oxygenase 1 (HO-1) and NAD(P)H-quione dehydrogenase 1 (NQO1) and xenobiotic exporters including multidrug resistance-associated protein (MRP) family members [8–13]. Importantly, Keap1 is highly sensitive to aklylation by electrophiles, making pharmacological intervention a viable strategy for the manipulation of Nrf2 activity and downstream antioxidant gene expression [14].

In addition to activating Nrf2, electrophiles can react enzymatically and non-enzymatically with the nucleophilic thiolate GSH−, decreasing intracellular GSH levels. GS-adducts of different electrophilic Nrf2 inducers have been described in vitro and in vivo [15,16], Bardoxolone (CDDO-Me) [17], and nitrated fatty acids (NO2-FA) [18–20]. However, the net effect of these compounds on intracellular GSH pools is not known, as activation of Nrf2 induces GSH biosynthesis.

Nitro-oleic acid (NO2-OA) is a therapeutically-promising drug and nitro-conjugated linoleic acid (NO2-cLA) is the most abundant endogenous NO2-FA. Both are electrophiles that post-translationally modify protein thiols through reversible Michael addition, exerting potent protective and anti-inflammatory effects [21–24] and demonstrating efficacy in diverse disease models [23, 25–29]. At the molecular level, specific NO2-OA binding to Cys273/288 of Keap1 via Michael addition activates Nrf2 [30–32,18]. These findings assign relevance to the study of NO2-OA in inflammatory cells.

In this context, this study sought to rigorously define the effects of NO2-OA on GSH biosynthesis, depletion, and cycling in murine RAW264.7 macrophages. NO2-OA exposure transiently decreased intracellular GSH concentration, followed by rapid induction of GSH biosynthesis and further GSH accumulation. Although this effect on GSH suggests a more reducing intracellular milieu, a paradoxical, oxidant-stress independent accumulation of GSSG was observed. Direct and indirect mechanisms by which electrophiles modulate GSSG cycling through GR were identified. Our findings show that GR activity is primarily determined by the intracellular level of GSH, establishing the ratio of GSH to GSSG.

2. Results

2.1. Lipid electrophiles upregulate GSH biosynthesis through Nrf2 activation in RAW264.7 macrophages

Basal intracellular GSH and GSSG measured in RAW264.7 macrophages by LC-MS/MS (Suppl. Fig. 1) yielded a mean of $56.4 \pm 4.2$ and $0.16 \pm 0.01$ nmol/mg protein for GSH and GSSG respectively and an overall ratio of GSH/GSSG of $366 \pm 24$ (n = 4–5 from 5 independent experiments; mean \pm standard error).

After $1 h$ of exposure to $5 \mu M$ electrophilic NO2-OA, GSH levels were reduced by 23%, returned to baseline level by $3 h$ and increased two-fold by $6 h$ (Fig. 1A, red trace). Surprisingly, the GSSG levels did not significantly change under this initial mild GSH depletion but increased 8.3-fold at $6 h$ (Fig. 1B, red trace). Both GSH and GSSG significantly decreased by $24 h$ but remained elevated compared to baseline. Notably, although GSH levels significantly increased at $6 h$ and $24 h$ post-NO2-OA exposure, a pronounced decrease in GSH/GSSG ratio was observed (Fig. 1C, red trace). These effects were then compared to those of the TLR4 activator lipopolysaccharide (LPS) and the GSH synthesis inhibitor buthionine sulfoximine (BSO) to evaluate the effect of macrophage activation and GSH depletion. Lipopolysaccharide induced an initial decrease in GSH that recovered to initial levels by $6 h$ while GSSG increased as a function of time, reaching a maximum over 10-fold above baseline at $24 h$.

In contrast, treatment with the irreversible $\gamma$-glutamylcysteine synthetase inhibitor BSO resulted in a linear reduction in intracellular GSH over $24 h$, with an overall loss of 55% of the GSH pool (Fig. 1A, blue trace). Interestingly, the loss in GSH resulted in a more pronounced (87%) reduction in GSSG (Fig. 1B, blue trace) suggesting that the decrease in GSH was not associated to oxidative stress.

Finally, we confirmed the Nrf2-dependent effects of NO2-OA treatment on GSH biosynthesis. The expression of the modulatory subunit of gamma-glutamyl-cysteine ligase (GCLM), which is the rate-limiting step in GSH biosynthesis, was increased at $6 h$ and was at its highest at $24 h$. By comparison, the NO2-OA modulated target HO-1 was upregulated between 3 and $6 h$ but trending towards baseline levels by $24 h$. Despite the presence of putative ARE sequences in its promoter sequence, GR expression was not modulated by NO2-OA treatment (Fig. 1D) [33].

2.2. NO2-OA induced upregulation of GSSG occurs independently of oxidative stress

The paradoxical increase in both intracellular GSH and GSSG in cells treated with NO2-OA motivated testing whether the increase in GSSG correlated with increased oxidative stress markers within the same time-frame (Fig. 2). To this end, changes in the relative abundances of monomeric vs. dimerized peroxiredoxins (Prdxs) were evaluated. The cytosolic and mitochondrial 2-Cys peroxiredoxins, Prdx1 and 3 respectively, are highly reactive antioxidant enzymes that dimerize upon oxidation by hydrogen peroxide or peroxynitrite (k ≈ $10^7$ M$^{-1}$ s$^{-1}$–10$^9$ M$^{-1}$ s$^{-1}$) and are biomarkers of cellular changes in peroxide levels [34–37].

Under basal conditions, RAW264.7 macrophages presented 35% of Prdx1 and 60% Prdx3 covalently dimerized (Fig. 2A–C). Treatment with NO2-OA had no effect on the proportion of covalent Prdx dimers compared to controls, despite the significant elevation in intracellular GSSG (Fig. 2A–C). In the setting of exacerbated oxidative stress, the peroxidatic cysteine in typical 2-Cys Prdx can be hyperoxidized to a SO3/A via a sulenic acid intermediate [37]. Before and after NO2-OA treatment, oxidized Prdx were detected only in the dimeric form, with absence of formation of monomeric hyperoxidized Prdx (Suppl. Fig. 2). In contrast, treatment of RAW264.7 cell with 400 μM H2O2 for 10 min led to significant formation of monomeric hyperoxidized peroxiredoxins. Taken together, no evidence for steady-state oxidant exposure was observed after NO2-OA treatment, indicating that the accumulation in intracellular GSSG is independent of oxidative stress.

To evaluate whether NO2-OA affected Prdx function or interfered with Prdx dimerization, RAW264.7 cells were incubated with 10 μM H2O2 in the presence or absence of 5 μM NO2-OA. Fig. 2D shows that both Prdx1 and 3 oxidized fully within 20 min of exposure regardless of the presence of NO2-OA. In line with its role as a TLR4 ligand and potent pro-inflamous mediator, LPS treatment caused significant Prdx oxidation with a similar time course as shown for GSSG formation (Fig. 2E). Exposure to high H2O2 levels consistently decreased the detection of loading control.

2.3. Inhibition of MRP1 is insufficient to increase intracellular GSSG

Cellular efflux of GSH-conjugated electrophiles and GSSG is mediated by the ATP-binding cassette (ABC) transporter class proteins including the multidrug resistance protein 1 (MRP1) [38]. Thus it is conceivable that the increases in GSSG levels induced by NO2-OA might be mediated by a direct effect on MRPs. To test this hypothesis, RAW264.7 were treated with the nonspecific MRP/organic anion transporter (OAT) inhibitor probenecid (250 μM) or the MRP1-selective inhibitor MK-571 (25 μM) and changes in GSSG levels determined. In contrast to NO2-OA, neither of these inhibitors was able to significantly increase intracellular GSSG (Fig. 3A–C). Although these results do not negate the possibility that NO2-OA might influence MRP function, they demonstrate that MRP inhibition is insufficient to increase the levels of GSSG.
2.4. NO2-OA is a covalent reversible inhibitor of GR

Since NO2-OA did not induce cellular oxidative stress (Fig. 2), and appreciating that Nrf2 activation upregulates GSH biosynthesis and NADPH production in vivo [39], it was hypothesized that NO2-FA may inhibit GR, reducing GSH regeneration and increasing intracellular GSSG. The GR active site contains two critical cysteines that form an internal disulfide during the catalytic reduction-oxidation cycle of the enzyme. Both NO2-OA and NO2-cLA inhibited \textit{S. cerevisiae} GR activity, with NO2-OA effecting a more pronounced inhibition (Fig. 4A). Interestingly, whereas incubation of GR with 3 μM NO2-OA in the presence of NADPH resulted in a complete inhibition of GR activity, GR pre-incubation with 3 μM NO2-OA in the presence of GSSG but absence of NADPH did not result in GR inhibition, which only became evident after NADPH addition (Fig. 4B).

To further characterize the inhibition and reactivity of GR with NO2-OA, rate constants were determined using pseudo-first-order conditions (Fig. 4C). A linear dependence was observed between the pseudo-first order rate constant (k_{obs}) and NO 2-OA concentration (Fig. 4D). The forward second-order rate constant (k_{on}) was determined from the slope of the k_{obs} versus NO2-OA concentration plot as \((3.45 ± 0.04) \times 10^3\) M\(^{-1}\) s\(^{-1}\). The non-zero y-axis intercept indicates a reversible reaction with a k_{off} = \((4.4 ± 0.4) \times 10^{-4}\) s\(^{-1}\) and \(K_{eq} = (1.3 ± 0.1) \times 10^{-7}\) M (25 °C, pH 7.4).

In order to compare the values obtained with other abundant thiol-containing intracellular enzymes, we kinetically characterized the reaction with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a known target for NO2-OA (Fig. 4D, inset) [18]. The values obtained were \(k_{on} = 389 ± 64\) M\(^{-1}\) s\(^{-1}\), \(k_{off} = (3.2 ± 0.9) \times 10^{-3}\) s\(^{-1}\) and \(K_{eq} = (8 ± 4) \times 10^{-8}\) M (25 °C, pH 7.4) indicating that GR reacts 10 times faster with NO2-OA than GAPDH. In the case of GSH, the forward rate constant with NO2-OA is \(64 ± 1\) M\(^{-1}\) s\(^{-1}\) (25 °C, pH 7.4), 50 times slower than GR [40].

2.5. NO2-OA modifies Cys61 of GR

Next, we sought to evaluate whether the inhibition involved covalent binding between GR and NO2-OA using biotin-labeled NO2-OA. Since the derivatization of NO2-OA carboxylic acid with biotin significantly impacts its size and charge, the inhibition of GR by biotin-NO2-OA was first confirmed (Suppl. Fig. 3). Incubation of purified GR with a 100-fold molar excess of biotin-NO2-OA in the absence of NADPH resulted in only low levels of adducted biotin-NO2-OA detected by non-reducing immunoblot. The adducted level of NO2-OA increased significantly in the presence of NADPH but absence of NADPH did not result in GR inhibition, which only became evident after NADPH addition (Fig. 4B).

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β-mercaptoethanol (BME, 5 mM) resulted in a rapid and complete recovery of GR enzymatic activity as shown by NADPH consumption (Fig. 6A). A dose-response was performed showing complete restoration of GR activity in the presence of 0.5 mM BME (Fig. 6B). Thiol-reversibility of the covalent inhibition of GR by NO2-OA was recapitulated with the endogenous thiol GSH. However, unlike BME, GSH elicited a hormetic response in which increasing concentrations (up to 2.5 mM) partially restored GR activity but higher levels resulted in a dose-dependent decline of the enzymatic activity (Fig. 6C). To test if this reduction was a result of product inhibition in vitro, GR activity was assayed as a function of [GSH] (Fig. 6D). GR activity was inhibited by >50% above 10 mM GSH suggesting that this mechanism is relevant at physiologic GSH concentrations and may affect GSH equilibria inside the cell.

Next, we evaluated the inhibitory effects of other electrophiles known to activate Nrf2 signaling and increase GSH levels. None of the three electrophiles tested inhibited the GR activity, indicating that this effect was specific for NO2-FA and not a general characteristic of electrophilic drugs (Fig. 6E).

Based on the product inhibition observed with GSH, it was hypothesized that electrophiles which do not inhibit GR directly may nonetheless exert indirect inhibition and induce GSSG accumulation through upregulation of GSH. CDDO-Im activated Nrf2 and strongly upregulated GCLM (Fig. 6F), an effect shared by DMF (data not shown). To compare temporal effects of CDDO-Im and NO2-OA, a time-course experiment was performed evaluating GSH and GSSG 0–24 h after electrophile exposure. Changes in GSH induced by CDDO-Im were comparable to those induced by NO2-OA. Despite the lack of direct GR inhibition in vitro, CDDO-Im treatment significantly increased GSSG (Fig. 6G, H). Notably, whereas all electrophiles had similar effects in GSH levels and all increased GSSG, NO2-OA resulted in significantly larger increases in GSSG levels and concomitant decreases in the GSH:GSSG ratio (Fig. 6I–K).

The large increase in GSSG induced by NO2-OA was found to be dose-dependent with respect to [NO2-OA], indicating that NO2-OA had a direct effect on GR activity in cells (Fig. 7A–B). In contrast, the increase in intracellular GSSG in response to CDDO-Im led to 50% increase in [GSH] and 4-fold increases in GSSG (Fig. 7C–D). In a separate experiment (Fig. 7E), CDDO-Im (200 nM) was not found to increase Prdx oxidation, suggesting that product inhibition by GSH and not oxidative stress was responsible for the observed GSSG accumulation.

Together these results implicate two separate and specific mechanisms by which electrophiles induce the accumulation of intracellular GSSG: covalent inhibition at catalytic Cys61 in the case of NO2-OA and product inhibition secondary to activation of Nrf2-induced biosynthesis and elevation of GSH levels for all electrophiles.
We use numerical integration of the governing rate equations to predict the relative concentrations of GSSG and GSH as the total [GS]T is increased. As time is gradually increased, enzymatic instantaneous steady-state conditions for each time point are achieved by employing enzymatic rate constants that are much faster than the rate of increase in [GS]T (reaction (10) in Suppl. Table 1). The values for K3 and K4 for noncompetitive parabolic inhibition are taken from published values for the rat liver enzyme and the values for K3o, K4o, and Vmax (for GR, v1) and [GPx]0 (for GPx, v2) are set to achieve optimal fit to the data (steady-state [GSSG], [GSH]) for all electrophiles except NO2-OA.

Fig. 8A presents the computed increases in [GSH] and [GSSG] with time using the rate constants shown in Suppl. Table 1. The results are identical whether [GS]T is increased by generation of GSH or GSSG (red vs. black), demonstrating rapid steady-state interconversion determined by the enzymatic mechanisms. Fig. 8B shows that steady-state is achieved very early during the calculation (within 5 s) and the inset shows that the rates of each enzymatic reaction are equal (v1 = v2) at every point throughout the calculation, a requirement for steady-state. In addition, this shows that the calculated results are identical to the values predicted by the steady-state derivation (Eq. (1)-(4)).

Fig. 8C shows that the model accurately predicts the experimental results for [GSSG] and [GSH] for all treatments with the exception of NO2-OA. It is clear that as the total [GS]T approaches 10 mM there is a dramatic increase in [GSSG] with only modest increases in [GSH]. To examine the mechanistic basis for this phenomenon, Fig. 8D shows the distribution of GR between different forms as [GS]T is increased. The noncompetitive parabolic binding of GSH to both GR and GR-GSSG results in conversion of active enzyme (GR) into inactive GRi forms; however, the overall rate of GSSG reduction to GSH (v1) remains relatively constant (Fig. 8B inset). This is because at [GSH] > 5 mM GPx is saturated (Km = k7/k8 = 0.5 mM, Eq. (4)) and so the rate v2 is maximal and constant, equal to k2[GPx]GSH.

Fig. 8D shows that even under baseline conditions the enzyme is substantially inhibited; the enzyme is 65% inhibited at [GSH] = 5 mM, 90% inhibited at [GSH] = 7.5 mM and increases to 98% at 9.5 mM. This is because of the steady-state condition v1 = v2 and so v1 is also constant. Therefore, the only way to counteract loss of active enzyme is to increase substrate [GSSG] for the remaining active enzyme. This illustrates the concept that major changes in the relative amounts of GSH and GSSG can occur independently of changes in oxidative conversion of GSH into GSSG (indeed, the rate of this conversion, v2, is essentially unchanged, Fig. 8B inset).

Fig. 8E superimposes experimentally measured [GSSG] as a function of [GSH] from RAW264.7 macrophages treated with NO2-OA data over the values obtained with compounds which do not inhibit GR, as well as the computational prediction. In contrast to the other electrophiles
Fig. 4. NO2-OA is a reversible covalent inhibitor of GR. A) Inhibition of GR activity by NO2-OA, NO2-cLA, or non-electrophilic OA (5 μM each) as measured by the DTNB-reduction assay after 10 min incubation. Results are mean ± SD from 3 to 5 independent experiments. For each experiment 3.36mU GR were incubated in 145 μL containing 5 μM NO2-FA, OA or vehicle control for 10 min at ambient temperature. **p < 0.0001 by one-way ANOVA vs. control. (B) TNB production by GR pre-incubated with vehicle control, NO2-OA + GSSG (purple) or NO2-OA + NADPH (red) for 10 min prior to addition of missing substrate. Results are summarized as mean ± SD from 3 to 5 independent experiments. (C) Reduced GR (2 nM) was incubated with 800 nM NO2-OA. At increasing times, aliquots (1.6 nM) were mixed with 0.1 mM NADPH and 1 mM GSSG, and the initial rate of absorbance decay at 340 nm and 25 °C was measured. The solid line represents the best fit to a single exponential equation. (D) kobs values at increasing concentrations of NO2-OA (200–1200 nM) were determined from kinetic traces as in (A); kobs values for the reaction between NO2-OA and GAPDH.

(Fig. 8C), the extent of [GSSG] increase (due to GR inhibition) is much more dependent on the NO2-OA concentration and not on the GSH concentration. This emphasizes our conclusion that the primary mechanism for NO2-OA is by covalent adduction rather than an increase of [GST].

In order to test how much tolerance there is in the values for the adjusted parameters K3 and K4, Suppl. Fig. 4 shows that the model provides a reasonable fit to the data only when these values vary by no more than approximately ±15%.

We utilized modeling to predict the quantitation of GR inhibition due to adduction by NO2-OA. From the steady-state assumption we presume increase in [GSSG] is due to decrease in the amount of active enzyme [GR] by the less than unity factor α, as predicted from the relationship v1 = v2.

\[
\alpha = \frac{k_2[GPx][GSH] + C^*[GSSG]}{k_j[GR]_1[GSSG] + C^*[GSSG]}
\]

(5)

Thus, for each experimental result using NO2-OA, the percent inhibition due to formation of the inactive adducted enzyme (GR,) is given by 100 * (1-α). Although not shown, obtaining α by decreasing [GR] with trial and error using the integrated rate approach provides results essentially identical to the steady-state value and also allows determination of the total enzyme present in the four inhibited forms from product inhibition ([GRi]_T = [GRi1] + [GRi2] + [GRi3] + [GRi4]). Fig. 8F shows the relative percent inhibition for increasing [NO2-OA]. In all cases the enzyme is greatly inhibited, between 94% and 97%, and surprisingly the relative proportion from adduction vs. product inhibition is not determined by increasing [NO2-OA]. These proportions do not correlate with [GSSG], but as Fig. 8G shows there is a linear inverse relationship between percent adducted (GRa) and [GSH]. This in vivo prediction validates the in vitro studies (Fig. 6C) demonstrating that increasing GSH has opposing effects on the activity of adducted enzyme, decreased activity from product inhibition and increased activity from reversal of adduction. Finally, Fig. 8H shows that increasing [NO2-OA] monotonically (although nonlinearly) decreases the total active GR, even with variable proportions of adduction vs. product inhibition (Fig. 8F).

3. Discussion

GSH, with intracellular concentrations in the millimolar range and numerous enzymatic and non-enzymatic modes of reactivity, is decisively positioned to act not only as a regulator of thiol oxidation status,
Fig. 5. NO₂-OA inhibits GR via Cys61 modification. (A) Catalytic cysteines are a main target of NO₂-OA as assessed by 10 min incubation with biotinylated NO₂-OA (8 μM) in the presence or absence of cofactor NADPH and GSSG (n = 2). (B) Sequence of tryptic peptide encompassing the GR catalytic site ALGGTC61VNVGC66VPK with b⁺ and y⁺ fragmentation patterns and potential Cys modifications. (C) Elution profile of the native and alkylated peptides containing Cys61. (D) Tabular summary of calculated (black) and identified (blue/red) b⁺ and y⁺ ions, respectively. MS/MS spectra of the GR catalytic site peptide obtained after control, IAM and NO₂-OA treated GR showing native (E), carbamidomethylated (F), and nitroalkylated (G) Cys61 peptide.
but also as a main target of electrophiles. The molecular pleiotropy of the GSH system is further affirmed by its ubiquity, penetrance and complexity in modulating cell signaling activities, detoxification and downstream post-translational protein modifications. The pharmacological manipulation of the GSH/GSSG couple allowed us to study its impact on temporal responses to oxidative and electrophilic stress. Cell exposure to NO$_2$-OA for 1 h leads to a slight decrease in GSH levels, likely caused by adduct formation, which has been previously observed. 

Fig. 6. GR is sensitive to product inhibition in context of electrophile-induced upregulation of [GSH] (A) Representative kinetic traces showing that addition of BME (5 mM) rapidly restores GR activity and NADPH consumption of pre-inhibited GR (34mU GR + 5 μM NO$_2$-OA). (B) GR activity restoration is dependent of BME concentration. Results are reported as mean ± SD. For some points, the error bars are shorter than the height of the symbol (C) Horner reversal of NO$_2$-OA inhibition of GR by GSH. GR inhibited with NO$_2$-OA was treated with increasing concentrations of GSH and activity was assayed. (D) GSH inhibits GR activity. GR activity was measured in absence or presence of physiologically relevant concentrations of GSH and displayed dose-dependent reduction in activity suggesting product inhibition. (E) Electrophiles CDDO-Im, DMP, and MMF do not inhibit GR activity in vitro. For each experiment 3.36mU GR was incubated in 145 μL 5 mM NO$_2$-OA or vehicle control for 10 min at ambient temperature. Rates are reported as % control ± SD (C–D) or nmol/sec GSSG (E) from 3 to 5 independent experiments; for some points, the error bars are shorter than the height of the symbol. (F) CDDO-Im (100 nM) induces GCLM expression in RAW264.7 macrophages. Vertical line indicates intervening lanes cropped to approximate non-adjacent bands from one blot showing two biological replicates. Increased levels of GSH (E) and GSSG (F) in RAW264.7 macrophages following treatment with 5μM NO$_2$-OA (red) or 100 nM CDDO-Im (dashed) for indicated times. (I) GSH, (J) GSSG, and the ratio of GSH:GSSG (K) in RAW264.7 cells after 6 h treatment with indicated electrophilic ligands. Statistical analysis by one-way ANOVA with Sidak correction for multiple comparisons; for some groups, error is less than thickness of the error bar.

Fig. 7. NO$_2$-OA but not CDDO-Im dose-dependently increases intracellular GSSG. RAW264.7 macrophages incubated with increasing concentrations of NO$_2$-OA (A–B) or CDDO-Im (C–D) for 6 h followed by measurement of GSH (A, C) and GSSG (B, D). Statistical analysis by one-way ANOVA with Dunnett correction for multiple comparisons, comparing treatment groups to control (DMSO), n = 3–8. (E) Immunoblotting for monomeric and dimeric Prdx1 and Prdx3 after 1 and 6 h treatment with 200 nM CDDO-Im revealed no increase in cytosolic or mitochondrial Prdx oxidation.
Evidence for product inhibition of GR by GSH was first published nearly three decades ago using isolated GR preparations but these observations were never further explored or extended to complex, dynamic cellular systems [42,43]. Of relevance, the range of concentration at which GSH inhibits GR activity is between 8 and 13 mM [42,43]; in our experiments IC50 (≈10 mM) directly relates to GSH concentrations found in cells (between 6 and 9 mM). Offering support to this regulatory process, depletion of GSH with BSO resulted in 2-fold greater reduction in GSSG than in GSH (Fig. 1).

Collective unbiased expression of [GSSG] as a function of [GSH] showed a positive non-linear correlation (Fig. 8C). BSO-treated cell populations clustered in the bottom-left quadrant of the distribution suggesting de-repression of GR, while electrophile-treated populations clustered in upper-right, signifying product inhibition. Computational simulation of the steady-state GSH system using a numeric integration approach with published parameters showed that product inhibition of GR was necessary to accurately fit the model to these experimental data. The simulations are not intended to provide a complete kinetic analysis of GR, which has been performed by others. Rather, it provides an illustration of how our experimental results are compatible with (indeed, predicted by) the consequences of the inhibition of GR by GSH. The results from both experiments and simulation are incompatible with a static concept of GSH cycling, in which the relative abundances of GSH and GSSG are solely dependent upon oxidant load and where GSH depletion via oxidation is prerequisite to GSSG accumulation. Instead, the observations suggest that in the absence of oxidative stress, [GSSG] is determined by flux through GR, which is subject to product inhibition by GSH.

It is possible that the responsiveness of GR to intracellular [GSH] evolved as a mechanism to optimize allocation of reducing equivalents. In a reducing or unstressed state, this mechanism could redploy energetically expensive reducing equivalents (NADPH) from generating unnecessaries to other biosynthetic pathways, such as production of lipids and nucleic acids. At the same time, this would permit cells to unneeded GSH to other biosynthetic pathways, such as production of glutathione peroxidases (GPX). While previous studies have not identified GRX as a target gene of Nrf2 [6,58], an electrophilic dithiocarbamate derivative has been previously identified to inhibit both GR [65] and GRX1 [66]. It is possible that NO2-OA may likewise inhibit GRX in addition to GR; indeed, our unpublished results suggest that mixed glutathione-protein disulfides are increased by NO2-OA. The biochemical characterization and biological sequelae of this interaction remain a topic of further study. In contrast to GRX, expression of GPX1 has been found to be dependent on Nrf2 activity and is responsive to electrophiles [58]. In addition, electrophilic pollutants from diesel exhaust and tobacco smoke have previously been found to inhibit GPX activity [67]. While expression of GPX is known to be dependent on Nrf2, in our experiments NO2-OA did not affect cell sensitivity to peroxide exposure, suggesting that activity of GPX is not impacted by this electrophile.

In conclusion, we have shown that electrophiles impact both the levels of GSH and GSSG through modulation of GSH synthesis to impact GR activity and, in the case of NO2-OA by reacting with catalytic Cy561.
This has broad implications, as changes in the GSH/GSSG couple and 
$E_{\text{GSH}}$ have been widely used as a marker for oxidative stress and in-
creased oxidative environments.

4. Experimental procedures

4.1. Materials

Primary antibodies were purchased from the following suppliers:

- GR (Abcam, Cambridge, MA),
- GCLM (Proteintech, Chicago, IL),
- HO-1 (Cell Signaling, Beverly, MA),
- GAPDH (Trevigen, Gaithersburg, MD),
- Histone H3 (Cell Signaling, Danvers MA),
- Peroxiredoxin 1 (R & D Systems, Minneapolis, MN),
- Peroxiredoxin 3 (R & D Systems, Minneapolis, MN),
- Peroxiredoxin1-4 SO$_2$/SO$_3$ (Abcam, Cambridge, MA).

Secondary antibodies were purchased from Santa Cruz Biotechnologies
(Dallas, TX).

LPS derived from E.coli O127:B8, purified GR from S. cerevisiae, DMF, MMF, GSH, $^{13}$C$_2$$^{15}$N GSH and GSSG were from Sigma-
Aldrich (St. Louis, MO). Oleic acid was obtained from Nu-Check Prep,
Inc. (Elysian, MN). NO2-OA, biotin-NO2-OA and NO2-cLA were synthesized as previously reported[21]. NO2-OA was used as a mixture of 9- and 10-NO2 regio-isomers while NO2-cLA was a mixture of 9- and 12-NO2 regio-isomers. CDDO-Im and -Me were from Toronto Research Chemicals (Toronto, Canada. Solvents were LC-MS quality and purchased from Burdick and Jackson (Morristown, NJ)). Formic acid was purchased from Fisher Scientific (Pittsburgh, PA).

4.2. Enzyme activity assays

Glutathione reductase activity was determined following the initial rates of dithionitrobenzoate (DTNB) reduction using an established biochemistry method adapted from Rahman et al.[68]. Briefly, purified GR from S. cerevisiae (3mU-12mU; consistent within experiments) was incubated with vehicle or inhibitor in the presence of NADPH (150 μM) or GSSG (1 mM) and reaction was started with either GSSG or NADPH respectively. Consumption of NADPH (ε 340 = 6.22 × 10³ M⁻¹ cm⁻¹) or production of chromogenic TNB (ε 412 = 1.42 × 10⁴ M⁻¹ cm⁻¹) were followed on a Agilent 8453 UV–Vis spectrophotometer.

4.3. Cell culture

RAW264.7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown per ATCC instructions in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

4.4. Immunoblotting

Cells were washed 2x with cold PBS and lysates were prepared in 4 °C RIPA buffer with MiniTab protease inhibitor cocktail (Roche, Switzerland). For Prdx experiments, cells were incubated in alkylation buffer (40 mM HEPES, 50 mM NaCl, 1 mM EGTA, 1 mM EDTA, 100 mM NEM, pH 7.4) for 10 min at room temperature before lysing with Triton X-100 and protease inhibitor cocktail. Protein was quantified by BCA assay (Pierce/Thermo Fisher) and samples containing 15–25 μg total cellular protein were loaded on 4–12% Bis-Tris gel. Proteins were resolved by reducing or non-reducing SDS-PAGE, transferred to nitrocellulose membrane, blocked with 5% non-fat dry milk in TBS-0.1% Tween 20 for 1 h. Primary and secondary antibodies were used at 1:1000 and 1:5000 dilutions unless otherwise indicated and visualized with Clarity ECL chemiluminescence kit and ChemiDoc imager (Bio-Rad, Hercules, CA).

4.5. Cellular GSH and GSSG determination

The method for simultaneous determination of GSH and GSSG was adapted from a previously published report[69]. Briefly, RAW264.7 macrophages were seeded in 24-well plates at a density of 300,000 cells/well and were cultured overnight prior to treatments. Media was aspirated and cells were washed 2x with sterile PBS and incubated with PBS containing 25 mM N-ethylmaleimide (NEM) for 15 min at 37 °C. The PBS/NEM solution was aspirated and 150 μL derivatizing solution (25 mM NEM, 40 mM HEPES/50 mM NaCl/1 mM EDTA; 2 μM 13C₂15N GSH; 2 μM 13C₄15N₂ GSSG) was added to each well and incubated for 15 min at room temperature. Cells were detached by scraping, followed by 3 cycles of sonication in a 4 °C water bath for 30 s each. The lysate was cleared by centrifugation at 21,000 g for 10 min at 4 °C. Protein content was assessed using the BCA assay. Proteins were precipitated by addition of 9 volumes of ethanol (200 proof), cooled to −80 °C overnight, centrifuged (21,000 g, 4 °C, 10 min) and the supernatant dried under N₂ stream at room temperature. Samples were reconstituted in dH₂O and 20 μL were injected for analysis by LC-MS/MS.

4.6. Reversed phase HPLC tandem mass spectrometry

A Shimadzu HPLC (Columbia, MD) coupled to a Thermo Scientific CTC HTS PAL autosampler (Waltham, MA) and an AB Sciex (Framingham, MA) 5000 triple quadrupole mass spectrometer were used for the quantification of GSH adducts and GSSG. Samples (20 μL) were resolved on a Phenomenex C18 column (2.0 x 100 mm, 5 μm particle size) using the following solvent system A) aqueous 0.1%
formic acid and B) 0.1% formic acid in acetonitrile at a flow rate of 600 µl/min. Chromatographic conditions were as follows: 1% solvent B for 0.1 min, followed by a linear gradient to 20.3% solvent B at 6 min, to then switch to 100% solvent B for 2 min and re-equilibration to return to the initial condition (1% solvent B) for 4 min. The following settings for the mass spectrometer were used: Source temperature 550 °C; ionization spray voltage 5500 V; CAD 4.0 arbitrary units; Curtain gas 40 arbitrary units; GS1 45 arbitrary units; GS2 50 arbitrary units; EP 5.00 V; CXP 10.00 V. Multiple reaction monitoring was performed with 65 ms dwell time and a declustering potential of 60 V using the following transitions in positive ion mode: GS-NEM (433.0/304.0, CE 38), 13C415N GS-NEM (436.0/307.0, CE 38), GSSG (613.2/355.2, CE 30), 13C415N2 GSSG (619.2/361.2, CE 30).

4.7. LC-MS/MS detection and analysis of GR post-translational modifications

Purified GR (S. cerevisiae; 10 µg) was incubated in the presence or absence of GSSG (2 mM), NADPH (3 mM), NO2-OA (8 µM) for 30 min in 50 mM phosphate buffer, pH 7.4 and the reaction was stopped by addition of iodoacetamide (10 mM final concentration, 10 min at room temperature). After alkylation, GR was digested using MS grade modified trypsin (trypsin/GR ratio of 1:50) for 16 h at 37 °C. The peptide digest was analyzed by LC-MS/MS for post-translational modifications using a Thermo Surveyor Plus HPLC coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Peptides (3 µg on column) were loaded onto a Phenomenex C18 (2.0 x 100 mm, 5 µm particle size; Torrance, CA) 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 650 µl/min flow rate. Chromatographic conditions were as follows: 5% solvent B for 2 min, followed by a linear gradient to 60% solvent B for 18 min, to then switch to 100% solvent B for 5 min and re-equilibration to return to the initial condition (5% solvent B) for 5 min. MS analysis was carried out in the positive ion mode with source parameters optimized for the detection of peptides containing nitroalkylated Cys. Instrument settings were as follows: source voltage, 3.75 kV; capillary temperature, 200 °C; source heater temperature, 450 °C; sheath gas flow and auxiliary gas flow, 25 arbitrary units; sweep gas flow, 20 arbitrary units; collision energy, 35 eV. MS/MS spectra was acquired using data-dependent acquisition in which the following transitions in positive ion mode: GS-NEM (433.0/304.0, CE 38), 13C415N GS-NEM (436.0/307.0, CE 38), GSSG (613.2/355.2, CE 30), 13C415N2 GSSG (619.2/361.2, CE 30).

4.8. Kinetic modeling of cellular GSH and GSSG

Steady-state conditions were assumed for both GR and GPx. For each enzyme, validation of the steady-state assumption was accomplished by numerical integration (Berkeley-Madonna www.berkeleymadonna.com using the Rosenbrock stiff algorithm) of the elementary rate equations for each mechanism. The rate constants were chosen to reflect published experimental values; for GPx k2, k9, and [H2O2] reported in [55], for GR k3 and k4 reported [42] and K_m in [59]. The parameters adjusted to optimize fit were V_max, K_m, and k4 for GR and GPx for GPx. Further detail is provided in Supplemental figures.

4.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism (La Jolla, CA, USA) version 7.01 by Student’s t-test, one-way or two-way analysis of variance (ANOVA) and multiple comparison testing as indicated in figure legends. P values less than 0.05 were considered statistically significant.

CRediT authorship contribution statement

**Soma Jobbagy:** Conceptualization, Investigation, Data curation, Formal analysis, Funding acquisition, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Dario A. Vitturi:** Conceptualization, Investigation, Data curation, Formal analysis, Funding acquisition, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Sonia R. Salvatore:** Investigation, Data curation, Formal analysis, Methodology, Visualization, Writing - review & editing. **Lucía Turell:** Conceptualization, Investigation, Data curation, Formal analysis, Funding acquisition, Methodology, Visualization, Writing - review & editing. **Emilia Kansanen:** Funding acquisition, Writing - review & editing. **Carlos Batthyany:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Jack R. Lancaster:** Conceptualization, Investigation, Data curation, Formal analysis, Software, Writing - original draft, Writing - review & editing. **Bruce A. Freeman:** Funding acquisition, Project administration, Supervision, Resources, Writing - review & editing. **Francisco J. Schopfer:** Conceptualization, Investigation, Data curation, Formal analysis, Funding acquisition, Methodology, Visualization, Project administration, Supervision, Resources, Writing - original draft, Writing - review & editing.

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Conflict of interest

BAF, DAV and FJS have financial interest in Complexa Inc. All other authors declare that they have no conflicts of interest with the contents of this article.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2018.11.008.

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