The labile iron pool attenuates peroxynitrite-dependent damage and can no longer be considered solely a pro-oxidative cellular iron source

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Abstract
The ubiquitous cellular labile iron pool (LIP) is often associated with the production of the highly reactive hydroxyl radical, which forms through a redox reaction with hydrogen peroxide. Peroxynitrite is a biologically relevant peroxide produced by the recombination of nitric oxide and superoxide. It is a strong oxidant that may be involved in multiple pathological conditions, but whether and how it interacts with the LIP is unclear. Here, using fluorescence spectroscopy, we investigated the interaction between the LIP and peroxynitrite by monitoring peroxynitrite-dependent accumulation of nitrosated and oxidized fluorescent intracellular indicators. We found that, in murine macrophages, removal of the LIP with membrane-permeable iron chelators sustainably attenuates the peroxynitrite-dependent oxidation and nitrosation of these indicators under simulated normal and oxidative conditions, in stark contrast to H₂O₂-induced oxidation. These observations could not be reproduced in cell-free assays, indicating that the chelator-enhancing effect on peroxynitrite-dependent modifications of the indicators depended on cell constituents, presumably including LIP, that react with these chelators. Moreover, neither free nor ferrous-complexed chelators stimulated intracellular or extracellular oxidative and nitrosative chemistries. On the basis of these results, LIP appears to be a relevant and competitive cellular target of peroxynitrite or its derived oxidants, and thereby reduces oxidative processes, an observation that may change the conventional notion that the LIP is simply a cellular source of pro-oxidant iron.

Most cellular iron is bound to prosthetic groups that are strongly associated with proteins, such as haem and Fe/S clusters, and engages in specific reactions. A ubiquitous, yet poorly characterized, small amount of cellular iron known as chelatable iron or the labile iron pool (LIP) also exists; this pool is methodologically defined as the fraction of cellular iron that is complexed by high-affinity metal chelators (1, 2). The LIP is thought to be weakly bound to cellular constituents with a small molecular mass and proteins (2), although the fundamental properties of cellular LIP complexes have yet to be determined. LIP reactivity towards strong chelators and nitrogen monoxide (nitric oxide, NO') (which yields dinitrosyl iron complexes [DNICs]) (3-5) and its presumed redox activity towards hydrogen peroxide offer important information about LIP chemical properties. Notably, the LIP undergoes rapid ligand substitution (it is labile), engages in redox reactions (1, 6, 7), and is primarily present in the ferrous state (8); the ferric state does not yield for DNICs (5).

Biologically, the LIP represents a dynamic cellular iron reservoir that traffic through different cell compartments and is readily available for incorporation into nascent metalloproteins (9, 10). However, as mentioned above, the LIP seems to exhibit redox activity towards hydrogen peroxide (H₂O₂) (6, 7); Among other reasons, this redox activity explains why organisms tightly control iron handling at the systemic and cellular levels in processes ranging from iron acquisition in the body to transport and incorporation into cells and cellular homeostasis (1, 11). Cellular LIP levels are maintained by iron regulatory proteins (IRPs) (10, 12) at a low concentration range that varies from 0.1 to 10 µM depending on the cell type.

Based on the reaction between the LIP and H₂O₂, we hypothesized that the LIP reacts with peroxynitrite (ONOOH/ONOO’; pKa = 6.9 (13)). This peroxide is formed by a diffusion-controlled reaction of nitric oxide with superoxide anion radicals (equation 1, k₁ = 1.9 x 10¹⁰ M⁻¹s⁻¹) (13), and it is a strong oxidant itself. In addition, upon protonation (14, 15) or reaction with CO₂ (equations 2-3), peroxynitrite ultimately generates even more aggressive species, such as OH’, nitrogen dioxide (NO₂’) and carbonate anion (CO₃²⁻) radicals (16-20).

\[ \text{NO’} + \text{O}_2^- \rightarrow \text{ONOO}^- \] (1)
\[ \text{ONOO}^- + \text{H}^+ \rightarrow 0.3(\text{NO}_2^\cdot + \text{OH}^-) + 0.7(\text{NO}_3^- + \text{H}^+) \] (2)
\[ \text{ONOO}^- + \text{CO}_2 \rightarrow \text{ONOOCO}_2^- \rightarrow 0.35(\text{NO}_2^- + \text{CO}_3^\cdot) + 0.65(\text{NO}_3^- + \text{CO}_2) \] (3)

Removal of the LIP from murine macrophages with cell membrane-permeable chelators increases nitrosation and oxidation of exogenous intracellular targets, indicating that the
LIP reacts with peroxynitrite and perhaps peroxynitrite downstream oxidants.

Results

We used two fluorescent indicators, 4,5-diaminofluorescein diacetate (DAF2-DA) and 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA), to monitor the intracellular peroxynitrite nitrosative and oxidative chemistry, respectively. These two indicators are trapped in cells through the actions of nonspecific esterases (21), which is a critical property for our goals. Although these probes do not react with peroxynitrite itself (they react with peroxynitrite downstream oxidants instead), both intracellular DAF2 nitros(yl)ation and H$_2$DCF oxidation depend on peroxynitrite under the conditions used in the present study (22) (see below and Supplementary Information, Figure S1). DAF2 nitros(y)lation is mechanistically complex; it involves two related and competitive radical mechanisms named nitrosylation (equations 4-5) and nitrosation (equations 6-7) (22, 23). Nitrosylation is the dominant mechanism, especially at low NO$^\bullet$ concentrations. We refer to these mechanisms together as nitros(yl)ation throughout the text.

$$\text{DAF2} + \text{NO}_2^\bullet \text{ (or CO}_3^{2-}) \rightarrow \text{DAF2}^\bullet + \text{NO}_2^- \text{ (or CO}_3^{2-}) \quad (4)$$

$$\text{DAF2}^\bullet + \text{NO}^\bullet \rightarrow \text{DAFT} \quad (5)$$

$$\text{NO}^\bullet + \text{NO}_2^\bullet \rightarrow \text{N}_2\text{O}_3 \quad (6)$$

$$\text{DAF2} + \text{N}_2\text{O}_3 \rightarrow \text{DAFT} + \text{NO}_2^- \quad (7)$$

Fluorescent boronate compounds (24), which otherwise would be the best choice for this study, were not able to be used since they are not trapped in cells, and, most importantly, boronates may outcompete the LIP for peroxynitrite at the required concentrations.

LIP removal by chelation increases nitrosative chemistry in cells

Figure 1A illustrates a typical experiment designed to monitor the intracellular nitros(y)lation of DAF2 using fluorescence spectroscopy. RAW 264.7 cells that had been previously loaded with DAF2-DA, as described in the experimental section, were suspended in pre-warmed working buffer (PBS containing 100 µM diethylene triamine pentaacetic acid (DTPA) and 100 U/mL bovine Cu,Zn-superoxide dismutase (SOD) at 37°C). When required, the cell membrane-permeable iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH) was added before NO$^\bullet$ donor introduction and the cell suspension was placed in a fluorimeter cuvette under constant stirring. Data acquisition was initiated immediately. The fluorescence does not increase until the NO$^\bullet$ donor sper/NO [(z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]diazen-1-ium1,2-diolate] is introduced (as designated by the arrows in Figure 1A and 1D). As reported in our previous study (22), intracellular DAF2 nitros(y)lation depends on NO$^\bullet$ (as confirmed here) and on peroxynitrite-derived radicals under the experimental conditions of the present study. Interestingly, DAF2 nitros(y)lation increased in the presence of the chelator SIH (Figure 1A and 1B). SIH rapidly obtains access to cells (2, 5) and is known to bind strongly and rapidly to ferrous (and ferric) iron in a 2:1 stoichiometry, yielding the [Fe(SIH)$_2$] complex (25-27). The effect of SIH was remarkable. The rate of DAF2 nitros(y)lation increased 4-fold in the presence of 15 µM sper/NO (approximately 180 nM NO$^\bullet$ under steady-state conditions, which were measured amperometrically using a NO$^\bullet$-selective electrode (Supplementary Information, Figure S2 panel C) (22)). The effect of SIH on the increase in fluorescence plateaued at approximately 100 µM SIH, suggesting the exhaustion of a SIH-reactive cell substrate (Figure 1C). The LIP concentration in RAW 264.7 cells was estimated to be approximately 8-10 µM using electron paramagnetic resonance (4, 5) (Data not shown). SIH was added in a large excess to guarantee saturation of the iron coordination sphere.

The classical membrane-permeable iron chelator 2,2’-bipyridine (Bipy) also accelerated the nitros(y)lation of DAF2 (Figure 1D). Importantly, the exclusion of the cell membrane-impermeable chelator DTPA from the working buffer did not affect the fluorescence, suggesting that the acceleration effect may be a general property of...
permeable chelators. Control experiments showed that neither SIH, Bipy nor their respective iron complexes fluoresce, and no fluorescence was observed when DAF2-DA was omitted from the experiments (data not shown).

Notably, DAF2 nitros(yl)ation depended on peroxynitrite formation and its downstream oxidants in the presence of SIH [see Supplementary Information (Figure S1) and the experiments using the peroxynitrite scavenger 2-phenyl-1,2-benzoselenazol-3-one (ebselen) described below]. In other words, the SIH chelator did not introduce additional chemical mechanisms of DAF2 nitros(yl)ation.

The simplest explanation for the chelator effect would be that free SIH or its respective LIP complex generates superoxide or radicals that stimulate nitrosative processes (22, 23). However, this possibility was excluded based on the results of the control experiments. Neither free SIH, Bipy nor their respective iron(II) complexes consumed oxygen or produced superoxide in cell-free assays (Supplementary Information, Figure S3, panels A, C and D). Additionally, SIH does not produce intracellular superoxide or oxidants, as no increases in the oxidation of dihydroethidine (DHE) (Figure S3, panel B) or H$_2$DCF (see below) were observed in the presence of SIH. Another plausible explanation is that SIH somehow increased the NO$^\bullet$ concentration, but we refuted this possibility. The steady-state nitric oxide concentration is not affected by SIH (Supplementary Information, Figure S2, panel C). We tested other possibilities that might explain the peroxynitrite-dependent chelator effect, but all of them were rationally and/or experimentally excluded. The spectroscopic data did not indicate that aqueous iron(II) and SIH, alone or in combination, interacted with DAF2 or DAFT and altered their fluorescence properties in the working buffer (supplementary Figure S2, panels A and B). We then hypothesized that the LIP reacts with peroxynitrite and/or peroxynitrite-derived reactive species to attenuate nitros(yl)ation and oxidation. LIP chelation with SIH would prevent these reactions. Accordingly, the [Fe(SIH)$_2$] complex does not directly react with peroxynitrite (Supplementary Information, Figure S4).

The acceleration of peroxynitrite-dependent DAF2 nitros(yl)ation by chelators depends on cells

We reasoned that if the effect of SIH on accelerating peroxynitrite-dependent DAF2 nitros(yl)ation is related to LIP chelation, the effect would not be reproduced in a reaction lacking cells. In a cell-free assay, a combination of a NO$^\bullet$ donor and xanthine oxidase/xanthine (XO/X) sufficient to produce a 10 nM/s flux of O$_2$$^\bullet^{-}$ increased the rate of DAF2 nitros(yl)ation compared to that observed in NO$^\bullet$ autoxidation (NO$^\bullet$ donor alone), and this increase was reversed by bovine superoxide dismutase (SOD) (1000 U/mL). This outcome is consistent with the experiments reported by Espey et al (23) and their conclusion that peroxynitrite-derived radicals stimulate nitrosative processes. In contrast to the cell-based experiments, SIH did not increase peroxynitrite-stimulated DAF2 nitros(yl)ation under these conditions (Figure 2A and 2B). Instead, SIH slightly decreased DAF2 nitros(yl)ation, indicating that free SIH actually scavenges peroxynitrite-derived radicals, which is also apparent in Supplementary information, Figure S4.

Peroxynitrite competition experiments

Peroxynitrite scavengers should decrease the effect of SIH on intracellular DAF2 nitros(yl)ation. The ferrous equivalent of the SOD mimic Mn(III) meso-tetrakis(N-ethylpyridinium-2-yl) porphyrin (MnTE-2-Pyp) has been suggested to catalytically and rapidly reduce peroxynitrite to NO$_2$$^\bullet$ (28). In our hands, MnTE-2-Pyp clearly prevented DAF2 nitros(yl)ation under the experimental conditions used in the present study (Supplementary Information, Figure S1, panel C), but this effect was not easily dissociated from O$_2$$^\bullet^{-}$ removal. In this set of experiments, we used ebselen and the non-fluorescent compound boronate 4-acetylphenyl boronic acid (APBA, from Boron Molecular), which are more specific peroxynitrite scavengers. Ebselen (29) and boronates react with peroxynitrite with high second order rate constants (k ~ 1 x 10$^6$ M$^{-1}$s$^{-1}$) (30, 31) according to equations 8-9.

\[
ebselen + \text{ONOO}^- \rightarrow \text{selenic acid} + \text{NO}_2^- \quad (8)
\]
APBA + ONOO$^-$ → 0.9[ACP + NO$_2^-$ + B(OH)$_3^-$] + 0.1[ACPB$^+$ + NO$_2^-$] \hspace{1cm} (9)

where ACP is 4-hydroxy-acetophenone, and ACPB$^+$ is a putative boronate-derived radical. DAF2-loaded RAW 264.7 cells were suspended in pre-warmed working buffer and placed in the fluorimeter cell, as described above. Ebselen, APBA and SIH were introduced in selected experiments, as required. Then, the NO$^*$ donor sper/NO was added, and data acquisition was initiated. Ebselen completely inhibited DAF2 nitrosylation (Figure 3A), regardless of the presence of SIH, indicating that intracellular DAF2 nitrosylation depends on peroxynitrite and that DAF2 nitrosylation follows the same peroxynitrite-dependent mechanisms in the presence of SIH.

The results using the boronate APBA were somewhat surprising. The boronate actually increased the rate of DAF2 nitrosylation in the absence of SIH (Figure 3B). The reason for this observation is not clear, but may be related to the observation that the boronate/peroxynitrite reaction phenomenologically partially produces NO$_2^-$ and a putative boronate-derived radical (equation 9). In fact, high APBA concentrations might increase the net yield of NO$_2^-$ by completely diverting peroxynitrite from reacting with other potential cellular targets [thiol peroxidases (32-34)] that reduce peroxynitrite to NO$_2^-$, thus increasing NO$_2^-$-dependent oxidative and nitrosative processes. These observations are germane to the use of boronates as antioxidants targeting peroxynitrite. More relevant to our purposes, the effect of SIH on accelerating DAF2 nitrosylation compared to the control decreased from 4.0 ± 0.1 in the absence of APBA to 2.5 ± 0.8 in its presence (Figure 3C). Thus, the boronate decreases the availability of the peroxynitrite that reacts with the LIP and subsequently decreases the effect of SIH on accelerating the rate of DAF2 nitrosylation. Based on the results of these experiments, the LIP is a competitor of ebselen and APBA for peroxynitrite. However, the observed boronate-resistant SIH-mediated acceleration effect (Figure 3C) may indicate that the LIP also reacts with NO$_2^-$ or CO$_2^-$.

Importantly, these competition experiments further strengthen the hypothesis that DAF2 nitrosylation depends on peroxynitrite-derived radicals.

**LIP removal enhances peroxynitrite-dependent DAF2 nitrosylation**

We tested the effects of SIH on cells exposed to peroxynitrite fluxes as positive controls. These experiments were also valuable for investigating the possible role of the LIP under simulated stress conditions. We used two approaches: cells were exposed to 3-morpholinosydnonimine hydrochloride (SIN-1, from Cayman Chemical) (35), which spontaneously releases both NO$^-$ and O$_2^{•−}$, both in the absence or in the presence of a NO$^*$ donor, and to a combination of a NO$^*$ donor and the quinone redox cycler 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), which catalytically generates intracellular O$_2^{•−}$ at the expense of cellular reducing agents (36). The SIN-1 experiments were performed in a conventional fluorimeter, as described above. For DMNQ, the procedure was essentially the same, but experiments were performed in a plate reader using DETA/NO (Z)-1-[N-(2-aminoethyl)-N-(2-aminooethyl)amino]diazene-1-ium-1,2-diolate) (DMNQ experiments) as a source of NO$^*$. As expected, the DAF2 nitrosylation rate increased in the presence of SIN-1 and DMNQ (Figure 4A and 4B) compared to that in the control. SIH clearly accelerated the DAF2 nitrosylation rate in the presence of these two compounds (Figure 4A and 4B). In particular, the rate of DAF2 nitrosylation stimulated by SIN-1 ± sper/NO was increased more than 10-fold in the presence of SIH compared to that in the controls excluding the chelator in the first 2 to 3 min. Later in the run, the DAF2-nitrosylation rate decreased when the LIP was removed by chelation, possibly due to DAF2 exhaustion.

**LIP removal enhances peroxynitrite-dependent oxidation of intracellular H$_2$DCF**

To test whether LIP chelation increases oxidation as well, the experiments described for DAF2 were repeated with cells previously loaded with H$_2$DCF-DA. These set of experiments were performed in 96 well plates. Cells were exposed to NO$^*$ or to peroxynitrite fluxes (DMNQ ± DETA/NO combination or SIN-1) with or without the iron chelators SIH or Bipy. The fluorescence
increases in experiments lacking DETA/NO and SIN-1 in the presence of SIH, Bipy, and Ebselen alone or in combination were not different from those of cell suspension alone, which shows again that SIH and the other chemicals used do not produce intracellular oxidants.

As expected, significant ebselen-preventable H$_2$DCF oxidation occurred in the presence of the NO$^*$ donor, NO$^*$ donor plus DMNQ (Figure 5A) and SIN-1 (Figure 5B). The H$_2$DCF oxidation rate increased in the presence of SIH (Figures 5A and 5B) or Bipy (Figure 5C) under all conditions tested, consistent with the working hypothesis. Ebselen again reduced the H$_2$DCF oxidation rate to control values in the presence of SIH, suggesting that H$_2$DCF oxidation occurring in the presence of the chelator depended on peroxynitrite. In cell-free experiments, SIH did not affect the H$_2$DCF oxidation induced by XO/X plus the NO$^*$ donor (Figure 2C), suggesting that the chelating effect on the probe oxidation depends on cellular substrate(s) that are reactive with metal chelators.

**Peroxynitrite-induced oxidation and nitroso(y)lation of intracellular indicators in iron-treated cells**

Some experiments were performed with LIP-enriched RAW 264.7 cells in another set of control runs. In this experiment, cells in tissue culture flasks were incubated with FeSO$_4$·7 H$_2$O (100 µM) for 1 hour and then rinsed extensively with the working buffer. After harvesting, cells were subsequently loaded with H$_2$DCF-DA or DAF2-DA as usual and plated in 96-well plates along with control cells. Control and iron-treated cell suspensions were exposed to NO$^*$ from DETA/NO or fluxes of peroxynitrite generated by the DMNQ and DETA/NO combination. The fluorescence of H$_2$DCF oxidation or DAF2 nitroso(y)lation reactions was measured as a function of the time in a plate reader, as described above. Iron enrichment decreased the oxidation of intracellular H$_2$DCF in RAW 264.7 cells under the two conditions tested, consistent with the protective effect of LIP on peroxynitrite (compare the open circle and filled square symbols in runs lacking SIH shown in Figures 6A and 6B). Remarkably, LIP removal by chelation with SIH normalized the rate of H$_2$DCF oxidation in control and iron-treated cells to essentially the same level (compare the open circles with filled squares in runs containing SIH symbols shown in Figure 6A and 6B). This result is expected if the behaviours of the control and iron-enriched cells are the same, with the exception of the different LIP concentrations, which SIH normalizes. The same experiments were performed with DAF2-loaded cells and produced overall similar results (Figure 6C and 6D).

**LIP removal accelerates intracellular nitrosative and oxidative processes mediated by activated macrophages**

Next, we tested whether chelators increased the extent of nitroso(y)lation and oxidation processes derived from endogenous NO$^*$. In this set of experiments, control or *Escherichia coli* 0111:B4 lipopolysaccharide (LPS)-challenged RAW 264.7 cells were loaded with either DAF2-DA or H$_2$DCF-DA as usual, but the culture included excess oxyhaemoglobin to scavenge NO$^*$ during the loading procedure to prevent a premature increase in fluorescence before the measurements were collected. Then, cells were plated on 96-well plates, and data acquisition was immediately initiated. Intracellular DAF2 nitroso(y)lation and H$_2$DCF oxidation were both restricted to LPS-activated RAW 264.7 cells and prevented by ebselen, regardless of the presence of SIH (Figure 7A and 7B). In LPS-challenged cells, the initial DAFT and H$_2$DCF fluorescence was higher in the presence of SIH, suggesting an acceleration of oxidation and nitroso(y)lation by the chelator before data acquisition was initiated due to endogenous NO$^*$ production. In Figure 8, the initial fluorescence was normalized to better show that the increase in fluorescence for both DAF2 nitroso(y)lation and H$_2$DCF oxidation processes was clearly accelerated in the presence of SIH.

**LIP removal enhances peroxynitrite-dependent protein carbonylation in macrophages**

Next, we tested whether SIH affects peroxynitrite-dependent protein oxidation. For this purpose, cells were treated with the NO$^*$ donor Sper/NO alone or in combination with the redox cycler DMNQ. The results show that the protein carbonylation content slightly increased in the presence of SIH in all cases (Figure 8).
Iron(II)/glutathione complexes react with peroxynitrite

One important aspect of the working hypothesis is that LIP reacts with peroxynitrite. We monitored peroxynitrite decomposition in a deoxygenated buffer (100 mM phosphate buffer) in the absence and presence of glutathione alone or iron(II)/glutathione (GSH) mixtures using UV-Vis spectroscopy to test this hypothesis. GSH and ferrous iron form a 2:1 stoichiometry complex, which is a likely cellular LIP complex (37-39). The decomposition of peroxynitrite in buffer alone followed first-order kinetics, with a rate constant of 0.19 ± 0.1 s⁻¹, a value that is close to the value expected using these experimental conditions (15, 40). The GSH thiol group itself directly reacts with peroxynitrite, with a rate constant of 6.6 x 10⁻⁶ M⁻¹s⁻¹ (41) (at pH 7.4 and 37°C). However, at the employed concentrations, GSH alone was much less efficient in accelerating peroxynitrite decomposition than the Fe(II)/GSH complex (Figure 9). The complete characterization of the Fe(II)/GSH and peroxynitrite reaction and its kinetic properties is beyond the scope of this study. Nevertheless, the results clearly show that likely LIP complexes directly react with peroxynitrite.

LIP removal attenuates hydrogen peroxide-induced intracellular H₂DCF oxidation.

Finally, we examined the different influences of LIP on hydrogen peroxide- and peroxynitrite-induced oxidation of H₂DCF. H₂DCF-loaded RAW 264.7 cells suspended in pre-warmed working buffer were portioned in a 96-well plate in the presence or absence of SIH or Bipy chelators. The experiment was initiated by the addition of H₂O₂ using a multi-channel pipet. Fluorescence was measured 30 minutes after the addition of H₂O₂ using a plate reader. As expected, H₂O₂ increased H₂DCF oxidation in a concentration-dependent manner, but, in contrast to the peroxynitrite experiments, both SIH and Bipy chelators attenuated this oxidation (Figure 10).

Discussion

In the present study, we extend the concept of LIP redox reactivity to show that the LIP may react with peroxynitrite. Although this reaction has been neglected to date, the hypothesis is somewhat expected because metals are among the preferential targets of peroxynitrite, and virtually all chemical species that react with H₂O₂ also react with peroxynitrite, typically at higher rate constants. For example, ebselen (29), boronate (24), general thiols (36), peroxiredoxin (32, 33) and glutathione peroxidases (34), and yeast cytochrome c peroxidase (42) all react with peroxynitrite faster than with H₂O₂.

Based on our findings, the LIP and peroxynitrite reaction is kinetically competitive with the cellular peroxynitrite targets cited above and sustainably attenuates the peroxynitrite-dependent oxidation and nitros(yl)ation of intracellular molecules under simulated normal and oxidative conditions, in stark contrast to H₂O₂-induced oxidation. We speculate that the ferrous LIP directly reduces peroxynitrite to NO₂⁻ (equation 10). The hypothetical oxoferryl product LIPFeIV=O shown in equation 10 may nonspecifically oxidize cellular constituents, which likely prevents this product from oxidizing the fluorescent indicators DAF2 and H₂DCF. Moreover, the oxoferryl species is probably less reactive than peroxynitrite downstream oxidants and may be neutralized by sacrificial reducing agents such as GSH and ascorbate. Consistent with these findings, cells loaded with ascorbate displayed dramatically decreased DAF2 nitroso(yl)ation (Supplementary Information, Figure S1, panel A).

\[ \text{LIPFe}^{2+} + \text{ONOO}^- \rightarrow \text{LIPFe}^{IV}=\text{O} + \text{NO}_2^- \] (10)

The hypothesis presented in equation 10 was actually inspired by the peroxynitrite redox reactions with divalent low molecular weight metal complexes (43, 44) and haemeproteins (42, 45-47). According to Radi (18) the antioxidant effects of Fe and Mn metalloporphyrin complexes on peroxynitrite depend precisely on the two-electron redox reaction of their divalent states (48, 49). Similarly, ferrous myeloperoxidase (45), deoxymyoglobin and deoxyhaemoglobin (46, 47) reduce peroxynitrite to nitrite rapidly (k \geq 10^6 M⁻¹s⁻¹). Thus, endogenous and exogenous Mn(II) and Fe(II) species have been shown to reduce peroxynitrite to nitrite with rate constants that are two orders of magnitude larger than the peroxynitrite reaction with CO₂ and presumably protect other biological targets from oxidation.
Rationally, equation 10 is the more likely explanation for our results and is consistent with preliminary results indicating that the Fe(II)/glutathione complexes react directly with peroxynitrite. However, we cannot currently exclude the possibility that the LIP also reduces downstream peroxynitrite oxidants (and NO\textsubscript{3}\textsuperscript{-} or CO\textsubscript{2}\textsuperscript{-}) to produce ferric LIP (LIPFe\textsuperscript{3+}) and the corresponding anion. These reactions would also be inhibited by LIP removal by chelators. Peroxynitrite-derived radicals rapidly react with Fe(II) compounds. For example, the model iron(II) small molecular weight complex hexacyanoferrate(II) reacts with NO\textsubscript{3}\textsuperscript{-} and CO\textsubscript{2}\textsuperscript{-} with high second-order rate constants (50-52) .

Another possible hypothesis is that peroxynitrite reacts with DNIC, which is formed by a rapid reaction of LIP with free nitric oxide. DNIC is formed in RAW 264.7 (5) cells, and an in vitro study has shown that diglutathione and dicysteine dinitrosyl iron complexes, as well as the bovine serum albumin protein, bound to DNIC react rapidly with peroxynitrite to produce nitrite anions (53).

Sequestration of LIP by chelators prevents DNIC formation in RAW 264.7 cells (5) and would prevent the DNIC/peroxynitrite reaction. These hypotheses are attractive since they all explain the finding that chelators increase peroxynitrite-dependent oxidative events in cells.

Clearly, the hypothesis apparently contradicts the generally accepted notion that the LIP is a cellular source of pro-oxidant iron. Similarly, according to the results of some cell-free (54) and cell-based studies (55) the binding of nitric oxide to iron complexes or LIP protects against oxidation through Fenton chemistry. However, this complete body of evidence revealing the pro-oxidative actions of the LIP is derived from studies using hydrogen peroxide. We only identified a few studies that used chelators under conditions where the oxidation or biological effects might have depended on peroxynitrite. As shown in the study by Sergent et al (56) iron supplementation attenuates lipid peroxidation in cultured rat hepatocytes exposed to LPS and γ-interferon (IFN), and the iron chelator deferiprone enhances this process. Fritsche et al (57) found that iron loading increases the survival of Plasmodium falciparum cells co-cultured with LPS stimulated-RAW 264.7 cells and that the iron chelator desferrioxamine decreases the survival.

Thus, LIP removal from macrophages and/or parasites increases damage to the parasite, perhaps by increasing peroxynitrite bioavailability. Similarly, Collins et al (58) observed that a desferrioxamine pretreatment exacerbates the experimental salmonellosis of LPS- and IFN-activated RAW 264.7 cells. The authors offered different plausible explanations, but their results are consistent with or at least do not refute the hypothesis that the LIP reacts with peroxynitrite or with its downstream oxidants. Interestingly, cytokines induce macrophages to scavenge iron in tissues during periods of inflammation. This action is thought to have the complementary roles of decreasing iron availability for invading microorganisms and avoiding the toxic effects of the Fenton reaction. Additionally, based on our results, macrophages may exhibit LIP-dependent increased resistance against peroxynitrite damage, which may represent a self-defense mechanism that helps macrophages sustain their functions during inflammation and other pathological conditions in which a long-lasting, macrophage-mediated immune response is important.

The consequences of the hypothetical reaction presented in equation 10 should be dissociated from those of the reactions of Fe\textsuperscript{3+} and Mn\textsuperscript{3+} metal complexes with peroxynitrite. These processes are one-electron reactions that increases the net yield of the oxidant species from peroxynitrite homolytic cleavage (43,59,60) and subsequently increase peroxynitrite-dependent oxidation and nitration. The very different outcomes of peroxynitrite reactions with ferrous and ferric species reveal the importance of the cellular and LIP redox status. We speculate that chronic conditions such as inflammation and iron overload may eventually overcome the capacity of cells to maintain the divalent status of the LIP, potentially increasing the ferric-LIP content and shifting the peroxynitrite and LIP reaction from an anti-oxidant to pro-oxidant effect. Therefore, our results are not in conflict with the well-known damage caused by iron homeostasis deregulation or iron overload conditions. However, an evaluation of the effects of chelator treatments only after a long exposure of cells to peroxynitrite may mask the initial protective effects of the LIP on this oxidant.

The effects of LIP oxidation on cellular iron homeostasis remain unclear. Does LIP...
oxidation decrease the LIP availability and activate IRPs? Experiments with H$_2$O$_2$ indicate that LIP oxidation produces these effects (61). In addition, cellular exposure to both NO$^*$ (61, 62) and peroxynitrite have been shown to activate mechanisms of iron homeostasis in the direction of iron acquisition. Although their effects have been linked to different mechanisms at the molecular level, based on the present results, we are tempted to hypothesize that peroxynitrite-dependent LIP oxidation reduces the availability of LIP, offering new insight into the links between LIP and nitric oxide/peroxynitrite in iron homeostasis.

We acknowledge that the evidence that ferrous LIP reacts with peroxynitrite is indirect. However, nitros(yl)ation and oxidation are well-known outcomes of peroxynitrite production, and both DAF2 nitros(yl)ation and H$_2$DCF oxidation were substantially or completely prevented by superoxide and peroxynitrite scavengers under the experimental conditions used in the present study. Additionally, ferrous/GSH complexes react directly with peroxynitrite. Thus, the simplest and most likely explanation for the observation that iron chelators enhance these processes is that the LIP reduces peroxynitrite and/or its downstream oxidants. Given the oxidative power of peroxynitrite itself and the indiscriminate reactivity of its downstream oxidants, LIP might also protect biological targets from peroxynitrite-induced oxidation, which was preliminarily observed for protein carbonylation.

Our finding changes the characterization of the LIP as an exclusively pro-oxidant species and emphasizes that generalization must be avoided when describing reactive radicals and oxidants. Our findings may provide additional insights into biological outcomes in situations where nitric oxide/peroxynitrite and LIP overlap.

Experimental Procedures

Chemicals

Unless specified otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and were of the highest purity available. SIH was synthesized by Schiff base condensation between 2-hydroxybenzaldehyde and isonicotinic acid hydrazide, as previously described (63). The peroxynitrite stock solution (> 25 mM) was synthesized, purified and stored frozen at -80°C, as described elsewhere (16, 64, 65). For the experiments, the peroxynitrite stock solution was incubated with MnO$_2$ to remove H$_2$O$_2$, centrifuged and kept in 1 M NaOH in an ice bath and protected from light. The peroxynitrite concentration was determined by determining the absorbance at 302 nm ($\lambda_{302} =$ 1670 L M$^{-1}$ cm$^{-1}$) (66) using UV-Vis spectroscopy. MnTE-2-PyP was a generous gift from Dr. Rebecca E. Oberley Deegan (Department of Medicine, National Jewish Health, Denver, CO, USA). Nitric oxide donors were purchased from Cayman Chemical Co (Ann Arbor, Michigan, USA).

Cell culture and treatment

RAW 264.7 cells (ATCC) were incubated and cultured at 37°C in Dulbecco’s Modified Eagle’s Medium supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin and 10% foetal bovine serum (FBS), as described previously (5). Cells were passaged, seeded onto 75 cm$^2$ T-flask culture dishes and grown overnight to reach 85 to 90% confluence. Then, cells were double-washed with PBS, harvested and centrifuged at 400 g for 5 min at 4°C. Finally, the cells were suspended in 10 mL of complete medium and incubated on an ice. The cell viability was within 85 to 95%, according to the results of the trypan blue exclusion assay conducted before and after some experiments.

For the specific set of experiments using endogenous NO$^*$ (Figure 7), the LPS challenged RAW 264.7 cells were prepared as described elsewhere (22). Briefly, cells were split, seeded onto T75 culture flasks and grown overnight to reach 85–90% confluence in the absence or presence of 1 µg/mL (LPS). Then, cells were harvested and treated with either DAF2-DA or H$_2$DCF in presence of HbO$_2$ to scavenge endogenous NO$^*$ being produced and to prevent substantive fluorescence development during the DAF2-DA and H$_2$DCF-DA loading procedure. A control experiment showed that inclusion of HbO$_2$ during the loading step substantially decreased fluorescence development before data acquisition was initiated.

Fluorescence experiments
DAF2-DA and H₂DCF-DA permeate biological membranes, but because their ester bonds are cleaved by non-specific esterases, the respective negatively charged products diaminofluorescein (DAF-2) and 2',7'-dichlorofluorescein (H₂DCF) no longer permeate the cell membrane and accumulate inside cells at high concentrations (21). Generally, the DAF2-DA and H₂DCF-DA loading procedures and experimental fluorescence assays were performed using previously reported methods (22). A suspension of RAW 264.7 cells in PBS/DTPA was loaded with 10 µM DAF2-DA or 30 µM H₂DCF for 30 min at 37°C under constant stirring. A total of 1.0 x 10⁶ cells was systematically used in this procedure to minimize differences in the loading and intracellular concentrations of the fluorescent indicators during the experiments. We were careful to avoid the presence of extracellular DAF2 and H₂DCF. Therefore, cells were subjected to two cycles of centrifugation and resuspension after treatment. In addition, immediately prior to the assays (22), cells were centrifuged again and suspended in pre-warmed working buffer. Then, suspended cells were placed in a fluorimeter cell under constant stirring for the conventional fluorimeter experiments (6 x 10⁶ cells in 2 mL) or placed in 96-well plates for the plate reader experiments (3 x 10⁶ cells in 250 µL). DAF2 or DCF fluorescence was measured at the time intervals designated in the figures.

DAF2 experiments were performed in open vessels in the conventional fluorimeter, as the results were virtually the same as those obtained in closed vessels with no headspace. Since the fluorescence response of the NO• donor depends on the intracellular DAF2 concentration, a control experiment using 15 µM sper/NO was conducted routinely before and between conventional fluorimeter experiments to minimize complications resulting from faulty DAF2 loading or leakage. Cells were discarded if the rate of DAF2 nitrosylation in this control varied beyond the standard deviation of previous data using the same concentration of the NO donor that have been accumulated, which only occurred occasionally. All these experiments were performed in a Shimadzu RF-5301pc spectrofluorimeter instrument. The temperature was controlled by a circulating water bath and maintained at 37°C. The experimental conditions and fluorescence acquisition parameter settings for DAF2 were as follows: T = 37°C, PBS, 100 µM DTPA, 100 U/mL Cu,Zn-SOD, pH 7.4, λex = 495 nm, λem = 520 nm, and ex and em slit widths = 5 nm.

The fluorescence measurements using multwell plates were performed in the Molecular Device SpectraMax M3 instrument with a total of 3 x 10⁶ cells per well. The NO• source in most of these experiments was DETA/NO (which has a half-life of 20 hours at 37°C and pH 7.4). (67) All cell treatments, experimental conditions and fluorescence acquisition parameter settings for DAF2 were the same as those described above. For H₂DCF experiments, the following fluorescence acquisition parameter settings were used: λex = 498 nm, λem = 523 nm, and ex and em slit widths = 9 nm and 15 nm, respectively.

For both the conventional or plate reader fluorescence measurements, some chemicals were added individually or in combination to the working buffer prior to the experiments, as designated in the figures, to investigate specific hypotheses as described throughout the text. SIH, Bipy, DMNQ, SIN-1, ebalsen, APBA, DAF2-DA and H₂DCF-DA stock solutions were produced in DMSO, whereas stock solutions of sper/NO and DETA/NO were generated in 0.01 M NaOH. Stock solutions of FeSO₄·7 H₂O were generated in 0.01 HCl and stock solutions of H₂O₂ were produced in the working buffer. Small volumes of these chemicals were added to the cell suspension in working buffer using automatic pipettes immediately prior to the experiments. The DMSO, 0.01 HCl and 0.01 M NaOH vehicles were added as controls in some experiments and had no effect on fluorescence.

Effects of an iron chelator on DAF2- and H₂DCF-stimulated nitrosylation and oxidation by XO/X in a cell-free system.

First, the flux of O₂•− by XO/X in PBS was determined using the standard method by monitoring the reduction of ferric cytochrome c at 25°C (68). Then, 1 µM DAF2 or H₂DCF was exposed to a flux of NO• (69) or to a combination of fluxes of NO• and O₂•− for at least 10 min in PBS containing 100 µM X in the presence or absence of 1000 U/mL Cu,Zn-SOD and the chelator SIH. Experimental conditions and fluorescence acquisition parameter settings were:
T = 37°C, PBS, 100 µM DTPA, pH 7.4, λex = 495 nm, λem = 520 nm, slit width = 5 nm.

**Kinetic analysis**

The rates of intracellular DAF2 nitrosylation and H₂DCF oxidation were determined from the slope of the fluorescence within the period of steady-state conditions of NO⁻, towards the end of the runs, which coincides with a period of a linear increase in fluorescence.

**Preparation of HbO₂**

A solution of 1 to 2 mM methaemoglobin in PBS (pH 7.4) was reduced with a large excess of sodium dithionite and purified size exclusion chromatography using Sephadex G-25 columns. The concentrated fractions were collected, flash-frozen in liquid nitrogen and stored at -80°C. The HbO₂ concentration was determined by monitoring the absorbance at 576 nm (ε = 14.6 mM⁻¹ cm⁻¹) (70) using a Shimadzu UV-1800 spectrophotometer.

**Analysis of the protein carbonyl content**

Cells (50 x 10⁶ /mL) were treated with sper/NO (1 mM) alone or in combination with the redox cycler DMNQ (20 µM) in the absence or presence of SIH (100 µM) for 60 min at 37°C. Then, cells were separated from the media by centrifugation and washed. The cell pellets were stored at -80°C until further analysis. Cell pellets were thawed, treated with 300 µL of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1 mM DTPA and protease inhibitors) and incubated on ice for 20 min. Lysates were centrifuged at 15,000 g for 10 min, the supernatants were collected, and their protein contents were determined by the Bradford method using a Bio-Rad Kit. An analysis of the protein carbonyl content was performed using the ELISA developed by Buss et al (71) and modified by Alamdari et al (72) with the following minor alterations. Triplicate blanks and solutions containing diluted standards or samples (5 µg protein/mL) were treated with the same solution used for protein derivatization, except that it lacked 2,4-dinitrophenylhydrazine (DNPH). The primary antibody (rabbit anti-DNPH, Sigma) was diluted 1/5,000 and the secondary antibody (goat anti-rabbit IgG-peroxidase conjugated, KPL) was diluted 1/500 with PBS containing 1% skim milk and 0.05% Tween 20. The substrate solution contained 420 µM 3,3',5,5'-tetramethylbenzidine (TMB) and 2 mM H₂O₂ in phosphate (50 mM):citrate (25 mM) buffer, pH 5.0. Colour development from the enzyme-substrate reaction was stopped after 5 min by the addition of 50 µL of 2 M H₂SO₄. The absorbance was read at 450 nm using a Tecan Infinite M200 microplate reader. The average absorbance of blank triplicates was subtracted from the absorbance values of the corresponding DNPH-derivatized samples or standards. The carbonyl concentration (nmol/mg protein) was calculated from the standard curve, and data are presented as the means ± standard deviations (S.D.) of three independent experiments. Statistical significance was determined using one-way ANOVA.

**Kinetics of peroxynitrite decomposition.**

Peroxynitrite decomposition was monitored by recording the absorbance at 302 nm in Shimadzu UV-1800 spectrophotometer coupled to a RX2000 rapid kinetics spectrophotometer accessory from Applied Photophysics using an asymmetric mixing procedure (2.0/0.2 mL). The peroxynitrite solution was always placed in the smaller syringe to minimize pH changes. All stock solutions were deoxygenated with a purge of argon gas before the experiments. The ferrous solution was prepared by transferring a deoxygenated acid solution (0.01 M HCl) through a Tygon tube directly to a sealed and previously deoxygenated screw cap vial containing solid FeSO₄·7 H₂O. Then, a small volume of the latter solution was transferred to a GSH solution that had been previously deoxygenated to prepare the Fe(II)/GSH complex stock solution for the experiment. The temperature was maintained within 0.2°C and the pH was measured at the outlet to monitor increases caused by the alkaline peroxynitrite solution (0.1 M NaOH).

**Statistical analysis**

All measurements are presented as the means ± S.D. of n ≥ 3 experiments. Means were compared between groups using an F test followed by a paired Student’s t test or one-way ANOVA. P values < 0.05 were considered to indicate statistical significance.

**Conflict of Interest**
The authors have no conflict of interests with the content of this article to declare.
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The abbreviations used in the present study are: APBA, 4-acetylphenylboronic acid, pinacol ester; Asc, ascorbate; Bipy, 2,2'-bipyridine; CBA, coumarin-7-boronic acid; DAF2, 4,5-diaminofluorescein; DAF2-DA 4,5-diaminofluorescein diacetate; DAFT, diaminofluorescein triazole; DCF, 2',7'-dichlorofluorescein; H$_2$DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate; DHE, dihydroethidine; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; DNIC, dinitrosyl iron complex; DTPA, diethylenetriaminepentaacetic acid; Ebs, ebselen; FCN, sodium hexacyanoferrate (II); HbO$_2$, oxyhaemoglobin; LIP, labile iron pool; LPS, *Escherichia coli* 0111:B4 lipopolysaccharide; MnTE-2-PYP, Mn(III) meso-tetrakis(N-ethylpyridinium-2-yl) porphyrin; PEG-SOD, bovine pegylated Cu,Zn-superoxide dismutase; Paraquat, 1,1'-dimethyl-4,4'-bipyridinium dichloride; proli/NO, disodium 1-[(2-carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate; SIH, salicylaldehyde isonicotinoyl hydrazone; SIN-1, 3-morpholinosydnonimine hydrochloride; SOD, bovine Cu,Zn-superoxide dismutase; sper/NO (z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]diazen-1-ium1,2-diolate; UA, uric acid; X, xanthine; and XO, xanthine oxidase.
Figure 1 – LIP removal by chelation accelerates intracellular DAF2 nitros(yl)ation. RAW 264.7 cells were loaded with 10 μM DAF2-DA as described in the experimental section. Then, 3 x 10^6 RAW 264.7 cells / mL were suspended in 2 mL of the working buffer in a fluorescence cuvette under constant stirring and placed in a conventional fluorimeter. When required, SIH (100 μM) or Bipy (50 μM) were added to the cell suspension just before initiation of data acquisition. The NO• donor sper/NO (2 and 15μM) was added partway through the experiments with the help of a syringe as designated in the panels A and D by the arrows. The vehicles DMSO (SIH and Bipy) and 0.01 M NaOH (sper/NO) used had no effect on fluorescence or pH when added to the working buffer as controls. A) Fluorescence traces of DAF2 nitros(yl)ation in the presence and absence of the iron chelator SIH (100μM); each trace is representative of at least three experiments. B) Rate of intracellular DAF2 nitros(yl)ation in the presence and in the absence of SIH (100 μM). The rate was calculated using the slope of the fluorescence data within the final minute of the run, a period of steady-state NO• and linear fluorescence increase. The SIH acceleration effect for each Sper/NO concentration used is statistically significant at the 95% confidence interval versus the respective control (p < 0.05; Paired Student t test)*. C) Rate of intracellular DAF2 nitros(yl)ation as a function of SIH concentration. The data represent the mean of three independent experiments ± S.D. D) Representative fluorescence traces of DAF2 nitros(yl)ation in the presence and in the absence of the iron chelator Bipy (50 μM). The experimental conditions and the fluorescence acquisition parameters were as follows: working buffer (PBS + DTPA 100 μM + 100 U/mL Cu,Zn-SOD, pH 7.4, T = 37°C; λex = 495 nm, λem = 520 nm, ex and em slit width = 5 nm.
**FIGURE 2**

**A** Free SIH does not inhibit peroxynitrite-dependent DAF2 nitrosylation in a cell-free system. DAF2 (1 µM) was mixed with sper/NO (100 µM) in PBS supplemented with a sufficient amount of XO/X to generate a flux of 10 nM/s of O$_2$•$^-$ in the absence and in the presence of SIH (100 µM) with or without SOD (1000 U/mL). The flux of O$_2$•$^-$ was first determined by the reduction of ferric cytochrome c assay under identical conditions.

**B** Rate of peroxynitrite dependent DAF2 nitrosylation under different conditions. Data represent the mean of three independent experiments ± S.D. The fluorescence acquisition parameter settings were as described in Figure 1.

**C** Rate of peroxynitrite dependent H$_2$DCF oxidation in cell-free system under different conditions. H$_2$DCF (1 µM); (sper/NO, XO/X and SIH as described in A). The fluorescence acquisition parameter settings were as described in the experimental section.

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**Figure 2** – Effects of SIH on peroxynitrite-dependent DAF2 nitrosylation and H$_2$DCF oxidation in a cell-free system. **A** Free SIH does not inhibit peroxynitrite-dependent DAF2 nitrosylation in a cell-free system. DAF2 (1 µM) was mixed with sper/NO (100 µM) in PBS supplemented with a sufficient amount of XO/X to generate a flux of 10 nM/s of O$_2$•$^-$ in the absence and in the presence of SIH (100 µM) with or without SOD (1000 U/mL). The flux of O$_2$•$^-$ was first determined by the reduction of ferric cytochrome c assay under identical conditions. **B** Rate of peroxynitrite dependent DAF2 nitrosylation under different conditions. Data represent the mean of three independent experiments ± S.D. The fluorescence acquisition parameter settings were as described in Figure 1. **C** Rate of peroxynitrite dependent H$_2$DCF oxidation in cell-free system under different conditions. H$_2$DCF (1 µM); (sper/NO, XO/X and SIH as described in A). The fluorescence acquisition parameter settings were as described in the experimental section.
**FIGURE 3**

**Figure 3** – Effects of peroxynitrite scavengers on the rate of intracellular DAF2 nitrosylation. Cells were treated as described in Figure 1 legend. SIH (100 μM), Ebselen (10 μM) and APBA (concentrations as designated in the Figure) and sper/NO (15 μM) were added to the cell suspension just before initiation of data acquisition. **A)** Ebselen inhibits intracellular DAF2 nitrosylation in the presence and in the absence of SIH. **B)** Effect of increasing concentrations of APBA on intracellular DAF2 nitrosylation in the presence and in the absence of SIH. **C)** APBA decreases the SIH acceleration ratio of DAF2 nitrosylation. The SIH-acceleration ratio was defined by the quotient of DAF2 nitrosylation rate in the presence and in the absence of SIH. Statistics (Paired Student t test): The data represent the mean of three independent experiments ± S.D. and were considered statistically significant at the 95% confidence interval (p < 0.05)* versus the respective control for ± Ebselen and for the paired comparisons designated in the figure. The fluorescence acquisition parameter settings were as described in Figure 1.
FIGURE 4

Figure 4 – SIH accelerates the intracellular DAF2 nitrosylation induced by peroxynitrite fluxes. A) SIH increases the rate of intracellular DAF2 nitrosylation induced by SIN-1 alone or in combination with sper/NO. The cell treatment were as described in Figure 1. SIH (100 µM) was added to the cell suspension just before initiation of data acquisition when required as designated in the Figure. Sper/NO (15 µM) or SIN-1 (300 µM) was added partway through the experimental run using a syringe as designated in the figure by the arrow. The traces are representative runs of at least three experiments. B) SIH increases the rate of intracellular DAF2 nitrosylation induced by the combination of DMNQ and the NO• donor DETA/NO. This NO• donor was necessary to follow fluorescence for longer periods without dramatic drop on NO• concentrations. This set of experiments was performed in a plate reader. Shortly, 3 x 10⁶ cells previously loaded with DAF2-DA as described in the experimental section were placed in 96 well plates in a final volume of 250 µL. SIH (100 µM), DMNQ (20 µM) and DETA/NO (1 mM) were added to selected plate wells just before initiation of data acquisition. Experimental conditions and fluorescence acquisition parameter settings were as follows: pH 7.4, T = 37°C, λex = 498 nm, λem = 523 nm, ex. and em. slit width = 9 nm and 15 nm, respectively. Statistics (Paired Student t test): the data represent the mean of at least three independent experiments ± S.D. and were considered statistically significant at the 95% confidence interval (p < 0.05)*.
**Figure 5** – The rate of peroxynitrite dependent oxidation of intracellular H$_2$DCF. RAW 264.7 cells were loaded with 30µM H$_2$DCF-DA as described in the experimental section. Then, 3 x 10$^6$ cells were placed in 96 well plates as described in the figure 4B. SIH (100 µM) or Bipy (500 µM), DMNQ (20 µM), SIN-1 (250 µM), and DETA/NO (1mM) were added to selected wells just before initiation of the data acquisition. 

**A)** Rate of intracellular H$_2$DCF oxidation by DETA/NO with or without DMNQ in the presence and in the absence of SIH. 

**B)** Rate of intracellular H$_2$DCF oxidation by SIN-1 in the presence and in the absence of SIH. 

**C)** Rate of intracellular H$_2$DCF oxidation by DETA/NO in the presence and in the absence of SIH or Bipy. 

The conditions and the fluorescence acquisition parameter settings were: pH 7.4, T = 37°C, λ$_{ex}$ = 498 nm, λ$_{em}$ = 523 nm, ex and em slit width = 9 nm and 15 nm, respectively. Statistics (Paired Student t test): The data represent the mean of at least four independent experiments ± S.D. and were considered statistically significant at the 95% confidence interval* (p < 0.05).
Figure 6 – Traces of DAF2 nitrosylation and H$_2$DCF oxidation in RAW 264.7 cells previously treated with iron. RAW 264.7 cells treated with 100 μM FeSO$_4$.7H$_2$O for one hour in full medium while adhered to 75-cm$^2$ culture T-flasks. Then, cells were extensively rinsed with the DTPA containing working buffer to remove extracellular iron and harvested as described in the experimental section. Subsequently, control and iron treated cells were loaded with 30 μM H$_2$DCF-DA or 10 μM DAF2-DA as usual and placed in 96 well plates (3 x 10$^6$ cell / well). SIH (100 μM) and DMNQ (20 μM) were added to selected wells as required. The experiment was initiated by addition of DETA/NO (1 mM) and fluorescence was measured every minute for at least one hour. A) Traces of intracellular DCF fluorescence in cells exposed to DETA/NO$^-$. B) Traces of DCF fluorescence in cells exposed to the combination of DMNQ and DETA/NO. C) Traces of DAFT fluorescence in cells exposed to DETA/NO$^-$. D) Traces of DAFT fluorescence in cells exposed to the combination of DMNQ and DETA/NO. Labels: (○) control cells; (●) iron treated cells. Conditions and Fluorescence acquisition parameter settings as described in Figure 4 and in figure 5 for DAF2 and H$_2$DCF experiments, respectively.
**Figure 7**

- **A)** Traces of intracellular DAF fluorescence in the presence and absence of SIH.
- **B)** Traces of intracellular DCF fluorescence in the presence and absence of SIH.

The control and LPS activated RAW 264.7 cells were treated with either DAF2-DA or H$_2$DCF in the presence of HbO$_2$ to scavenge endogenous NO$^\cdot$ and prevent premature fluorescence increase before the acquisition of data was initiated. Then cells were portioned into 96 well plates. The data acquisition was initiated immediately and collected every minute for at least 30 minutes. The initial fluorescence for both DAF and DCF was normalized to start at the same level for control and LPS treated cells. The data represent the mean of 4 independent experiments ± S.D. The conditions and fluorescence acquisition parameter settings were as described in the figure 4 and the figure 5 for DAF2 and H$_2$DCF experiments, respectively.
**FIGURE 8**

**Figure 8** – Content of protein carbonylation in cells treated with DMNQ and a NO• donor. For this set of experiments, 50 x 10⁶ cells / mL were challenged with the combinations of NO’ (Sper/NO 1 mM) and the redox cycler DMNQ (20 µM) for 60 minutes under constant stirring. The sample manipulation and carbonyl content analysis were as described in the experimental section. Statistics (ANOVA): Data represent the mean of 9 independent experiments ± S.D. and are statistically significant at the 95% confidence interval (p < 0.05)*.
**FIGURE 9**

![Graph of Figure 9](image-url)

**Figure 9**—Traces of peroxynitrite decomposition. Peroxynitrite (200 μM) was mixed with phosphate buffer (100 mM, pH 7.4, T = 25°C) alone (---) or in the presence of 0.66 mM GSH (----) or Fe(II) (0.25 mM) plus 0.66 mM GSH (-----). Traces represent averages of at least four experimental runs. The rate constant of the decomposition of peroxynitrite in the phosphate buffer was determined by non-linear least-square fitting of the data to a single first-order exponential equation using the OriginPro 8.5 software. The rate constant was measured as being 0.19 ± 0.10 s\(^{-1}\).
FIGURE 10

**Figure 10** - Oxidation of intracellular H$_2$DCF by hydrogen peroxide. RAW 264.7 cells were previously loaded with 30μM H$_2$DCF-DA as described in the experimental section. Then, 3 x 10$^6$ cells were placed in a 96 well plates, and SIH (100 μM) or Bipy (500 μM) and hydrogen peroxide were added to selected wells. DCF fluorescence was measured after 30 minutes. The conditions and the fluorescence acquisition parameter settings were as described in Figure 5. Statistics (Paired Student t test): The data represent the mean of at three independent experiments ± S.D. and are statistically significant at the 95% confidence interval* (p < 0.05).
The labile iron pool attenuates peroxynitrite-dependent damage and can no longer be considered solely a pro-oxidative cellular iron source

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