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Dinitrosyliron complexes are the most abundant nitric oxide-derived cellular adduct: Biological parameters of assembly and disappearance

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Abstract

It is well established that nitric oxide ($\bullet$ NO) reacts with cellular iron and thiols to form dinitrosyliron complexes (DNIC). Little is known, however, regarding their formation and biological fate. Our quantitative measurements reveal that cellular concentrations of DNIC are proportionally the largest of all $\bullet$ NO-derived adducts (900 pmol/mg protein (45–90 μ M)). Using murine macrophages (RAW 264.7), we measured the amounts, and kinetics of, DNIC assembly and disappearance from endogenous and exogenous sources of $\bullet$ NO in relation to iron and O₂ concentrations. Amounts of DNIC were equal to or greater than measured amounts of chelatable iron and depended on the dose and duration of $\bullet$ NO exposure. DNIC formation paralleled the upregulation of iNOS and occurred at low physiologic $\bullet$ NO concentrations (50–500 nM). Decreasing the O₂ concentration reduced the rate of enzymatic $\bullet$ NO synthesis without affecting the amount of DNIC formed. Temporal measurements revealed that DNIC disappeared in an oxygen-independent manner ($t_{1/2}$ = 80 min) and remained detectable long after the $\bullet$ NO source was removed (>24 h). These results demonstrate that DNIC will be formed under all cellular settings of $\bullet$ NO production and that the contribution of DNIC to the multitude of observed effects of $\bullet$ NO must always be considered.

Keywords

Dinitrosyliron Complexes; Nitric oxide; Chelatable iron pool; S-nitrosothiols

INTRODUCTION

Nitric oxide ($\bullet$ NO, nitrogen monoxide) is an endogenously produced diatomic free radical and biological signaling molecule [1]. Its unique physical and chemical properties dictate

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that under biological conditions it only reacts with a minority of chemical species, i.e., other radicals and transition metals [2]. Of these biological targets, one of the potentially most significant and least studied is the chelatable iron pool (CIP) [3]. This small methodologically defined population of redox-active iron is associated with a diverse population of both high and low molecular weight cytosolic ligands. It has recently been demonstrated that when cells are exposed to exogenous •NO, the CIP is quantitatively converted into paramagnetic dinitrosyliron complexes with thiol-containing ligands (DNIC) [4]. Although little is known biologically about DNIC, they were detected in living systems as early as 1964, long before the discovery of endogenous •NO synthesis in humans [5–7]. Since then, a wealth of excellent data has accumulated that describe the chemistry of DNIC assembly and degradation under synthetic laboratory conditions [8–10]. *In vitro* studies have demonstrated that DNIC can be generated in cells cocultured with activated macrophages [11]. To date, however, parameters of DNIC metabolism under conditions of endogenous •NO production have never been characterized.

DNIC possess •NO-mimetic properties with regard to guanylyl cyclase activity and phenotypic responses [12, 13]. However, unlike *S*-nitrosothiols, iron-nitrosyls, 3-nitrotyrosine and other bioactive •NO-derived cellular adducts, the fate of DNIC in cells is not known. Our data indicate that quantitatively, they represent the largest intracellular pool of •NO-derived cellular adducts and are more physiologically important than previously realized. These studies are the first to quantify the formation and disappearance of DNIC from endogenously produced •NO (LPS-stimulated RAW 264.7 cells). We measured the magnitude and rates of cellular DNIC formation and disappearance as a function of oxygen concentration, onset and rates of •NO production, •NO concentration, and iron availability. We noted that changes in O₂ concentration had much less of an effect on DNIC formation and degradation than it had on the overall magnitude of •NO synthesis or •NO degradation. This implies that the effect of •NO on iron homeostasis will be significant under a diverse set of cellular conditions and persist long after enzymatic •NO synthesis has ceased. We conclude that the CIP is, therefore, a primary and immediate cellular target of •NO in all •NO-producing cells and DNIC will be formed under wide-ranging cellular conditions. Physiological and pathological implications of these findings are discussed.

EXPERIMENTAL PROCEDURES

Chemicals

Sper/NO and DETA/NO were generous gifts. •NO gas was purchased from Scott Gas. All cell culture reagents were purchased from Invitrogen with the exception of the Arginine (R) free DMEM media (AthenaES). All other reagents were purchased from Sigma.

Cell Culture

All experiments were performed on RAW 264.7 macrophages grown in DMEM supplemented with 10% FBS and 1% Pen/Strep. For DNIC measurements, cells were plated at 15×10^6 cells per 15 cm culture dishes. To upregulate iNOS, cells were treated with LPS (1 µg/mL). For hypoxic experiments (O₂ < 21%), cells were cultured in a hypoxic chamber glove-box (Coy Scientific) and maintained at 37°C, 5% CO₂. Ambient oxygen concentration (%O₂) was continuously monitored and adjusted (+/–0.2% O₂) by purging with nitrogen gas.

Cell viability

RAW 264.7 viability was monitored by the reduction of alamarBlue™ to its fluorescence product ($\lambda_{\text{Ex/Em}} = 550/595$) [14] after 24 hours.

Western blots

Protein was isolated with CellLytic (Sigma) containing 1 mM phenylmethylsulfonyl fluoride and 10 μ L/mL Protease Inhibitor Cocktail Set III (Calbiochem). Lysates were centrifuged at 12,000 $\times$ g for 15 min. After electrophoresis, proteins were transferred to PVDF membranes and blocked with 5% nonfat dry milk in TGST buffer (Tris-Glycine-SDS containing 0.1% Tween 20) for 1 h at room temperature. Membranes were incubated overnight at 4 °C with 1:500 iNOS antibody (Santa Cruz Biotechnology). Blots were incubated for 1 h with 1:2,000 horseradish peroxidase-conjugated secondary anti-rabbit antibody (Cell Signaling Technology, Beverly, MA). Transferred proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and Images were captured with Flourchem HD2 chemimager (Alpha Innotech).

3-nitrotyrosine

3-nitrotyrosine was quantified using an OxiSelect™ Nitrotyrosine ELISA Kit according to manufacturer protocols. Treated samples from 15 cm culture dishes were harvested with CellLytic (Sigma) lysis solution containing 1mM PMSF and 10 μ L/mL Protease Inhibitor Cocktail III (calbiochem). Protein concentrations were determined by Bradford assay, and 50 μ L of 1.5 μ g/ μ L protein were added to each well (n=3).

Preparation of an •NO-saturated solution

•NO gas was scrubbed of higher nitrogen oxides by passage through NaOH pellets followed by 1 M deaerated (bubbled with 100% argon) KOH solution. The purified •NO was collected by saturating a deaerated phosphate buffer solution (0.1 M potassium phosphate, pH 7.4). The saturated •NO solution was assayed by •NO electrode and chemiluminescence to ensure no significant contamination with higher nitrogen oxides and to determine the •NO concentration.

Cellular •NO metabolism

RAW 264.7 cells were trypsinized and put into a reaction chamber at 6×10^6 cells/mL of serum-free media. Cell suspensions were constantly stirred in a sealed, water-jacketed, temperature-controlled (37°C) chamber. The reaction chamber was equipped with both •NO and O₂ electrodes connected to an Apollo 4000 Free Radical Analyzer (WPI, Sarasota, FL). Headspace in the vessel was negligible compared to the vessel volume to assure that the rate of •NO volatilization was insignificant compared with its reaction in solution. Reactions were initiated by injection of a saturated •NO solution with a gas-tight syringe, and •NO metabolism was measured by using an •NO-specific electrode (amino-700, response time of < 0.2 sec, sensitivity of 25 nM, Innovative Instruments, Tampa, FL). Upon disappearance of the •NO, the cells were immediately removed and processed for EPR analysis.

Real-time •NO measurements

From LPS stimulated RAW 264.7 cell: cells in 15 cm plates at 21% or 1% O₂ were treated with 1 μ g/mL LPS. After 12 hours the cells were washed (3 $\times$ PBS) and the media was replaced with arginine-free media. An •NO-electrode was then positioned using a stereotactic apparatus ~1mm from the monolayer and allowed to equilibrate for 2 hours. •NO synthesis was initiated by addition of 1 mM Arginine to the media. From NONOate treated cells: •NO electrode was positioned ~1mm from the monolayer and allowed to equilibrate for 2 hours followed by addition of NONOates (n=3).

Nitrate, nitrite, and S-nitrosothiol measurements

NO₃⁻, NO₂⁻, and RSNO were measured by chemiluminescence (Sievers Nitric Oxide Analyzer, NOA 280i) according to the manufacturer's protocols and as previously

described. Briefly, aliquots of media from treated cells and the supernatant from lysed cells were injected into the reaction chamber containing either HCl/I_3^- for nitrite or vanadium chloride HCl to measure $\text{NO}_3^-/\text{NO}_2^-$. RSNO concentrations were measured by the Tri-iodide method [15].

EPR Determination of DNIC and CIP

DNIC quantification: Cells were trypsinized and resuspended in equal volumes of PBS. DNIC signals were measured by EPR at $g = 2.04$ using a Varian E109 spectrometer equipped with a TM 102 cavity at 9.41 GHz (X-band), fitted with a liquid nitrogen dewar (at 77°K) with the following settings: microwave power = 2mW, modulation amplitude = 20 G and time constant = 1 sec. A total of 4 scans were obtained for each spectrum. DNIC concentrations were estimated by comparing the signal to that of a known standard of nitrosylmyoglobin ($\bullet\text{NO-myoglobin}$) [4]. For quantitative studies a microwave power saturation profile for EPR signal amplitudes was conducted to ensure we were operating below the saturation. Protein samples were collected from each experiment so that DNIC could be quantitatively compared between individual runs (pmol DNIC/mg protein). For CIP quantification cells were treated with 1mM desferrioxamine (DFO) for 4 h and then harvested for EPR analysis. The concentration of the CIP was calculated by comparing the signal to that of known concentrations of a DFO-Fe(III) standard prepared as previously described [16]. EPR settings: $g = 4.3$, microwave power = 2 mW, modulation amplitude = 25 G, time constant = 1 sec. A total of 16 scans were obtained for each spectrum.

$\bullet\text{NO-myoglobin}$ standard was prepared as follows: met-myoglobin was suspended in PBS and concentration was determined by absorbance at $\lambda=409.5\text{nm}$ ($\epsilon=186\text{ mM}^{-1}\text{cm}^{-1}$). Sample was then reduced with sodium dithionite to form ferrous deoxymyoglobin ($\lambda=435$, $\epsilon=121\text{ mM}^{-1}\text{cm}^{-1}$). Nitric oxide ($\bullet\text{NO}$ saturated solution) was then added in excess and absorbance of the resulting $\bullet\text{NO-myoglobin}$ was measured at $\lambda=421$ ($\epsilon=147\text{ mM}^{-1}\text{cm}^{-1}$).

RESULTS

DNIC are formed in $\bullet\text{NO}$ -producing macrophages

We induced $\bullet\text{NO}$ production in RAW 264.7 macrophages cultured at 1% and 21% O_2 by stimulation with LPS ($1\mu\text{g/mL}$). The accumulation of $\bullet\text{NO}$ -metabolites ($\text{NO}_2^-/\text{NO}_3^-$) in the media were then measured over 24 h (Fig. 1A). The rates of $\bullet\text{NO}$ synthesis increased throughout the course of the experiment as measured by $\bullet\text{NO}_2^-/\text{NO}_3^-$ accumulation. However, due to the requirement of O_2 for enzymatic $\bullet\text{NO}$ synthesis, there was a decrease in the overall magnitude of $\text{NO}_2^-/\text{NO}_3^-$ (nmols) generated at 1 vs. 21 % O_2 , similar to what others have reported [17, 18]. Changes in iNOS expression over time at 21% O_2 and as a function of % O_2 concentration were also measured (Fig. 1B & D). This agrees with previous reports that have shown that murine iNOS contains a Hypoxia Response Element (HRE) in its promoter, and that its expression is synergistically up regulated by $\text{IFN}\gamma$ and hypoxia in ANA1 macrophages [18, 19]. Nevertheless, steady-state $\bullet\text{NO}$ concentrations do not necessarily correlate to either the amount of $\text{NO}_2^-/\text{NO}_3^-$ accumulated or the amount of iNOS expressed. Thus we used electrochemical detection to measure, in real-time, steady-state $\bullet\text{NO}$ concentrations in the media (Fig. 1C). Interestingly, despite a greater amount of $\bullet\text{NO}$ synthesis at 21% than at 1% O_2 , the steady-state concentrations of $\bullet\text{NO}$ at both O_2 concentrations were approximately equivalent ($[30\text{--}50\text{ nM}]_{\text{ss}}$).

In parallel plates the formation of intracellular DNIC and CIP was measured at 1% and 21% O_2 (Fig. 2A). DNIC formation was rapid and paralleled the onset of iNOS induction and $\text{NO}_2^-/\text{NO}_3^-$ accumulation. To ensure that changes in DNIC were not due to LPS-induced fluctuations in the CIP, the CIP was measured in LPS-stimulated RAW 264.7 cells grown in

arginine-free media (0–24h, 1% & 21% O₂). The CIP remained constant under all conditions. At both O₂ concentrations, the amount of DNIC reached a maximum at approximately 10–14 h and remained elevated greater than 24 h. Also, despite a greater amount of •NO being synthesized at 21% O₂, the amount of DNIC detected at both O₂ concentrations was not statistically different at any time point ($p=0.05$ level, Student's *t* test). This indicates that DNIC formation will be much less affected than •NO synthesis over a range of physiologic O₂ concentrations.

It has been reported that DNIC are formed from the reaction of •NO with the CIP. For the purposes of comparing the amount of DNIC to the amount of chelatable iron (CIP), we measured the CIP by treating cells with desferrioxamine and then measuring the concentration of the resultant intracellular ferrous iron complex by EPR (CIP = 260 ± 50 pmol/mg protein, Fig 2A). In this figure it can be seen that initially (6 hours), the concentration of DNIC is equivalent to that of the chelatable iron. At longer time points, however, DNIC levels exceeded the CIP indicating that prolonged •NO exposure mobilizes iron from other sources. To rule out iron uptake from the media as the source of iron for DNIC assembly, we added the cell-impermeable metal chelator DTPA to the media. Under these conditions we did not detect any decrease in the amount of DNIC formed (data not shown). To reconfirm that the CIP is an initial source of iron for DNIC assembly, however, we treated RAW 264.7 cells with the metal chelator desferrioxamine concurrent with LPS stimulation. Under these experimental conditions we were unable to detect any DNIC (Fig 2B).

These cells were also treated with the iNOS inhibitor aminoguanidine, or grown in the absence of arginine (a substrate for NOS). Figure 2B demonstrates that endogenous DNIC formation requires both the synthesis of •NO and chelatable iron. Thus, while the CIP appears to be the initial source of iron for DNIC assembly, prolonged •NO exposure mobilizes iron from other intracellular stores.

DNIC formation is determined by both the concentration and duration of •NO exposure

Nitric oxide donor compounds (diazoniumdiolates) allow precisely controlled *in vitro* delivery of •NO [20]. We used these compounds to compare differences in steady-state •NO concentrations and exposure times to the magnitude of DNIC formation in cells. RAW 264.7 cells grown in monolayer were treated with the •NO-donor DETA/NO at 500 μ M or 1,000 μ M for 18 h, and both the concentration of •NO in the Media (Fig. 3A) and the resultant intracellular DNIC were quantified (Fig. 3B). Maximal DNIC were observed by 4 h, after which the duration of •NO exposure had no effect on DNIC accumulation. For both treatments the amount of DNIC actually exceeded the measured amount of chelatable iron similar to what was observed when DNIC were formed during prolonged endogenous •NO production. In comparison to the CIP, the 1,000 μ M DETA/NO treatment yielded 2–3 times the amount of DNIC (~500–700 pmol DNIC/mg protein) as the CIP and the 500 μ M treatment was also elevated (~350pmol DNIC/mg protein).

Nitric oxide exposure and cell viability

Because iron availability is essential for many cellular processes including DNA synthesis and cell division, we measured the viability of •NO-exposed cells in relation to the amount of chelatable and complexed (DNIC) iron. Figure 3C demonstrates a dose-dependent effect of •NO concentration on cell viability. At low physiologic •NO concentrations, where the amount of DNIC was less than the amount of chelatable iron (100–250 μ M DETA/NO), cell viability was greater than 95%. There was a threshold concentration of •NO, however, where increasing amounts correlated to decreases in cell viability. Interestingly, this cytotoxicity threshold occurred when the amount of •NO resulted in the concentrations of

DNIC being greater than, or equal to, the concentration of the CIP. Cytotoxicity correlated to both the concentration of •NO and the degree to which the amounts of DNIC exceed the CIP. Prolonged sequestration of cellular iron via the formation of DNIC leads to a disruption in iron homeostasis which could be the driving force behind the observed cytotoxicity at higher •NO concentrations.

•NO metabolism and DNIC formation

The amount of •NO that enters a closed system must, by conservation of mass, accumulate within the system. In addition to the formation of DNIC, there are a multitude of other possible cellular •NO adducts. For this reason, we measured the proportion of •NO added to a suspension of cells that ended up in DNIC. Cells were treated with increasing amounts of pure •NO (aliquots from a •NO-saturated solution). Cellular metabolism of •NO was then measured in real-time using an •NO-selective electrode (Fig. 4A). The rate of •NO metabolism by cells is a function of the cell density and the oxygen concentrations [21]. Thus, we kept the cell density constant and O₂ concentrations uniform throughout the experiment. Immediately upon •NO disappearance, cells were processed for DNIC analysis (Fig. 4B). We found a linear relationship between moles of •NO added and moles of DNIC formed (Fig 4C). Based on the hypothesized stoichiometry of DNIC assembly [8], we approximate that at a minimum 7–8% of the total •NO exposed to a cell becomes DNIC at 21% O₂.

Since the O₂ concentration is a determinant of the rate of •NO metabolism, we examined the magnitude of DNIC formation at 21% or 0.5% O₂. RAW 264.7 cells were exposed to identical doses of •NO (98 nmols) at both O₂ concentrations and DNIC were quantified. As predicted, the metabolism of •NO was much slower at 0.5% O₂ than at 21% O₂. This difference in the rate of •NO-metabolism extended the exposure time of cells to •NO from 3 min to 16 min (Fig. 4D). At 0.5% O₂ the addition of •NO resulted in a dramatic increase in DNIC formation compared to an equivalent amount of •NO added at 21% O₂ (Fig. 4E). These data demonstrate the rapid kinetics of DNIC formation (seconds) and highlight the importance of both •NO dose and exposure time.

The rates of DNIC Disappearance in cells

Kinetic measurements of DNIC disappearance in live cells from endogenous or exogenous sources of •NO have not been reported. To determine the cellular life-time of DNIC, we treated RAW 264.7 cells with 1mM Sper/NO for 1 hour or 1µg/mL LPS for 10 hours. Sper/NO treated wells were washed and put in fresh media. LPS stimulated cells were washed and placed in Arginine free media. Under both experimental conditions, the loss of the EPR $g=2.04$ signal was measured over time. We determined the half-life of DNIC to be approximately 78 ± 5 min regardless of the means of •NO-exposure (Fig. 5). When these decay experiments were repeated at 1% O₂, no difference in the rate of DNIC disappearance was observed (data not shown) suggesting the mechanism is O₂-independent.

The relationship between DNIC and S-nitrosothiols

There are several reports indicating S-nitrosothiols (RSNO) can be formed via DNIC-dependent mechanisms [22, 23]. Since none of these studies used endogenous •NO-producing cells to examine this association, we set out to measure total cellular RSNO and DNIC concentrations over time in •NO-producing RAW 264.7 cells (Fig. 6A). We found that the onset of RSNO formation lagged behind that of DNIC formation. Like DNIC, however, endogenous RSNO were stable and continuously elevated for >24 h. Although greater amounts of RSNO were detected at 1% than at 21% O₂, in all cases the maximal concentrations of RSNO were an order of magnitude less than that of DNIC. We also measured rates of RSNO disappearance in relation to DNIC disappearance in •NO

producing (data not shown) and •NO treated cells (Fig. 6B). We observed that the initial rate of RSNO decay was rapid in comparison to that of DNIC while the rate of RSNO decay at later time points was similar to that of DNIC. This biphasic RSNO decay rate suggests both DNIC-dependent and independent mechanisms of RSNO formation.

DNIC decay products

We treated non-LPS stimulated RAW 264.7 cells with •NO under conditions to maximize DNIC levels (1 mM Sper/NO for 1 h). After washing the cells, we were unable to detect any free •NO given off over time from the cells (data not shown). 3-nitrotyrosine (3-NT) is an important •NO-derived cellular adduct that can be formed under various conditions of •NO exposure. We measured the formation of 3-NT in response to changes in •NO dose and •NO exposure time. Under these conditions we were unable to detect any amount of 3-NT. These results solidify DNIC as quantitatively the largest •NO-derived cellular adduct that is formed upon •NO exposure (Table 1).

DNIC are formed via autocrine actions of •NO

Nitric oxide is highly diffusible and capable of acting as an autocrine or paracrine signaling molecule [24]. Therefore, we set out to distinguish which of these mechanisms was the dominant mode for DNIC formation. We treated RAW 264.7 cells with LPS to stimulate •NO production in the presence or absence of an extracellular •NO-scavenger (oxymyoglobin). In parallel plates, non-LPS stimulated cells were treated with the 500µM DETA/NO also in the presence or absence of oxymyoglobin (Fig. 7). When •NO was synthesized intracellularly, oxymyoglobin scavenged any •NO that diffused out of the cell into the surrounding media, thereby preventing its reentry into an adjacent cell. Under these conditions the amount of intracellular DNIC formation was unchanged in comparison to non-oxymyoglobin treated cells. However, when the •NO source was extracellular, oxymyoglobin completely inhibited the ability of •NO to form intracellular DNIC. This strongly suggests that •NO acts in an autocrine fashion by rapidly reacting with the CIP to form DNIC before it diffuses out of the cell.

DISCUSSION

This study has identified several fundamental, previously uncharacterized, biochemical properties of cellular DNIC. We have demonstrated that DNIC are produced under physiologic conditions from low concentrations of •NO (~50 nM) and they are the largest intracellular •NO-derived adduct under all conditions we examined (Table 1). In LPS stimulated RAW 264.7 cells, DNIC formation paralleled iNOS upregulation and the onset •NO production as measured by $\text{NO}_2^-/\text{NO}_3^-$ accumulation. The concentrations of DNIC at both 21% and 1% O_2 were approximately equivalent. During prolonged •NO exposure, the absolute amounts of DNIC plateau at a concentration that was similar to, but often in excess of, the amount of iron that can be chelated by desferrioxamine. Nevertheless, cellular pretreatment with this chelator completely inhibited the formation of DNIC during endogenous •NO production implying that DNIC assembly utilizes the CIP as the initial iron source.

Although O_2 is not a substrate for chemical DNIC assembly [8], under biological conditions it will affect the amount of DNIC formed. Nitric oxide is metabolized by cells in an O_2 -dependant manner (the greater the O_2 concentration, the faster •NO is metabolized) [21]. Therefore, at low O_2 the biological half-life of •NO is extended. This consequently lengthens the exposure time of cellular targets, like iron, to •NO. This is exemplified in figures 4D & E where it can be seen that for a given amount of •NO (nmols), the exposure time to •NO and the amounts of DNIC detected was much greater at low O_2 (<1%)

than at 21% O₂. •NO not only will decreasing the O₂ concentration affect the rate of •NO metabolism (Fig 4D), but it will also result in a concomitant decrease in the rate of enzymatic •NO synthesis (Fig. 1A). This decrease in the rate of •NO synthesis at low O₂, however, is proportionally less dramatic than the decrease in the rate of •NO metabolism at low O₂. Because •NO metabolism is slowed to a greater extent than •NO synthesis under these conditions, the end result is equivalent steady-state •NO concentrations at both 1% and 21% O₂ (Fig. 1C). Furthermore, this equalizing of steady-state •NO concentrations augments the exposure of •NO-targets, like iron, such that equivalent DNIC levels are observed at both O₂ concentrations (Fig. 2A). This has important implications not only for DNIC metabolism but for the effects of O₂ gradients on the chemical biology of •NO in general.

To understand the effects of prolonged •NO exposure on DNIC formation, we used •NO-donors to generate two steady-state •NO concentrations that would simulate both physiological (100–800 nM) and pathological (800–2,500 nM) conditions. Under both conditions we noted maximal DNIC formation by 4 hours, while at later time points we saw no further increase in the concentrations of DNIC. At the lower •NO dose, DNIC concentrations were similar to those observed during endogenous •NO production. However, upon exposure to higher steady-state •NO concentrations, DNIC accumulated at levels in 2–3 fold excess of the CIP. When we looked at DNIC levels over a range of •NO concentrations, we noted that there was a threshold concentration of •NO where the CIP no longer became the limiting source of iron for DNIC assembly. Interestingly, it was at this threshold •NO concentration and above where cytotoxicity ensued. Therefore, in addition to other well-known modes of •NO cytotoxicity, we speculate that one consequence of pathologic •NO concentrations may be the accumulation of non-CIP iron in the form of DNIC. Other potential sources of cellular iron, in addition to the CIP, are iron storage proteins, iron-sulfur clusters, heme and non-heme iron proteins.

Several lines of evidence indicate that cellular DNIC assembly is a rapid process, which suggests that the CIP is a predominant target for •NO [4, 8]. The data in Fig. 4 demonstrate that DNIC are detectable within minutes of •NO exposure. Increases in DNIC were also observed that directly paralleled the production of •NO from both LPS stimulated RAW 264.7 cells and •NO-donor compounds (Figs. 2 & 3). The most compelling evidence that DNIC assembly is rapid comes from Fig. 7 which demonstrates that •NO reacts intracellularly to form DNIC before it escapes out of the cell by diffusion. When oxymyoglobin, a potent extracellular •NO-scavenger, was present, the amount of DNIC detected in •NO-producing cells was identical to what was observed without oxymyoglobin. This implies that DNIC assembly is an autocrine function of •NO. In other words, DNIC formation is not dependent upon •NO exiting the cell and then reentering the same or neighboring cell.

The exact chemical or cellular mechanisms of DNIC assembly are not known. However, the stoichiometry of •NO to iron is purported to be 3:1[8]. In Fig. 4 we were able to examine the approximate flux of •NO that entered a cell(s) in relation to the amount of DNIC formed. We saw a linear relationship between the amount of •NO added and the amount of DNIC measured. However, because of the rapid concentration-dependent kinetics of higher ordered •NO reactions, we may be potentially underestimating the amount of •NO that ends up in DNIC. We suspect that the actual quantitative relationship between the amount of •NO and the fractional yield of DNIC may not be linear. At a minimum, however, 7–8% of all •NO exposed to a cell will end up as ligands of DNIC.

The stability of DNIC, like most •NO-adducts, should be an integral determinant of their biological effects. We found that once the •NO source was removed, DNIC disappeared with

a half-life of approximately 80 min independent of the oxygen concentration, and were still detectable > 24 h post •NO removal. We favor the term “disappearance” over “decay” because at this stage of our investigations we cannot make inferences into the mechanism(s) of DNIC disappearance. Decrease in the $g = 2.04$ signal can occur by several means: formation of non-paramagnetic •NO-iron complexes (MNIC), dimerization into diamagnetic forms, decay of DNIC, and even cellular export as some have reported [8, 25, 26].

Work by Turella *et al.* found that Glutathione Transerferase (GST) extended the $t_{1/2}$ of DNIC in cell lysates from 2.8 min to 4.5 – 8 h [27]. Exposure of 0.2 mM monomeric Cys-DNIC to ambient air leads to complete loss of paramagnetism within 40 min while Cys-DNIC maintained in a deoxygenated vessel persist for hours [28]. Preliminary work on other cell types suggests that rates of DNIC disappearance are cell type-specific (data not shown). This highlights the complexity of the system and suggests that cell type might dictate protein-specific DNIC composition and/or clearance leading to varying phenotypic consequences.

Recent work done by Lancaster’s group demonstrated that manipulation of the CIP affected both DNIC and nitrosothiol (RSNO) formation implying that DNIC may be the source of RSNO formation [23]. Figure 6 demonstrates that the formation of RSNO from endogenous •NO production lags behind the earliest detectable DNIC, and like DNIC, RSNO remain continually elevated throughout the duration of the experiment. When we looked at RSNO decay subsequent to •NO removal, the rate of RSNO decay was biphasic-- rapid at first then paralleling the rate of DNIC disappearance. What is potentially more important to note in these figures is the concentration differences between the two species. Upon •NO exposure there are 1 to 2 orders of magnitude greater DNIC formed than there is RSNO. This certainly doesn’t rule out the involvement of DNIC (or iron) in RSNO formation. It does suggest, however, that the direct decay of DNIC into RSNO is not the dominant mode of DNIC disappearance.

A consequence of DNIC formation is the sequestration of chelatable iron and the induction of an iron-starved phenotype. These perturbations in cellular iron availability will have both short- and long-term effects on cell behavior. From a chemical standpoint, this chelation-like chemistry could serve an antioxidant function by rendering the CIP non-reactive, thereby preventing iron-driven oxidative stress. Biologically, however, alterations in cellular iron accessibility will have other consequences such as regulating iron homeostasis and modifying the activities of iron-containing/requiring proteins.

SUMMARY

We have shown that the magnitude of DNIC formation and disappearance in response to changes in O_2 concentrations is much less affected than the overall magnitude of •NO synthesis or •NO degradation in response to changes in O_2 . For these reasons the formation of DNIC may function by buffering the dramatic effects of O_2 on •NO synthesis and degradation. Because the half-life of DNIC are long compared to free •NO, DNIC may serve as a sort of “•NO capacitor” capturing •NO and channeling it onto thiols to form *S*-nitrosothiols. These *S*-nitrosothiols could go on to release free •NO, degrade into NO_2^- , or transnitrosate other thiols, thereby extending the half-life of •NO or •NO-like activity long after enzymatic •NO synthesis has ceased. Even though the amounts of RSNO are small compared to the amounts of DNIC, these levels of RSNOs are reportedly sufficiently high to exert numerous biological effects. Furthermore, since NO_2^- has bioactivity, mainly through its reduction to •NO under hypoxia, DNIC could extend •NO activity by serving as a NO_2^- generator. This may explain results by others who have shown that DNIC have •NO-like activity via activating sGC or inducing vasorelaxation [13, 29–32]. Whatever the biological or pathological functions of DNIC are revealed to be, our data indicate that in comparison to

other •NO-derived adducts, DNIC quantitatively represent the largest intracellular pool. Their high abundance, long half-life, and ubiquitous nature indicate that they will likely be formed under all cellular settings of •NO production.

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LIST OF ABBREVIATIONS

CIP	chelatable iron pool
CysNO	S-nitroso-L-cysteine
DETA/NO	(Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate
DFO	desferrioxamine
DMEM	Dulbecco's Modified Eagle Medium
DNIC	dinitrosyliron complexes
DTPA	Diethylenetriaminepentaacetic acid
Fe	iron
iNOS	inducible nitric oxide synthase
IRE	iron responsive elements
IRP	iron regulatory protein
LPS	lipopolysaccharide
MbO₂	oxymyoglobin
•NO	nitric oxide
NO₂⁻	nitrite
NO₃⁻	nitrate
RSNO	S-nitrosothiol
Sper/NO	(Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]diazen-1-ium-1,2-diolate

References

1. Ignarro LJ. Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. *J Physiol Pharmacol.* 2002; 53:503–514. [PubMed: 12512688]
2. Thomas DD, Ridnour LA, Isenberg JS, Flores-Santana W, Switzer CH, Donzelli S, Hussain P, Vecoli C, Paolucci N, Ambs S, Colton CA, Harris CC, Roberts DD, Wink DA. The chemical biology of nitric oxide: implications in cellular signaling. *Free Radic Biol Med.* 2008; 45:18–31. [PubMed: 18439435]
3. Petrat F, de Groot H, Sustmann R, Rauen U. The chelatable iron pool in living cells: a methodically defined quantity. *Biol Chem.* 2002; 383:489–502. [PubMed: 12033438]
4. Toledo JC Jr, Bosworth CA, Hennon SW, Mahtani HA, Bergonia HA, Lancaster JR Jr. Nitric oxide-induced conversion of cellular chelatable iron into macromolecule-bound paramagnetic dinitrosyliron complexes. *J Biol Chem.* 2008; 283:28926–28933. [PubMed: 18480062]

5. Mallard JR, Kent M. Differences Observed between Electron Spin Resonance Signals from Surviving Tumour Tissues and from Their Corresponding Normal Tissues. *Nature*. 1964; 204:1192. [PubMed: 14264374]
6. Vanin AF, Nalbandian RM. Free Radicals of a New Type in Yeast Cells. *Biofizika*. 1965; 10:167–168. [PubMed: 14332030]
7. Vithayathil AJ, Ternberg JL, Commoner B. Changes in electron spin resonance signals of rat liver during chemical carcinogenesis. *Nature*. 1965; 207:1246–1249. [PubMed: 4287143]
8. Vanin AF. Dinitrosyl iron complexes with thiolate ligands: physico-chemistry, biochemistry and physiology. *Nitric Oxide*. 2009; 21:1–13. [PubMed: 19366636]
9. Vanin AF, Sanina NA, Serezhenkov VA, Burbaev D, Lozinsky VI, Aldoshin SM. Dinitrosyl-iron complexes with thiol-containing ligands: spatial and electronic structures. *Nitric Oxide*. 2007; 16:82–93. [PubMed: 16979919]
10. Vanin AF, Serezhenkov VA, Mikoyan VD, Genkin MV. The 2.03 signal as an indicator of dinitrosyl-iron complexes with thiol-containing ligands. *Nitric Oxide*. 1998; 2:224–234. [PubMed: 9851363]
11. Drapier JC, Pellat C, Henry Y. Generation of EPR-detectable nitrosyl-iron complexes in tumor target cells cocultured with activated macrophages. *J Biol Chem*. 1991; 266:10162–10167. [PubMed: 1645341]
12. Mokh VP, Poltorakov AP, Serezhenkov VA, Vanin AF. On the nature of a compound formed from dinitrosyl-iron complexes with cysteine and responsible for a long-lasting vasorelaxation. *Nitric Oxide*. 22:266–274. [PubMed: 20067839]
13. Mayer B, Kleschyov AL, Stessel H, Russwurm M, Munzel T, Koesling D, Schmidt K. Inactivation of soluble guanylate cyclase by stoichiometric S-nitrosation. *Mol Pharmacol*. 2009; 75:886–891. [PubMed: 19114587]
14. Ahmed SA, Gogal RM Jr, Walsh JE. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [³H]thymidine incorporation assay. *J Immunol Methods*. 1994; 170:211–224. [PubMed: 8157999]
15. MacArthur PH, Shiva S, Gladwin MT. Measurement of circulating nitrite and S-nitrosothiols by reductive chemiluminescence. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2007; 851:93–105.
16. Woodmansee AN, Imlay JA. Quantitation of intracellular free iron by electron paramagnetic resonance spectroscopy. *Methods Enzymol*. 2002; 349:3–9. [PubMed: 11912920]
17. Robinson MA, Baumgardner JE, Good VP, Otto CM. Physiological and hypoxic O₂ tensions rapidly regulate NO production by stimulated macrophages. *Am J Physiol Cell Physiol*. 2008; 294:C1079–1087. [PubMed: 18272818]
18. Melillo G, Taylor LS, Brooks A, Cox GW, Varesio L. Regulation of inducible nitric oxide synthase expression in IFN-gamma-treated murine macrophages cultured under hypoxic conditions. *J Immunol*. 1996; 157:2638–2644. [PubMed: 8805668]
19. Melillo G, Musso T, Sica A, Taylor LS, Cox GW, Varesio L. A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. *J Exp Med*. 1995; 182:1683–1693. [PubMed: 7500013]
20. Thomas DD, Miranda KM, Espey MG, Citrin D, Jourdain D, Paolocci N, Hewett SJ, Colton CA, Grisham MB, Feelisch M, Wink DA. Guide for the use of nitric oxide (NO) donors as probes of the chemistry of NO and related redox species in biological systems. *Methods Enzymol*. 2002; 359:84–105. [PubMed: 12481562]
21. Thomas DD, Liu X, Kantrow SP, Lancaster JR Jr. The biological lifetime of nitric oxide: implications for the perivascular dynamics of NO and O₂. *Proc Natl Acad Sci U S A*. 2001; 98:355–360. [PubMed: 11134509]
22. Boese M, Mordvintsev PI, Vanin AF, Busse R, Mulsch A. S-nitrosation of serum albumin by dinitrosyl-iron complex. *J Biol Chem*. 1995; 270:29244–29249. [PubMed: 7493954]
23. Bosworth CA, Toledo JC Jr, Zmijewski JW, Li Q, Lancaster JR Jr. Dinitrosyliron complexes and the mechanism(s) of cellular protein nitrosothiol formation from nitric oxide. *Proc Natl Acad Sci U S A*. 2009; 106:4671–4676. [PubMed: 19261856]

24. Lancaster JR Jr. A tutorial on the diffusibility and reactivity of free nitric oxide. *Nitric Oxide*. 1997; 1:18–30. [PubMed: 9701041]
25. Vanin AF, Stukan RA, Manukhina EB. Physical properties of dinitrosyl iron complexes with thiol-containing ligands in relation with their vasodilator activity. *Biochim Biophys Acta*. 1996; 1295:5–12. [PubMed: 8679673]
26. Watts RN, Hawkins C, Ponka P, Richardson DR. Nitrogen monoxide (NO)-mediated iron release from cells is linked to NO-induced glutathione efflux via multidrug resistance-associated protein 1. *Proc Natl Acad Sci U S A*. 2006; 103:7670–7675. [PubMed: 16679408]
27. Turella P, Pedersen JZ, Caccuri AM, De Maria F, Mastroberardino P, Lo Bello M, Federici G, Ricci G. Glutathione transferase superfamily behaves like storage proteins for dinitrosyl-diglutathionyl-iron complex in heterogeneous systems. *J Biol Chem*. 2003; 278:42294–42299. [PubMed: 12871931]
28. van Faassen, E.; Vanin, A., editors. *Radicals for Life The Various Forms of Nitric Oxide*. Amsterdam: Elsevier; 2007.
29. Severina IS, Bussygina OG, Pyatakova NV, Malenkova IV, Vanin AF. Activation of soluble guanylate cyclase by NO donors--S-nitrosothiols, and dinitrosyl-iron complexes with thiol-containing ligands. *Nitric Oxide*. 2003; 8:155–163. [PubMed: 12826064]
30. Vanin AF, Kleshchev AL, Mordvintsev PI, Sedov KR. Hypotensive activity of the nitrosyl complexes of nonheme iron with different anionic ligands. *Dokl Akad Nauk SSSR*. 1985; 281:742–745. [PubMed: 4017859]
31. Mordvintsev NI, Vanin AF. Dinitrosyl complexes of nonheme iron in the blood plasma of animals and man. *Izv Akad Nauk SSSR Biol*. 1988;942–946. [PubMed: 2854145]
32. Galagan ME, Oranovskaia EV, Mordvintsev PI, Medvedev OS, Vanin AF. Hypotensive effect of dinitrosyl iron complexes in experiments on waking animals. *Biull Vsesoiuznogo Kardiolog Nauchn Tsentra AMN SSSR*. 1988; 11:75–80. [PubMed: 2852943]
33. Baker PR, Schopfer FJ, Sweeney S, Freeman BA. Red cell membrane and plasma linoleic acid nitration products: synthesis, clinical identification, and quantitation. *Proc Natl Acad Sci U S A*. 2004; 101:11577–11582. [PubMed: 15273286]
34. Albakri QA, Stuehr DJ. Intracellular assembly of inducible NO synthase is limited by nitric oxide-mediated changes in heme insertion and availability. *J Biol Chem*. 1996; 271:5414–5421. [PubMed: 8621396]

Highlights

- DNIC are the most abundant NO-derived species in NO-producing RAW 264.7 macrophages
- The rate of DNIC metabolism is O₂ independent, with a biological half-life of 80 min
- DNIC form within seconds and can be detected >24 hours after the NO source is removed
- DNIC formation is dependent upon the dose and duration of NO exposure
- DNIC concentrations can exceed that of intracellular chelatable iron

Figure 1. Indices of •NO production in LPS stimulated RAW 264.7 macrophages
 RAW 264.7 cells cultured at 21% or 1% O_2 were stimulated with LPS at T = 0 h **A**) Chemiluminescent measurements of NO_2^- and NO_3^- accumulation in the media. **B**) Western blot for iNOS expression at 21% O_2 . **C**) Real-time electrochemical measurement of steady-state •NO concentrations in the media (•NO-selective electrode ~1mm above monolayer) at 21% and 1% O_2 . Cells were treated with LPS for 12 hours in arginine-free media. To initiate •NO production, 1 mM arginine was added, T=0 h. The experiment was terminated by addition of the •NO-scavenger oxymyoglobin to verify electrode response and presence of •NO. **D**) Western blot of iNOS expression after LPS treatment (1 $\mu\text{g}/\text{mL}$) at 1%, 5%, 10% and 21% O_2 (T=12 h). For all experiments $n \geq 5$.

Figure 2. Endogenous DNIC formation in •NO-producing cells

A) RAW 264.7 cells in culture were treated with LPS at both 1% and 21% O₂ (identical conditions of Figure 1A). At the indicated time points the cells were harvested for DNIC quantification by EPR. Iron in the CIP was measured in separate plates by EPR (gray band). **B)** EPR measurements of DNIC in RAW 264.7 cells. All cells (except untreated) were exposed to LPS for 10 hours under the indicated conditions. Representative data $n \geq 5$.

Figure 3. Dose and time-dependent effects of •NO exposure on DNIC formation

A) Real-time electrochemical measurement of steady-state •NO concentrations in the media of •NO-donor (DETA/NO 1,000 & 500 μ M) treated RAW 264.7 cells. The experiment was terminated by addition of the •NO-scavenger oxymyoglobin to verify electrode response and presence of •NO. B) Temporal EPR measurements of DNIC from cells treated with DETA/NO identically to those in panel A. The gray horizontal band indicates the measured CIP. C) Cells were treated for 18 h with DETA/NO at the indicated concentrations. $\circ$ = DNIC concentration divided by the CIP concentration, $n=4$. $\blacksquare$ = cell viability, $n=8$. Viability was assessed via alamarBlue® dye reduction assay.

Figure 4. The amount of •NO and the exposure time determines the magnitude of DNIC formation

A) Cells were placed in suspension and stirred in a reaction vessel with an •NO-selective electrode. •NO metabolism (disappearance) was measured after the additions of bolus amounts of an •NO-saturated stock solution. Following •NO disappearance, the cells were immediately removed from the reaction chamber and processed for EPR analysis. **B)** EPR measurements of DNIC formation from the cells in panel A. **C)** The linear relationship between the amount of DNIC formed and the amount of •NO added. **D)** •NO metabolism after bolus addition of •NO at a starting O₂ concentration of either 21% or 0.5%. **E)** Bar graph indicating amount of DNIC formed from samples in panel D.

Figure 5. Rates of DNIC disappearance after endogenous or exogenous •NO exposure
RAW 264.7 cells in culture were treated with either **A**) LPS for 10 hours followed by replacement with arginine-free media or **B**) The •NO-donor Sper/NO (1 mM) for 1 hour followed by replacement with fresh media. Samples were harvested at the indicated time points for DNIC analysis (n=3).

Figure 6. Simultaneous measurements of DNIC and RSNO concentrations over time

A) Formation of RSNO and DNIC from endogenously produced •NO. RAW 264.7 cells in culture were stimulated with LPS (T=0) at 21% and 1 % O₂. Samples were harvested at the indicated time points for total DNIC (EPR) and RSNO (chemiluminescence) quantification. Combined data is presented for DNIC at 21% and 1% O₂ because no statistical differences are observed between the two conditions **B)** Measurements of the rates of RSNO and DNIC disappearance. Cells were treated with the NO-donor Sper/NO (1 mM) for 1 h. The media was replaced with fresh media and samples were harvested at the indicated time points for DNIC and RSNO quantification, n = 3.

Figure 7. The effect of extracellular •NO scavenging on DNIC formation from endogenous or exogenous •NO sources

RAW 264.7 cells were treated with either DETA/NO (500 μ M) for 4 hours or LPS for 12 hours (8 hours in arginine-free media + 4 hours complete media) in the presence or absence of oxymyoglobin (100 μ M, last 4 hours). Samples were collected for DNIC analysis (n=3).

Table 1
Cellular concentrations of major •NO adducts

Heme values from the study cited in the table are from RAW 264.7 cells. LNO₂ values are from packed red blood cells with no treatment. Ranges of RSNO and DNIC are the range of values detected under the various conditions in this study. 3-Nitrotyrosine (3NT) was measured in RAW 264.7 cells treated with 500 μM DETA/NO for 24 hrs and with 100, 250, 500, 750, and 1000 μM DETA/NO for 10 h (as in Fig 3). 3NT was not detectable in any of the samples.

Intracellular Compound	Concentration	Source
3-Nitrotyrosine	Not detectable (< 1.5 pmol/mg protein)	This Study
LNO ₂	249 ± 104 nM*	[33]
Total heme [†]	15–25 pmol/mg protein	[34]
RSNO	1–100 pmol/mg protein	This Study
DNIC	100–900 pmol/mg protein	This Study

* For comparison with LNO₂ data, 100 pmol/mg protein = 5–10 μM for our DNIC samples.

[†] The value for total heme is included for the purposes of estimating the potential maximal concentration of •NO nitrosyl heme complexes.