Nitrosothiol Formation and Protection against Fenton Chemistry by Nitric Oxide-induced Dinitrosyliron Complex Formation from Anoxia-initiated Cellular Chelatable Iron Increase*

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Background: Dinitrosyliron complexes (DNIC) have been found in a variety of pathological settings associated with ‘NO. However, the iron source of cellular DNIC is unknown. Previous studies on this question using prolonged ‘NO exposure could be misleading due to the movement of intracellular iron among different sources. We here report that brief ‘NO exposure results in only barely detectable DNIC, but levels increase dramatically after 1–2 h of anoxia. This increase is similar quantitatively and temporally with increases in the chelatable iron, and brief ‘NO treatment prevents detection of this anoxia-induced increased chelatable iron by deferoxamine. DNIC formation is so rapid that it is limited by the availability of ‘NO and chelatable iron. We utilize this ability to selectively manipulate cellular chelatable iron levels and provide evidence for two cellular functions of endogenous DNIC formation, protection against anoxia-induced reactive oxygen chemistry from the Fenton reaction and formation by transnitrosation of protein nitrosothiols (RSNO). The levels of RSNO under these high chelatable iron levels are comparable with DNIC levels and suggest that under these conditions, both DNIC and RSNO are the most abundant cellular adducts of ‘NO.

Results: Cellular DNIC formation upon brief nitric oxide (‘NO) exposure is dramatically increased by anoxia pretreatment. DNIC is rapidly generated from ‘NO reaction with chelatable iron.

Conclusion: This experimental system provides a useful tool to manipulate the levels of DNIC and probe their cellular functions.

Significance: Cellular DNIC formation requires free ‘NO and provided evidence for the conversion of chelatable iron to DNIC. That this cellular iron pool is responsible for DNIC formation was claimed previously (16–18). In cells, the majority of iron is "captured" in iron-sulfur clusters, heme and non-heme iron proteins (19). However, there is a small (0.2–3%) proportion of total cellular iron that is relatively "free" and moves in transit between iron-containing components, such as iron-sulfur clusters (Fig. 1) (20, 21). Because the nature of this iron-containing entity is poorly understood, it is currently defined as "labile iron" or "chelatable iron" by the methods utilized to measure it, specifically the ability to be chelated in cells by metal chelators (Fig. 1). Because the properties of the chelatable iron that are chelated by different chelators could be different, we here will specifically refer to the chelators that we used.

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Nitric oxide (nitrogen monoxide, ‘NO) is implicated in virtually every physiological and pathophysiological process (1). It has been reported that the most abundant cellular adducts of ‘NO is dinitrosyliron complexes (DNIC). These complexes contain one iron, two nitrosyl groups, and two other ligands, all binding to iron ((L)2Fe(NO)2) (2). In cells, low molecular weight thiols and cysteine-containing proteins (RSH) constitute the majority of DNIC ligands (3–5). DNIC exhibit a unique EPR spectrum characterized by a principal g value of 2.03. Long before the demonstration of ‘NO synthesis in mammalian systems (6–8), DNIC were identified in animal tissues as an early indicator of tumor development (9–11). Later, DNIC were found in a variety of pathological settings associated with endogenous ‘NO production, such as diabetes (12), organ transplant rejection (13), and sepsis (14).

However, how DNIC are formed in cells is unknown. We have shown previously that cellular DNIC formation requires free ‘NO and provided evidence for the conversion of chelatable iron to DNIC (15). That this cellular iron pool is responsible for DNIC formation was claimed previously (16–18).

In cells, the majority of iron is "captured" in iron-sulfur clusters, heme and non-heme iron proteins (19). However, there is a small (0.2–3%) proportion of total cellular iron that is relatively "free" and moves in transit between iron-containing components, such as iron-sulfur clusters (Fig. 1) (20, 21). Because the nature of this iron-containing entity is poorly understood, it is currently defined as "labile iron" or "chelatable iron" by the methods utilized to measure it, specifically the ability to be chelated in cells by metal chelators (Fig. 1). Because the properties of the chelatable iron that are chelated by different chelators could be different, we here will specifically refer to the chelators that we used.

5 The abbreviations used are: DNIC, dinitrosyliron complex(es); RSH, low molecular weight thiols and cysteine-containing proteins; PROLI/NO, disodium 1-[2-(carboxylato)pyrrolidin-1-yl]diazien-1-ium-1,2-diolate-methanolate; RSNO, nitrosothiol; ROS, reactive oxygen species; DFO, deferoxamine mesylate salt; NEM, N-ethylmaleimide; DTPA, diethylenetriamine pentaacetic acid; H2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; SIH, salicylaldehyde isonicotinoyl hydrazone; poly-HEMA, poly(2-hydroxyethyl methacrylate); Mb, myoglobin; oxyMb, oxymyoglobin; MbNO, nitrosylmyoglobin; ANOVA, analysis of variance.
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FIGURE 1. Reactions of cellular chelatable iron involved in this study. Metal chelators, including SIH and DFO, coordinate with chelatable iron, establishing reversible equilibria (double-direction arrows), whereas NO may have higher affinity for chelatable iron because DNIC formation is not a simple coordination but requires a one-electron oxidation. These reactions are rapid. The movement between chelatable iron and other cellular iron-containing components takes multiple steps (broken arrows) and, therefore, is not as rapid. Prolonged presence of NO or metal chelators will act as a “sink” and result in net movement of iron from other stores into this sink (DNIC or iron-chelate complexes).

The conclusion that chelatable iron was the iron source of DNIC was largely based on the finding that chelatable iron was decreased by prolonged (1-h) NO treatment and that prolonged incubation with a metal chelator inhibited DNIC formation (15). There are, however, two major caveats to this approach. First, as shown in Fig. 1, the prolonged presence of NO or metal chelators will act as a “sink” and result in net movement of iron from other stores (iron-containing proteins) into this “sink” (DNIC or iron-chelate complexes). Thus, the amount of DNIC or iron-chelate complexes will be in excess of the amount immediately prior to the addition of NO or a chelator. Second, although longer term than the mass action phenomenon above, complexation of iron (whether by NO or by a chelator) will be sensed by the iron regulatory protein/iron-responsive element cellular iron homeostasis system and induce an “iron starvation” response (22). Through primarily posttranscriptional mechanisms, the cell responds by inducing increased available iron (primarily via mobilizing iron from ferritin and increasing iron uptake) (22). Again, the measured amount of DNIC or iron-chelate complexes will be artificially high.

With this realization, we initiated studies using only brief exposure to NO, which, based on model chemical studies, should bind rapidly to chelatable iron and result in DNIC formation. To our surprise (and dismay), we initially found that treatment of murine macrophage RAW 264.7 cells for 2 min with disodium 1-[2-(carboxylato)pyrrolidin-1-yl]diazene-1,2-diolate-methanol (PROLI/NO), a donor releasing NO with a half-life of 2 s at 37 °C and pH 7.4, resulted in no significant amount of DNIC. A biologically and clinically relevant condition that displays elevated levels of chelatable iron is hypoxia/anoxia (23–32), and then we discovered that the incubation of cells in a sealed vial (or in a hypoxic chamber) resulted in a dramatic increase in the DNIC signal upon subsequent brief NO exposure. Here we utilize this method to selectively manipulate the levels of DNIC and probe their cellular functions.

Upon NO exposure, RSH can be nitrosated, forming nitrivers (RSNO). There have been numerous reports claiming that nitrosation of critical proteins initiates post-translational signaling by a principle similar to phosphorylation (33, 34). The specificity of phosphorylation is implemented by kinases; however, the mechanism of cellular RSNO formation from NO is not understood. We have recently suggested that DNIC might mediate cellular RSNO formation (35), which is consistent with previous in vitro studies (36). Again, prolonged NO exposure was applied in our previous study (35). NO plays such diverse roles that over time, downstream reactions become more complex, and they may interact with the formation and the metabolism of DNIC and RSNO. Therefore, we here have studied the relationship between DNIC and RSNO by brief NO exposure to cells.

Chelatable iron can be harmful and function as a pro-oxidant via Fenton reaction (37), where ferrous iron (Fe2+) reacts with hydrogen peroxide (H2O2), generating a highly reactive oxygen species (ROS) with the reactivity of hydroxyl radical (·OH) (38). NO acts as an antioxidant protecting against iron-induced oxidative stress (39–41). Among possible mechanisms, it has been proposed that NO may inhibit Fenton reaction via chelatable iron sequestration, although to our knowledge, this mechanism has not been tested experimentally in cells (39–41). The NO reaction with cellular chelatable iron forming relatively stable DNIC represents a rapid and persistent iron sequestration by NO. Therefore, we also take advantage of our experimental system to test this possible antioxidant function of NO.

EXPERIMENTAL PROCEDURES

Materials—Deferoxamine mesylate salt (DFO), N-ethylmaleimide (NEM), 30% H2O2 solution without stabilizer, ferric chloride (FeCl3), poly(2-hydroxyethyl methacrylate) (polyHEMA), myoglobin (Mb) from equine skeletal muscle, diethyl-2- (dithionite, Na2S2O4) were purchased from Sigma-Aldrich. 2’,7’-Dichlorodihydrofluorescein diacetate (H2DCFDA) was from Invitrogen. Sodium hydrosulfite (Na2S2O4) and sodium phosphate were obtained from Fisher Scientific (Fair Lawn, NJ). PROLI/NO was from Alexis Biochemicals (San Diego, CA). The concentration of PROLI/NO in 20 mM KOH was determined by an oxymyoglobin (oxyMb) assay, and oxyMb was made as described previously (42). PROLI/NO releases NO with a half-life of 2 s at 37 °C and pH 7.4. Salicylaldehyde isonicotinoyl hydrazone (SIH) was a generous gift from Dr. José C. Toledo, Jr. (Universidade de São Paulo, Ribeirão Preto, Brazil).

Cell Culture, Anoxia, Reoxygenation, Normoxia, and Treatments—Murine macrophage-like RAW 264.7 cells were purchased from ATCC (Manassas, VA) and were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. At 80–90% confluence, cells were washed twice with PBS and were collected by scraping in Hanks’ balanced salt solution (Invitrogen, catalog no. 14175) containing 20 mM sodium phosphate, pH 7.4. Sodium phosphate was added to maintain the pH during anoxia. To induce anoxia, 1 × 107 cells/ml cell suspension was filled in an amber vial with no headspace. The vial was sealed with a rubber septum and then incubated at 37 °C. The O2 level falls below the detection limit of the O2 electrode (<100 nm or 0.1%) within 5–10 min due to the O2 consumption by cells (35). Reoxygen-
At various periods of anoxia, PROLI/NO (expressed in concentrations of ‘NO that it liberates) was injected by a Hamilton gas-tight syringe into the sealed vial through the rubber septum. Similarly, to avoid any loss of ‘NO due to its volatility, cells undergoing normoxia (or reoxygenation) were transferred from poly-HEMA-coated plates into sealed vials (without headspace) before the addition of PROLI/NO. The transfer was made as quickly as possible because we have found that unintended hypoxia/anoxia is induced with time by concentrated cells in vials where the gas exchange surface is limited (data not shown). PROLI/NO was injected immediately once the vial was sealed to reduce any unintended hypoxia/anoxia due to rapid concentration of ‘NO-DFO as standards.

Sample Processing for the Determination of Chelatable Iron and DNIC—Chelatable iron was determined as Fe³⁺-DFO adduct by EPR. At the end of the treatments, as indicated, the cell suspension was immediately transferred to DFO on ice for a final concentration of 1 mM. As discussed under “Results,” because reoxygenation is inevitable during sample processing, the immediate addition of DFO at the end of anoxia is required to prevent the DFO-chelatable iron from decreasing during reoxygenation (Fig. 5). Following brief centrifugation at 4 °C, the cell pellet was resuspended in cold PBS containing 1 mM DFO. Samples were then lysed by freeze-thaw followed by sonication (60 Sonic Dismembrator from Fisher) at a power of 4 watts (root mean square) for 15 s. The whole cell lysate was divided for the simultaneous determination of protein concentration and Fe³⁺-DFO adduct by EPR.

For DNIC determination, the reaction was stopped by the immediate addition of oxyMb (twice the ‘NO concentration added) during simultaneous cooling on ice. OxyMb quickly scavenges any excess of ‘NO present in the suspension (42). Following brief centrifugation at 4 °C, the cell pellet was washed twice by cold PBS containing 100 μM DTPA, pH 7.4 (PBS) and was then resuspended in PBS. The cell suspension was separated for the simultaneous determination of DNIC by EPR and protein concentration following the addition of lysis buffer (PBS containing 0.1% Triton X-100 and protease inhibitors).

EPR Measurement—Samples were transferred into quartz EPR tubes, frozen in liquid N₂, and stored at −80 °C until analysis. All EPR measurements (Bruker Elexys E500) were performed at 150 K. The instrumental settings were as follows: 10-G modulation amplitude; 160-ms time constant; 320-ms conversion time; 1 milliwatt of power for DNIC and 10 milliwatts for Fe³⁺-DFO. Four scans were accumulated. The DNIC concentration was quantitated by the double integration of the signal at 2.03 G, using nitrosylmyoglobin (MbNO) as standards. Fe³⁺-DFO concentration was quantitated by the peak to trough of the signal at 4.3 G and using known concentrations of Fe³⁺-DFO as standards.

Preparation of Standards for the Quantitation of EPR Spectra—MbNO can be made from metmyoglobin by first adding ‘NO to form nitrosylmetmyoglobin followed by the addition of dithionite to reduce nitrosylmetmyoglobin to MbNO. It could also be made from oxyMb by adding dithionite to reduce it to deoxymyoglobin followed by the addition of ‘NO (44). Both methods require sparging inert gas through a protein solution to maintain a deoxygenated state (44), which, however, is very likely to cause denaturation of the protein. Dithionite reduces oxygen and can therefore be used to temporarily maintain a deoxygenated condition. Here we used dithionite to reduce metmyoglobin to deoxymyoglobin, which is then converted to MbNO by the addition of ‘NO. OxyMb and MbNO have very similar UV spectra. To avoid the interference of oxyMb, an excess of dithionite is important to temporarily remove the oxygen from the solution, preventing oxyMb formation before the ‘NO addition. However, dithionite itself absorbs at about 315 nm (45), and overuse of it will interfere with the absorption of MbNO at 420 nm, causing overestimation of the MbNO concentration. Here we prepared MbNO in a spectrophotometric cuvette, so that the real-time reaction was monitored by a Shimadzu UV-2501PC spectrophotometer (Kyoto, Japan). The convenient protocol of making MbNO was developed as follows.

The stock solution of 100 mM dithionite in a 100 mM potassium phosphate buffer containing 100 μM DTPA, pH 7.4, was prepared immediately prior to use. A high concentration of phosphate is necessary to maintain the pH of the stock solution. To 10–20 μM Mb (based on mass and molecular weight of 17,600) in PBS was added 1 mM dithionite; the UV spectrum showed a red shift of the Soret band to 435 nm, indicating the presence of excess dithionite. 30 μM ‘NO from PROLI/NO was immediately added to the solution, and the Soret band shifted to 420 nm, indicating the conversion of deoxymyoglobin to MbNO, and the peak at 315 nm decreased due to the dithionite reaction with oxygen. Another addition of PROLI/NO (5–10 μM ‘NO) showed no significant increase of the Soret band of MbNO, confirming the completion of the reaction. The Soret band of MbNO may slightly decrease due to the disappearance of dithionite; therefore, it is important to have dithionite low enough not to interfere with the absorbance of MbNO at 420 nm. On the other hand, it is also important that dithionite is present during the ‘NO addition to avoid oxyMb formation as described above. The concentration of MbNO was calculated using ε 420 nm = 132 mM⁻¹ cm⁻¹ (44). A yield of ≥80% based on the amount of Mb can be obtained by this method.

The previous method for preparing Fe³⁺-DFO standards (46) was slightly modified. 0.4 mM FeCl₃ was incubated with 20 mM DFO in PBS at 37 °C for 10 min, and the UV-visible spectrum of the product Fe³⁺-DFO was monitored for no further change of the spectrum to confirm the completion of the reaction. This is important because we have found that the spectrum still increases after a 10-min incubation of FeCl₃ with 1 mM DFO. The concentration of Fe³⁺-DFO was calculated based on the extinction coefficient ε = 2.865 mM⁻¹ cm⁻¹ at λmax = 420 nm. A yield of ≥85% based on the amount of FeCl₃ was obtained.
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All stock solutions of standards were diluted to at least five concentrations between 0 and 10 μM. All standards were immediately transferred to EPR tubes and were stable for at least 1 month at −80 °C.

Real-time ‘NO Measurement by ‘NO-Electrode—The ‘NO concentration was followed by an ‘NO-specific electrode (ISO-NOPF200) connected to an Apollo 4000 analyzer (World Precision Instruments Inc., Sarasota, FL). The electrode was placed into a thermostatic vessel through a glass capillary (inner diameter 1 mm), which was inserted through the rubber septum of the vessel. Another glass capillary of the same size was also inserted through the septum for ‘NO injection. Although the sealed vessel has access to the atmosphere through the glass capillaries, the oxygen exchange and ‘NO volatilization were negligible due to the small diameters of the capillaries. Anoxia was performed as described above except for using the amber vial as vessel. For normoxia, once the cell suspension was transferred from poly-HEMA plates and the vessel was filled and sealed without headspace, ‘NO could not be added until a stable baseline of the electrode was reached. This stabilization took at least 3 min; therefore, “normoxia” is relative due to the rapid O2 consumption by cells (35). However, as shown in Fig. 3A, the rapid ‘NO consumption indicates that significant anoxia has not been achieved under this condition. As explained under “Discussion,” this could explain the overestimation of DNIC formation from the bolus ‘NO addition under “normoxia” (2).

Continuous stirring was maintained, and ‘NO concentration was monitored throughout the experiments. Following the addition of oxyMb and the complete disappearance of ‘NO, the cell suspension was collected and processed for DNIC determination as described above.

Simultaneous Determination of DNIC and RSNO—Cells were pretreated with anoxia and briefly treated with ‘NO and SIH as described above for DNIC determination. After washing, cells were separated and resuspended in PBS for DNIC determination and in 50 mM potassium phosphate buffer (pH 7.4) containing 100 μM DTPA and 50 mM thiol-blocking reagent NEM for RSNO determination, respectively. RSNO samples were lysed by freeze-thaw followed by brief sonication. The whole cell lysate was stored on ice until it was measured on the same day to avoid any loss due to the freeze-thaw cycle, and the method of tri-iodide-coupled chemiluminescence was applied as described previously (47). To investigate the effect of NEM pretreatment on the DNIC content for comparison with RSNO, part of the whole cell lysate containing NEM was also used for the determination of DNIC (DNIC + NEM).

ROS Detection by H2DCF Oxidation—2 h of anoxia pretreatment and 2 min of ‘NO and SIH treatment were conducted using the same method as described above except that 25 μM H2DCFDA was added 30 min before the end of anoxia or normoxia. The cell suspension was immediately transferred to H2O2 (under open air) for a final concentration of 1 mM. 2 min later, the intracellular fluorescence of the oxidation product of H2DCF, DCF, was measured by flow cytometry (BD Biosciences) at λex = 488 nm and λem = 530 nm. It is not necessary to remove the extracellular DCF because flow cytometry measures intracellular fluorescence. The extra ‘NO has not been removed because ‘NO volatilizes and ‘NO consumption by cells dramatically increases when the cell suspension is transferred to open air (reoxygenation); thus, there will not be a significant amount of ‘NO left in the suspension to interfere with ROS generated by 1 mM H2O2.

Protein Assay—All protein concentrations were measured by the bicinchoninic acid assay as instructed by the manufacturer (Fisher).

RESULTS

DNIC from Brief ‘NO Exposure Is Increased by Anoxia Pretreatment—As mentioned above, when RAW 264.7 cells are exposed to PROLI/NO for 2 min, DNIC formation is barely detectable. However, as shown in Fig. 2, when cells are pretreated with anoxia for various times and then briefly exposed to ‘NO, DNIC formation increases dramatically and then slows after 2-h anoxia. We noticed that cell death (measured by trypan blue staining) initiates after 2-h anoxia (data not shown), which could contribute to the slower increase of DNIC. Because the ‘NO treatment is under anoxia, Fig. 2 also demonstrates that, similar to RSNO (35), DNIC formation does not require oxygen (O2). O2-independent DNIC formation has also been reported from cells exposed to endogenous ‘NO (2).

Although, strictly, our experimental condition in a sealed vial (< 0.1% O2) is anoxia, we have found the same increase of DNIC formation upon brief ‘NO exposure (same as in Fig. 2) in cells incubated in a hypoxic chamber with the oxygen level maintained at 1–2%. Therefore, our results reported as follows very likely apply to hypoxia (0.5–5% O2).

The Increase of DNIC Is Not Due to Higher ‘NO Concentration under Anoxia—‘NO consumption by cells is O2-dependent (35, 48), and thus under anoxia, cells consume ‘NO more slowly, resulting in increased ‘NO concentration within the solution (35). Similar to our result shown in Fig. 2, higher DNIC production from bolus ‘NO addition in hypoxic cells has been reported previously, and this increase was attributed to the higher ‘NO level (2). To test this possibility, we used an ‘NO electrode to follow the real-time ‘NO level after addition to the cell suspension pretreated with 1 h of anoxia or normoxia. As shown in Fig. 3A, with the same amount of ‘NO addition (10 μM), the ‘NO consumption of cells under normoxia is faster than that under anoxia, consistent with previous findings (35, 48). Consequently, there is more ‘NO present in the anoxic solution during the 2-min incubation, which could result in more DNIC.

FIGURE 2. EPR spectra of DNIC formed by brief ‘NO exposure in cells pretreated with anoxia. Into cells pretreated with anoxia for indicated times was injected 10 μM ‘NO from PROLI/NO. 2 min later, cells were processed for the determination of DNIC by EPR. Representative EPR spectra from more than 10 independent experiments are shown.

FIGURE 3. ‘NO concentration within the solution under anoxia. EPR was used to follow the ‘NO level after addition to the cell suspension pretreated with 1 h of anoxia or normoxia. As shown in Fig. 3A, with the same amount of ‘NO addition (10 μM), the ‘NO consumption of cells under normoxia is faster than that under anoxia, consistent with previous findings (35, 48). Consequently, there is more ‘NO present in the anoxic solution during the 2-min incubation, which could result in more DNIC.
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FIGURE 3. Real-time 'NO detection in cell suspension (A) and the DNIC formed consequently (B). After 1 h of anoxia (A) or normoxia (N), PROLI/NO releasing 2 or 10 μM 'NO was injected into cell suspension (defined as time 0), and 20 μM oxyMb was injected 2 min later. The 'NO level was monitored by the 'NO-specific electrode and was expressed in nmol/mg protein based on 1 mg/ml protein in the cell suspension (A); after 'NO was completely scavenged by oxyMb, the reaction mixture was immediately processed for DNIC measurement by EPR (B). Shown are representative 'NO polarographs (A) and DNIC quantitation (B) from at least three independent experiments. **, p < 0.001 compared with normoxia + 10 μM NO by one-way ANOVA. Error bars, S.D.

formation, as shown in Fig. 3B (0.26 ± 0.04 nmol/mg protein under anoxia versus 0.02 ± 0.02 nmol/mg protein under normoxia). However, when less 'NO (2 μM) is added to the anoxic cell suspension to provide the same overall level of 'NO as (or even a lower level than) that present in normoxic suspension (Fig. 3A), the DNIC formed (0.18 ± 0.03 nmol/mg protein) is still significantly higher than that formed under normoxia (Fig. 3B). Therefore, it can be concluded that the increase of DNIC formation is not due to the higher 'NO concentration under anoxia.

Actually, anoxia is achieved within 5–10 min (<0.1% O₂) under our experimental conditions (35) as described under “Experimental Procedures”; any change of O₂ concentration after 10 min would not be significant enough to affect the 'NO consumption. In other words, after 10 min, the same amount of 'NO addition at any time point will give rise to the same 'NO level. Therefore, the increase of DNIC with duration of anoxia beyond 10 min (Fig. 2) also indicates that it is not due to the change in 'NO concentration.

DFO-chelatable Iron Increases during Anoxia and Decreases during Reoxygenation—It has been reported that chelatable iron increases dramatically during hypoxia/anoxia (23–32). To test under our conditions whether the increase during anoxia of DNIC is due to the increase of chelatable iron, we simultaneously quantified the cellular level of chelatable iron and of DNIC from brief 'NO exposure after anoxia pretreatment.

The metal chelator DFO has been used to quantify chelatable iron because its iron adduct, Fe³⁺-DFO, shows a characteristic EPR signal at g = 4.3, which can be quantified by standard concentrations of Fe³⁺-DFO (2, 15, 46). However, as illustrated in Fig. 1, the prolonged incubation of cells with DFO could mobilize chelatable iron from other sources, resulting in an overestimation of the authentic level. Moreover, a rapid measurement is needed because, as described below, anoxia-induced chelatable iron disappears during reoxygenation (which is inevitable during the incubation with DFO), and DFO is only slowly taken up by cells (46). Therefore, we employed a modified method involving disrupting cell membranes in the presence of DFO to ensure the immediate access of DFO to cellular iron (24). As shown in Fig. 4, DFO-chelatable iron increases from 0.04 ± 0.02 to 0.19 ± 0.06 nmol/mg protein after 1 h of anoxia and more than 7 times to 0.29 ± 0.05 nmol/mg protein after 2 h of anoxia. It does not change significantly under normoxia up to 2 h (0.07 ± 0.04 nmol/mg protein).

It has also been reported that chelatable iron decreases during reoxygenation following the increase during hypoxia/anoxia (23, 24, 26, 27), although the time course has not been determined. Knowing how rapidly it decreases is important for defining the time frame of sample processing after hypoxia/anoxia. As shown in Fig. 5, a gradual decrease is found during reoxygenation following the increase during hypoxia/anoxia. As illustrated (Fig. 5, inset) gives a rate constant of 8.8 × 10⁻⁴ s⁻¹ and a half-life of 13 min. To our knowledge, this is the first time this decrease is first order, a logarithmic plot of the data (Fig. 5, inset) gives a rate constant of 8.8 × 10⁻⁴ s⁻¹ and a half-life of 13 min. To our knowledge, this is the first kinetic study on the disappearance of chelatable iron during reoxygenation.

The Increase of DNIC Correlates with That of Chelatable Iron—We further quantified the DNIC concentration using MbNO as
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FIGURE 5. Cellular level of DFO-chelatable iron during reoxygenation following anoxia. After 2-h anoxia and the indicated periods of reoxygenation, cells were processed for the determination of DFO-chelatable iron. Data are presented as the percentage using reoxygenation at 0 min as 100% (A2h in Fig. 4) and normoxia for 2 h as 0% (N2h in Fig. 4) and are from three independent experiments. Inset data were replotted based on the first order disappearance of DFO-chelatable iron and fitted to a linear regression as described under “Results.” The solid line in the main figure is based on the linear regression in the inset.

FIGURE 6. SIH inhibition of the formation of RSNO and DNIC. After 2 h of anoxia, cells were treated with or without 100 μM SIH for 2 min followed by 10 μM NO from PROLI/NO for 2 min. Then cells were processed and separated for simultaneous determination of DNIC by EPR and RSNO by tri-iodide-coupled chemiluminescence. The SIH effect was presented as the percentage of the amount of DNIC or RSNO without SIH addition. n = 4; *p < 0.05; **, p < 0.001 compared with RSNO or DNIC without SIH addition (100%) by one-way ANOVA. Error bars, S.D.

**SIH-chelatable Iron Is an Important Source of Iron for DNIC Formation**—We also examined the effect of metal chelator on DNIC formation. To avoid the movement between chelatable iron and other iron sources during extended incubation with metal chelator, we utilized SIH, which has been shown to enter the cell and bind calcine-chelatable iron within 1 min (15). SIH-chelatable iron is an important source of iron for DNIC formation. We further followed the kinetics of DNIC formation upon NO addition to examine how fast DNIC are formed. As shown in Fig. 7, once PROLI/NO is added to cells pretreated with 1 h of anoxia, DNIC appear as rapidly as a few s and increase dramatically within 20 s, and immediately after that, the rate of formation slows down, and the DNIC level reaches a plateau. Interestingly, this time course replicates that of NO liberation from PROLI/NO under the same condition. It can be seen in Fig. 7 that DNIC increases as NO concentration increases and reaches a plateau when PROLI/NO has released all NO, and the NO level remains relatively constant. The fact that DNIC is formed at a rate similar to the rate of NO release implies that DNIC formation is so rapid that it is limited by NO liberation from PROLI/NO.

More interestingly, the two time courses also replicate the time course of NO (also from PROLI/NO) reaction with calcine-chelatable iron that we have shown in our previous report (15), which also indicates that NO reaction with chelatable iron (forming DNIC) is limited by NO liberation from PROLI/NO. We consider that the rapidity and the concurrency of the three time courses argue against the possibility that a significant portion of DNIC is formed from the reactions of NO with other iron sources, because the movement between chelatable iron and other iron sources is unlikely to be as rapid.

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a standard. As shown in Fig. 4, DNIC from brief NO exposure increases from 0.006 ± 0.005 to 0.26 ± 0.04 nmol/mg protein after 1 h of anoxia and reaches 0.47 ± 0.06 nmol/mg protein after 2 h of anoxia. However, the same incubation under normoxia up to 2 h does not result in significant change in DNIC formation (0.03 ± 0.03 nmol/mg protein). More importantly, DNIC from brief NO exposure correlates well with the cellular level of chelatable iron under all conditions, indicating that chelatable iron is the iron source of DNIC. However, this is only correlative evidence. The increase of chelatable iron and DNIC by anoxia pretreatment allows accurate quantitation, providing a useful tool for further causative studies as follows. It should be mentioned here that we have shown previously that cellular DNIC formation requires free NO (15); therefore, chelatable iron is not reacting directly with PROLI/NO to form DNIC.

**DNIC Formation Is Simultaneous with NO Reaction with DFO-chelatable Iron**—In parallel to the determination of DNIC, we compared the level of DFO-chelatable iron before and after brief NO exposure in cells pretreated with 2 h of anoxia. As shown in Fig. 4, brief NO exposure reduces DFO-chelatable iron to a level that is not statistically different from that in normoxic cells (0 h and N2h in Fig. 4), indicating that NO rapidly reacts with the increased DFO-chelatable iron so that it is not available for DFO binding. This result is consistent with our previous data suggesting that NO reacts with calcine-chelatable iron (calcine is a metal chelator) within 2 min (15).

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The basal iron detected by DFO that is not prevented by cell NO treatment may be due to an iron species that is unavailable to NO added to intact cells (which is removed by oxyMb addition before lysis) and which is then liberated (accessible to DFO) by the cell disruption process.
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**TABLE 1**

DNIC and RSNO (nmol/mg protein) formed by brief 'NO exposure in cells pretreated with anoxia

| Pretreatment | DNIC (pmol/mg protein) | RSNO (pmol/mg protein) |
|--------------|------------------------|------------------------|
| 0 h          | Under detection limit  | Under detection limit  |
| Anoxia 2 h   | 0.47 ± 0.06            | 0.22 ± 0.09            |

After 0 and 2 h of anoxia, cells were treated with 10 μM 'NO from PROLI/NO for 2 min. Then cells were separated for the determination of DNIC by EPR, and the rest were freeze-thawed in the presence of NEM. Samples with NEM treatment were then separated for the determination of DNIC by EPR (DNIC + NEM) and RSNO by chemiluminescence. Data represent mean ± S.D. from 3–6 independent experiments. *, **p < 0.05; ***, **p < 0.001 compared with DNIC by one-way ANOVA.

Fig. 7 also shows that the amount of 'NO that is liberated by PROLI/NO is in great excess of the amount consumed by DFO-chelatable iron and/or in DNIC formation. 'NO that is liberated by PROLI/NO is 10 nmol/mg protein because we measured 1 mg/ml protein for 1 × 10⁷ cells/ml suspension. On the other hand, assuming a 2:1 ratio of 'NO to chelatable iron or DNIC, 'NO consumed by DFO-chelatable iron (0.19 nmol/mg protein) is only 0.38 nmol/mg protein; 'NO consumed in DNIC formation (0.26 nmol/mg protein by 2-min incubation) is 0.52 nmol/mg protein. Due to the relatively small amount of 'NO consumed, we are unable to quantitatively obtain the 'NO/chelatable iron stoichiometry by comparing the difference in peak height of 'NO liberation with and without cells. In addition, we cannot be certain that all of the 'NO that disappears is due only to DNIC formation.

**Simultaneous Formation of DNIC and RSNO**—After anoxia pretreatment and brief 'NO exposure, we simultaneously measured both DNIC and RSNO in cells. Surprisingly, RSNO is formed in parallel with DNIC, and its abundance (0.22 ± 0.09 nmol/mg protein) is comparable with that of DNIC (Table 1). This abundance is even higher than that formed by prolonged 'NO exposure under either hypoxia or normoxia conditions (1–100 pmol/mg protein) (2, 35). These levels are also comparable with those induced by transnitrosation from nitrosocysteine and competitive with DNIC for the most abundant cellular adduct of 'NO (49).

We have previously shown that DNIC and RSNO correlate well under various conditions (35). Besides the conversion of DNIC to RSNO that has been suggested (35), it has also been suggested that the standard method of determining RSNO (47) may actually detect DNIC or a portion of it. Pretreatment with NEM, a thiol blocker, is necessary for RSNO detection in order to avoid false RSNO generation during sample preparation from unreacted RSH and nitrite in an acidic solution (47). This procedure includes lysing cells in the presence of NEM by freeze-thaw immediately after cell collection. In addition to RSNO, we also determined DNIC content from these samples. As shown in Table 1, NEM pretreatment disrupts more than half of DNIC (DNIC versus DNIC + NEM). The rest of DNIC (0.20 ± 0.02 nmol/mg protein) is at about the same level as RSNO. Therefore, there are two types of DNIC, NEM-reactable and NEM-resistant; the standard method of RSNO measurement may also detect NEM-resistant DNIC.⁷

Also, similar to DNIC, the RSNO generated from brief 'NO exposure to normoxic cells is under our detection limit (Table 1). The rapidity of RSNO formation and its increase by anoxia pretreatment indicate that, similar to DNIC formation, this RSNO formation also requires chelatable iron. In parallel to DNIC formation, we also examined the SIH effect on RSNO formation. Fig. 6 shows that the addition of 100 μM SIH 2 min prior to 'NO addition inhibits about 30% of RSNO formation in cells pretreated with 2 h of anoxia. In contrast, more than 60% of DNIC formation is inhibited by SIH. As presented under “Discussion,” this result suggests that, at least to some extent, DNIC are responsible for RSNO formation.

**Iron Sequestration by ‘NO Inhibits Fenton-induced ROS Generation**—To induce oxidative chemistry from the Fenton reaction, we added H₂O₂ to cells with elevated levels of chelatable iron by anoxia pretreatment. ROS was detected by the oxidation of H₂DCF that is cleaved from H₂DCFDA by intracellular esterases (50). H₂O₂ does not directly react with H₂DCF (50, 51); therefore, it will not interfere with the detection. In addition, the ROS referred to here are those reactive with H₂DCF that are induced by but do not include H₂O₂. The H₂O₂ addition is important because, in the absence of H₂O₂, the ROS...

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⁷The possibility that a major portion of the chemiluminescence signal that we identify as RSNO is actually DNIC is not supported by the finding that when 'NO is removed from cells, the chemiluminescence signal (RSNO) rapidly declines, whereas the DNIC EPR signal only slowly decays (2).
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FIGURE 8. NO and SIH effects on H₂DCF oxidation in the presence of H₂O₂. After 2 h of anoxia (A) or normoxia (N), cells were treated with 10 μM NO from PROLI/NO or 100 μM SIH for 2 min. 25 μM H₂DCFDA was added 30 min prior to the end of anoxia or normoxia. Cells were transferred to H₂O₂ or vehicle (H₂O₂) for a final concentration of 1 mM. 2 min later, intracellular DCF fluorescence was measured by flow cytometry. Data represent mean ± S.D. (error bars) from three independent experiments. *, p < 0.05; **, p < 0.01 compared with anoxia + H₂O₂ by one-way ANOVA.

generation is not significantly affected by anoxia pretreatment under our conditions (Fig. 8). This is different from the work by Duranteau et al. (52), where an increase of intracellular DCF fluorescence was found in hypoxic cardiomyocytes. In addition to the difference in cell type, it could be that they applied ≈1% O₂, whereas under our conditions, O₂ is depleted (<0.1%) to the limit of mitochondrial oxygen extraction. To minimize ROS generation via non-Fenton mechanisms, especially when a decrease in chelatable iron occurs rapidly during reoxygenation, as shown in Fig. 5, we measured ROS 2 min after H₂O₂ addition. As shown in Fig. 8, H₂O₂-induced ROS generation is increased significantly by anoxia pretreatment, consistent with the increase of chelatable iron during anoxia and Fenton chemistry as the major mechanism for ROS production. SIH addition 2 min prior to H₂O₂ addition completely inhibits the increase, which demonstrates that the increase of ROS generation is due to the increase of chelatable iron, which catalyzes Fenton chemistry. More importantly, brief NO treatment prior to H₂O₂ addition also abolishes the increase of ROS, simulating the SIH effect, thereby demonstrating that NO prevents Fenton-induced ROS generation by iron sequestration.

DISCUSSION

The DFO-chelatable iron in RAW 264.7 cells has previously been reported as 0.190 ± 0.055 nmol/mg protein from a 1-h incubation with 2 mM DFO (15) and 0.260 ± 0.050 nmol/mg protein from a 4-h incubation with 1 mM DFO (2). As mentioned in the Introduction, prolonged incubation with DFO at such high concentrations could mobilize iron from other sources into the chelatable iron pool (Fig. 1), thereby resulting in an overestimation of the basal level of chelatable iron. Indeed, relatively long term incubation with DFO (and also NO) elicits an “iron starvation” response that interrupts iron homeostasis (53). To avoid this prolonged incubation, we applied a method involving disruption of cell membrane, thereby allowing DFO to access iron rapidly and avoiding effects on cellular iron homeostasis (24). We detected only 0.04 ± 0.02 nmol/mg protein of DFO-chelatable iron in RAW 264.7 cells using this method.

Many studies have indicated the increase of chelatable iron during hypoxia/anoxia/ischemia (23–32); however, quantitation has only been reported by two groups. One group used molar concentrations of nitrite in an unbuffered solution in order to form DNIC for quantitation (54), a treatment that is likely to perturb and liberate iron from normally masked pools. Similar to our method, another group lysed cells in the presence of DFO and measured the Fe³⁺-DNIC adduct by high performance liquid chromatography (23–28). Using this method, up to a 5-fold increase of chelatable iron was reported, depending on the cell type and the length of hypoxia/anoxia/ischemia (23–28). Consistently, we find that DFO-chelatable iron increases about 5 times to 0.19 ± 0.06 nmol/mg protein after 1 h of anoxia and more than 7 times to 0.29 ± 0.05 nmol/mg protein after 2 h of anoxia. These values are similar to those obtained from prolonged incubation with DFO under normoxia (2, 15), suggesting that the increased chelatable iron under anoxia comes from the same source as mobilization with prolonged DFO incubation. This could also explain why the DFO-chelatable iron measured by prolonged incubation was maintained at the same level during hypoxia (2), which is inconsistent with many other reports (23–32).

The source of the increased chelatable iron during hypoxia/anoxia is unknown (54). A recent study based on fluorescent imaging has suggested that during hypoxia, lysosomes release chelatable iron to the cytosol, which in turn translocates into mitochondria (29). Our data on H₂DCF oxidation are consistent with translocation of chelatable iron from lysosomes to the cytosol (29). We point out that although the use of DCF as a probe has been seriously criticized (51), the major complication in our case is in fact a major advantage, namely the dependence of response on the presence of cellular iron as catalyst (50). H₂DCF has been shown to be oxidized only by a Fenton-type reaction or by cytochrome c (50). The SIH effect shown in Fig. 8 rules out the involvement of cytochrome c in the increase of ROS generation by anoxia pretreatment.

If subcellular translocation is the mechanism of the increase of chelatable iron, the total cellular level of chelatable iron should remain the same during hypoxia/anoxia. Instead, Fig. 4 has shown an increase of DFO-chelatable iron. As mentioned in the Introduction, the properties of the chelatable iron that are chelated by different chelators could be different; therefore, DFO-chelatable iron does not represent the total level of chelatable iron. In addition, our method of freeze-thawing and rather mild sonication may not be sufficient to release lysosomal iron to become chelated by membrane-impermanent DFO. If chelatable iron, despite its subcellular location (lysosome, cytosol, or mitochondria), is equally reactive to NO forming DNIC, there will be no DNIC increase after anoxia pretreatment. In contrast, we have shown an increase in Fig. 2, which indicates that the chelatable iron in lysosomes may not be active for DNIC formation, possibly due to its acidic environment.

The decrease of chelatable iron during reoxygenation following an increase during hypoxia/anoxia has also been reported.
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(23, 24, 26, 27); however, how fast chelatable iron decreases has not been studied. Here we have shown that under our conditions, the half-life of this decrease is ∼13 min. In light of the demonstration of increased Fenton-mediated ROS formation resulting from the increase of chelatable iron during anoxia, the mechanism(s) of chelatable iron reuptake upon reoxygenation may have critically important protective roles in conditions such as ischemia/reperfusion injury.

We have shown that ¹⁷⁷⁷NO reacts with DFO-chelatable iron as rapidly as we can measure, and cellular DNIC is formed in parallel, which can be inhibited by a metal chelator added 2 min prior to ¹⁷⁷⁷NO addition. Our kinetic study shows that the DNIC formation is so rapid that it is limited by ¹⁷⁷⁷NO liberation from donor. Overall, we here provide strong evidence for cellular DNIC generation from the ¹⁷⁷⁷NO reaction with chelatable iron, which has been claimed previously (15–18). In addition to RAW 264.7 cells, we have also observed the increase of DNIC formation upon brief ¹⁷⁷⁷NO exposure by anoxia pretreatment with isolated hepatocytes, bovine aortic endothelial cells, and human tumor cell model Tr7H cells (55) (data not shown), suggesting that this mechanism of DNIC formation may be ubiquitous. The rapidity of DNIC formation indicates that it is very likely an iron chelation by ¹⁷⁷⁷NO similar to the coordination between iron and chelators. Moreover, our result implies that DNIC formation in vivo is so rapid that it is limited by the availability of ¹⁷⁷⁷NO and chelatable iron. It has been reported that extracellular oxyMb almost completely inhibits DNIC formation from exogenous ¹⁷⁷⁷NO but not from endogenous ¹⁷⁷⁷NO, which indicates that DNIC formation is so rapid that the rate of ¹⁷⁷⁷NO reaction to form DNIC is rapid enough to compete with the escape from the cell by diffusion (2, 56) (although opposite results using hepatocytes have been presented (57)). We hasten to add that paramagnetic DNIC formation from chelatable iron and ¹⁷⁷⁷NO is more complex than a simple binding reaction because it requires one-electron oxidation (36). The mechanism and identity of the oxidant are unknown.

We have previously shown that ¹⁷⁷⁷NO and SIH rapidly restore calcein fluorescence that is quenched by calcein-chelatable iron (15). We here show the sequestration of DFO-chelatable iron by ¹⁷⁷⁷NO and the inhibition of DNIC formation by SIH. All of these findings indicate that ¹⁷⁷⁷NO and metal chelators, including calcein, DFO, and SIH, share the same chelatable iron pool.

SIH did not completely inhibit DNIC formation, similar to our previous report using prolonged ¹⁷⁷⁷NO exposure (15, 35). A likely explanation for this result lies in the different binding equilibria for ¹⁷⁷⁷NO compared with SIH (Fig. 1). As we report here (and also as shown previously by Hickok et al. (2)), formation of DNIC is rapid, essentially stoichiometric with the chelatable iron pool, and only slowly decays upon ¹⁷⁷⁷NO removal. Chelation by SIH, however, although of high affinity (58), is reversible, so the ¹⁷⁷⁷NO will, by Le Châtelier’s principle, result in extraction of iron from the SIH complex into DNIC. It is also possible that ¹⁷⁷⁷NO binds initially to one transiently free ligation site of the iron when it is partially coordinated by SIH, and subsequent binding of a second ¹⁷⁷⁷NO results in complete dissociation of the iron from SIH and formation of DNIC. Either of these possibilities is evidenced by our previous finding that ¹⁷⁷⁷NO will rapidly extract iron from its calcein complex (15). Another explanation is that about 40% of DNIC formation is not mediated by SIH-chelatable iron; however, other data that we report here argue against this.

The levels of DNIC from short term administration of exogenous ¹⁷⁷⁷NO that we measure here are significantly different from those reported previously by Hickok et al. (2). Specifically, substantially higher levels were observed (up to 0.5 nmol/mg protein) and a linear response to increasing ¹⁷⁷⁷NO concentration. We do not have a definitive explanation for these differences; however, we have observed that cells become anoxic quite quickly when placed in a sealed vial without headspace, which was utilized by Hickok et al. (2) in the bolus ¹⁷⁷⁷NO titration experiments.

From all of the above findings, this study has shown a series of chemical and biological differences between prolonged and brief exposure to either ¹⁷⁷⁷NO or metal chelators. Due to the rapid conversion of chelatable iron to DNIC upon brief ¹⁷⁷⁷NO exposure, we have developed a method of DNIC detection following brief ¹⁷⁷⁷NO exposure for the quantitation of chelatable iron. This method is simple, rapid, real-time, and quantitative and only minimally perturbs iron homeostasis. In addition, a comparison can only be made between samples containing the same oxygen level when ¹⁷⁷⁷NO is added because the ¹⁷⁷⁷NO consumption by cells (which will influence the ¹⁷⁷⁷NO concentration) is oxygen-dependent.

We utilized the ability to manipulate DNIC levels to probe two potential cellular functions, RSNO formation and inhibition of Fenton oxidative chemistry. We find a dramatic increase in RSNO levels concomitant with anoxia-induced DNIC increase, providing strong evidence that in cells this process involves transnitrosation from DNIC to RSH (which is consistent with previous in vitro studies (36)). The levels of RSNO are quantitatively similar to DNIC, making both RSNO and DNIC the most abundant cellular adducts of ¹⁷⁷⁷NO (at least post-anoxic treatment).

SIH inhibits DNIC and RSNO formation to different extents. Comparison of these numbers might seem to suggest that the majority of RSNO formation is not mediated by SIH-inhibitable DNIC; however, without mechanistic knowledge of the process of transnitrosation from DNIC to RSH, it is hazardous to assume a 1:1 stoichiometric requirement and that, for example, a 50% decrease in DNIC should result in a 50% decrease in RSNO. In addition, especially in light of the heterogeneity in both DNIC and RSNO intracellular species, it is possible that the portion of DNIC that is not inhibited by SIH is responsible for major RSNO formation. This highlights the need for studies on the heterogeneity in DNIC and RSNO. A similar SIH effect has been shown on DNIC and RSNO formation from prolonged ¹⁷⁷⁷NO exposure (35). Again, however, ¹⁷⁷⁷NO plays such diverse roles that over this prolonged time, the downstream reactions become more complex and may interact with the formation and metabolism of DNIC and RSNO.

Also using this system, we show that the rapid chelatable iron sequestration by ¹⁷⁷⁷NO inhibits Fenton-induced ROS production. This result suggests that iron sequestration by ¹⁷⁷⁷NO is an important mechanism for ¹⁷⁷⁷NO protection against iron-induced oxidative stress. In particular, a biologically and clinically relevant condition that our experimental system simulates is hypoxia/reoxygenation or ischemia/reperfusion (59). Oxida-

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tive stress is largely involved in hypoxia/reoxygenation-induced injury (60, 61). Metal chelation has been found to ameliorate ischemia/reperfusion-induced injuries (29, 61–63), indicating the important role of the elevated iron during hypoxia (23–32). In addition, NO has been shown to prevent hypoxia/reoxygenation-induced injury (64–67).

Finally, these results suggest possible biological functions during early carcinogenesis of the g = 2.03 EPR signal first reported 50 years ago (9–11), specifically increases in cellular chelatable iron and RSNO levels. The mechanistic importance of these phenomena in cancer development is unknown.

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