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Original Contribution

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 (•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 •NO-derived adducts (900 pmol/mg protein, or 45–90 μM). Using murine macrophages (RAW 264.7), we measured the amounts, and kinetics, of DNIC assembly and disappearance from endogenous and exogenous sources of •NO in relation to iron and O_2 concentration. Amounts of DNIC were equal to or greater than measured amounts of chelatable iron and depended on the dose and duration of •NO exposure. DNIC formation paralleled the upregulation of iNOS and occurred at low physiologic •NO concentrations (50–500 nM). Decreasing the O_2 concentration reduced the rate of enzymatic •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 •NO source was removed (>24 h). These results demonstrate that DNIC will be formed under all cellular settings of •NO production and that the contribution of DNIC to the multitude of observed effects of •NO must always be considered.

Nitric oxide (•NO, nitrogen monoxide) is an endogenously produced diatomic free radical and biological signaling molecule [1]. Its unique physical and chemical properties dictate that under biological conditions it reacts with only 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 O_2 concentration had much less of an effect on DNIC formation and degradation than they 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

Abbreviations: CIP, chelatable iron pool; 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; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MbO_2, oxymyoglobin; •NO, nitric oxide; NO_2^-, nitrite; NO_3^-, nitrate; RSNO, *S*-nitrosothiol; Sper/NO, (*Z*)-1-[*N*-(3-aminopropyl)-*N*-(4-(3-aminopropylammonio)butyl)amino]diazen-1-ium-1,2-diolate.

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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-free DMEM (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% fetal bovine serum and 1% penicillin/streptomycin. For DNIC measurements, cells were plated at 15×10^6 cells per 15-cm culture dish. To upregulate iNOS, cells were treated with LPS (1 μg/ml). For hypoxic experiments (O_2<21%), cells were cultured in a hypoxic chamber glove-box (Coy Scientific) and maintained at 37 °C, 5% CO_2. Ambient oxygen concentration (%O_2) was continuously monitored and adjusted (±0.2% O_2) by purging with nitrogen gas.

Cell viability

RAW 264.7 viability was monitored by the reduction of alamarBlue to its fluorescent product ($\lambda_{ex/em}$ = 550/595) [14] after 24 h.

Western blots

Protein was isolated with CelLytic (Sigma) containing 1 mM phenylmethylsulfonyl fluoride and 10 μl/ml Protease Inhibitor Cocktail Set III (Calbiochem, San Diego, CA, USA). Lysates were centrifuged at 12,000 *g* for 15 min. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes and blocked with 5% nonfat dry milk in 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, Santa Cruz, CA, USA). Blots were incubated for 1 h with 1:2000 horseradish peroxidase-conjugated secondary anti-rabbit antibody (Cell Signaling Technology, Beverly, MA, USA). Transferred proteins were visualized using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) and images were captured with a Fluorchem HD2 Chemimager (Alpha Innotech).

3-Nitrotyrosine

3-Nitrotyrosine was quantified using an OxiSelect nitrotyrosine ELISA kit according to the manufacturer's protocols. Treated samples from 15-cm culture dishes were harvested with CelLytic (Sigma) lysis solution containing 1 mM phenylmethanesulfonyl fluoride and 10 μl/ml Protease Inhibitor Cocktail Set III (Calbiochem). Protein concentrations were determined by Bradford assay, and 50 μl of 1.5 μg/μl protein was 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 O_2 electrodes connected to an Apollo 4000 free radical analyzer (World Precision Instruments, Sarasota, FL, USA). Headspace in the vessel was negligible compared to the vessel volume to ensure 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 using an •NO-specific electrode (Amino-700, response time of <0.2 s, sensitivity of 25 nM; Innovative Instruments, Tampa, FL, USA). Upon disappearance of the •NO, the cells were immediately removed and processed for electron paramagnetic resonance (EPR) analysis.

Real-time •NO measurements

LPS-stimulated RAW 264.7 cells

Cells in 15-cm plates at 21 or 1% O_2 were treated with 1 μg/ml LPS. After 12 h the cells were washed (3× with phosphate-buffered saline (PBS)) and the medium was replaced with arginine-free medium. An •NO electrode was then positioned using a stereotactic apparatus ~1 mm from the monolayer and allowed to equilibrate for 2 h. •NO synthesis was initiated by addition of 1 mM arginine to the medium.

NONOate-treated cells

An •NO electrode was positioned ~1 mm from the monolayer and allowed to equilibrate for 2 h, followed by addition of NONOates (n = 3).

Nitrate, nitrite, and S-nitrosothiol measurements

NO_3^-, NO_2^-, and *S*-nitrosothiols (RSNO) were measured by chemiluminescence (Sievers nitric oxide analyzer, NOA 280i) according to the manufacturer's protocols and as previously described [15]. Briefly, aliquots of medium 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 NO_3^-/NO_2^-. RSNO concentrations were measured by the tri-iodide method [15].

EPR determination of DNIC and CIP

For DNIC quantification, cells were trypsinized and resuspended in equal volumes of PBS. DNIC signals were measured by EPR g = 2.03 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 2 mW, modulation amplitude 20 G, and time constant 1 s. A total of four scans were obtained for each spectrum. DNIC concentrations were estimated by comparing the signal to that of a known standard of nitrosylmyoglobin (•NO-myo) [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 1 mM 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 were g = 4.3, microwave power 2 mW, modulation amplitude 25 G, time constant 1 s. A total of 16 scans were obtained for each spectrum.