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Induction of iron regulatory protein 1 RNA-binding activity by nitric oxide is associated with a concomitant increase in the labile iron pool: implications for DNA damage

Pawel Lipinski[a,*], Rafal R. Starzynski[a], Jean-Claude Drapier[b], Cecile Bouton[b], Teresa Bartlomiejczyk[c], Barbara Sochanowicz[c], Ewa Smuda[a], Agnieszka Gajkowska[d], Marcin Kruszewski[c,d]

[a] *Department of Molecular Biology, Institute of Genetics and Animal Breeding, Polish Academy of Sciences, Jastrzebiec, ul. Postepu 1, 05-552 Wolka Kosowska, Poland*
[b] *Institut de Chimie des Substances Naturelles, CNRS, 91190 Gif-sur-Yvette, France*
[c] *Department of Radiobiology and Health Protection, Institute of Nuclear Chemistry and Technology, 03-195 Warszawa, ul. Dorodna 16, Poland*
[d] *Department of Experimental Hematology and Cord Blood Bank, Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, 02-781 Warszawa, ul. W.K. Roentgena 5, Poland*

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Abstract

Iron regulatory protein 1 (IRP1) is a bifunctional [4Fe–4S] protein that controls iron homeostasis. Switching off its function from an aconitase to an apo-IRP1 interacting with iron-responsive element-containing mRNAs depends on the reduced availability of iron in labile iron pool (LIP). Although the modulation of IRP1 by nitric oxide has been characterized, its impact on LIP remains unknown. Here, we show that inhibition of IRP1 aconitase activity and induction of its IRE-binding activity during exposure of L5178Y mouse lymphoma cells to NO are associated with an increase in LIP levels. Removal of NO resulted in a reverse regulation of IRP1 activities accompanied by a decrease of LIP. The increased iron burden in LIP caused by NO exacerbated hydrogen peroxide-induced genotoxicity in L5178Y cells. We demonstrate that the increase in LIP levels in response to chronic but not burst exposure of L5178Y cells to NO is associated with alterations in the expression of proteins involved in iron metabolism.

Keywords: Cytosolic aconitase; IRP1; LIP; Iron metabolism; Nitric oxide; L5178Y cells

A stringent control of cellular iron metabolism involves co-ordinated reciprocal changes in the expression of H- and L-chain ferritin (Ft), and transferrin receptor (TfR) via regulation of mRNA translation and transcript stability, respectively. The underlying molecular mechanism is based on specific interaction of cytosolic *trans*-acting iron regulatory proteins (IRP1 and IRP2) with highly conserved stem-loop RNA structures called iron-responsive elements (IREs) found in the relevant mRNA transcripts [1]. Iron-dependent regulation of the IRE-binding activity of IRP1 seems to be determined by labile iron pool (LIP), a cytosolic collection of low molecular-weight iron complexes [2]. It represents the primary source of metabolic iron to be imported into mitochondria for heme synthesis and incorporated in Fe–S proteins. On the other hand, LIP also constitutes a pool of cellular iron able to catalyze the formation of highly toxic oxygen-derived species [2]. At high LIP levels, IRP1 undergoes modification through assembly of its [4Fe–4S] cluster, and switches from an IRE-binding protein to an aconitase, a cytosolic form of

* Corresponding author. Fax: +48 22 756 14 17.
E-mail address: P.Lipinski@ighz.pl (P. Lipinski).

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the mitochondrial enzyme. Reactivation of IRE-binding activity following depletion of iron from LIP is due to the deficient synthesis and/or assembly of [4Fe–4S] clusters and corresponds to the accumulation of newly synthesized apo-IRP1 [1].

Apart from iron deficiency, NO proved to be the first biological factor able to increase binding of IRP1 to IRE [3,4] and consequently, to repress Ft [4,5] and increase TfR mRNA expression [5]. Two hypotheses have been put forward to explain NO-induced IRP1 activation. The first states that NO rapidly activates IRP1 through direct interaction with its [4Fe–4S] cluster [6]. An alternative proposal suggests that NO, like desferrioxamine (DFO), an iron chelator, may have an indirect effect on IRP1 RNA-binding activity by depleting intracellular iron [7]. DFO is known to both activate IRP1 and decrease LIP level [2,8] but the relationship between NO and LIP has thus far received little attention [9,10]. Here, in order to clarify the relationship between NO, IRP1 activities, and LIP level, we exploited two closely related mouse lymphoma L5178Y (LY) sublines (LY-R and LY-S cells) that display distinct patterns of iron metabolism regulation [8]. We found that irrespective of their different steady-state LIP and IRP1 statuses, NO induced, in both LY-R and LY-S cells, a rise in LIP levels correlated with activation of the IRE-binding activity of IRP1. Furthermore, we showed that this NO-induced rise in LIP is a major factor strongly aggravating hydrogen peroxide (H_2O_2)-induced genotoxicity, especially in LY-R iron-rich cells. We also made an attempt to identify likely regulatory mechanisms contributing to NO-mediated movement of iron to cytosolic LIP under conditions of burst or chronic exposure to NO.

Materials and methods

Reagents. SpermineNONOate, (*Z*)-1-[*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]-amino]diazen-1-ium-1,2-diolate (SPER/NO) and diethylenetriame NONOate, (*Z*)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA/NO) were from Cayman Chemical (Ann Arbor, MI). Calcein acetoxymethyl ester was from Molecular Probes Inc (Eugene, OR). [^{32}P]CTP and [^{32}P]dCTP were from NEN Life Science Products-DuPont (Bad Homburg, Germany). All other chemicals were from Sigma (St. Louis, MO).

Cells. The L5178Y (LY) cellular model consists of two murine leukemic T lymphoblast lines. A maternal, LY-R line is a transplantable murine leukemia cell line derived from a thymic tumor induced in DBA/2 mouse by methylholantrene. A mutant, LY-S line was established from the maternal line after long-term in vitro culture [11]. LY cells were maintained in suspension cultures in Fischer's Medium (Gibco, UK) supplemented with 8% adult bovine serum and antibiotics, as described previously [12].

Exposure of LY cells to NO donors. The mouse L5178Y lymphoblasts were incubated in medium supplemented with 25 μM SPER/NO at a density of $3–4 \times 10^5$ for the indicated periods. After the end of 2-h exposure to SPER/NO, LY cells were extensively washed and further cultured in a fresh medium for the indicated periods. Incubation of cells with 100 μM DETA/NO was performed for 16 h. Control cells were cultured in parallel in the absence of DETA/NO and collected for analysis after 16-h incubation.

Determination of aconitase activity. Aconitase activity in cellular cytosolic extracts was measured spectrophotometrically by following the disappearance of *cis*-aconitate at 240 nm at 37 °C, as described previously [13].

Electrophoretic mobility shift assay. IRP1–IRE interactions were examined as described previously [14] by incubating 2 μg of the cytosolic protein extracts with a molar excess of [^{32}P]CTP-labelled H-ferritin IRE probe. In parallel experiments, cytosolic extracts were treated with 2-mercaptoethanol (2-ME) at a final concentration of 2% before addition of the IRE probe to produce maximal IRE-binding activity [15]. IRE–protein complexes were then separated by electrophoresis on 6% non-denaturing polyacrylamide gels. The signals representing the IRE-IRP1 complexes were quantified with a Molecular Imager using Quantity One software (Bio-Rad, Munich, Germany).

Determination of LIP. LIP was measured using isonicotinoyl salicylaldehyde hydrazone (SIH), a highly permeant iron chelator (kindly provided by Prof. P. Ponka, Montreal, Canada), and the fluorescent metal sensor calcein as previously described [16].

Comet assay. Initial DNA damage was determined by the alkaline version of the 'comet' assay, performed as described previously [17]. Briefly, approximately 0.5 ml of cell suspension was mixed with low melting point agarose (final concentration 1%) and cast on a microscope slide. After cell lysis the slides were allowed to unwind in fresh electrophoretic buffer (1 mM Na_2EDTA and 300 mM NaOH, pH > 13) and electrophoresed (1.2 V/cm, 30 min, 10 °C). Pictures of 100 randomly selected comets per slide were captured. Image analysis was performed using a Comet v.3.0 (Kinetic Imaging, Liverpool, UK). DNA damage is expressed as a tail moment (fraction of DNA in the comet tail multiplied by tail length).

Western blotting. Samples of 50 μg of protein from cell cytosolic extracts were resolved on 12% SDS/polyacrylamide gels and electroblotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Ft subunits were detected with rabbit antisera specific for recombinant mouse H-Ft- and L-Ft, respectively. Recombinant mouse H-Ft and L-Ft were used as controls. Dr. P. Santambrogio, Milan, Italy, kindly provided anti-Ft antisera as well as the recombinant proteins. Ft peptides were detected using an ECL chemiluminescence kit (Amersham Life Sciences, Little Chalfont, UK).

Northern blotting. Total RNA was obtained using TRIzol (Gibco, UK) according to the manufacturer's protocol. RNA samples (10 μg) were electrophoresed through denaturing agarose gels (1%), transferred onto a Hybond-N nylon membrane (Amersham), and UV cross-linked. After prehybridization, the membranes were hybridized overnight at 42 °C with random-primed [^{32}P]dCTP-labelled cDNA probe: for mouse heme oxygenase 1 (HO-1) (provided by Dr. R. Tyrrell, University of Bath, Bath, UK), TfR (kindly provided by Dr. L.C. Kühn, ISREC, Epalinges, Switzerland). Membranes were stripped and reprobed with random-primed [^{32}P]dCTP-labelled cDNA probe for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH). After overnight exposure, all blots were visualized using a Molecular Imager (Bio-Rad).

Statistical analysis. Statistical evaluation was done with Statistica software (StatSoft, Tulsa, OK). Significance of the difference in mean values was estimated using Student's *t* test for independent measurements.

Results

The relationship between NO, IRP1 activities, and LIP level was first assessed under conditions of the burst exposure of the two cell lines to NO using SPER/NO, which spontaneously decomposes in solu-

tion to release bioactive NO· with a half-life of 39 min [18]. We performed a kinetic experiment, monitoring in parallel IRP1 activities and LIP levels throughout a short-term, 2-h exposure of LY cells to SPER/NO. In order to further characterize the relationship between NO-dependent modulation of IRP1 activities and LIP levels, we designed another kinetic study to examine the period following the removal of SPER/NO from the LY cell culture medium. As shown in Fig. 1, NO induced an increase in IRP1 binding to IRE (Fig. 1A) with concomitant inhibition of aconitase activity in LY cells (Fig. 1B). This NO-mediated modulation of IRP1 activities was associated with a parallel rise in LIP levels (Fig. 1C). A comparison of the results obtained with LY-R and LY-S cells reveals two distinct temporal and quantitative patterns of close correlation connecting the NO-mediated increase in IRP1/IRE complex formation activity with the changes in LIP levels. Modulation of IRP1-binding activity by NO in LY-R cells was observed after only 30 min of incubation with SPER/NO and continued to be gradually enhanced at later time-points, i.e., 1 and 2 h. In contrast, in LY-S cells a significant increase in IRP1 IRE binding was achieved after only 2 h and inhibition of c-aco activity after 1 h. In the two cell lines, the increase in LIP levels followed the respective changes in the RNA-binding activity of IRP1. After stopping NO exposure, we consistently observed a synchronized return to the normal state, i.e., deactivation of IRP1–IRE binding, restoration of aconitase activity, and normalization of LIP levels in the two LY cell lines. However, differences between LY-R and LY-S cells were revealed with respect to the time-course of the resumption of basal IRP1 activities after removing the supply of NO. In LY-S cells, a return to normal activity was achieved within 6 h after the end of NO exposure vs 12 h in LY-R cells. The decline in LIP strictly paralleled the decrease in IRP1 IRE-binding activity and the resumption of its aconitase activity.

Two-hour exposure of LY cells to SPER/NO resulted in little DNA damage. However, in LIP-rich LY-R cells, pre-exposure to NO potentiated the DNA damage induced by subsequent treatment with H_2O_2 (Fig. 2A). In contrast, in LIP-scarce LY-S cells the effect was not statistically significant (Fig. 2B). Treatment with an iron chelator (SIH) diminished the DNA damage induced by H_2O_2, in both NO-pre-exposed and control cells.

The effect of NO on IRP1 activities and LIP in the two LY cell lines was also assessed under conditions of chronic exposure to NO. For this purpose we used DETA/NO, which spontaneously decompose in solution to release bioactive NO· with a half-life of 20 h [18], and has previously been used to mimic a chronic exposure of mammalian cells to NO [9,19]. As shown in Fig. 3, 16-h incubation of LY cells with DETA/NO resulted in a concerted pattern of changes including

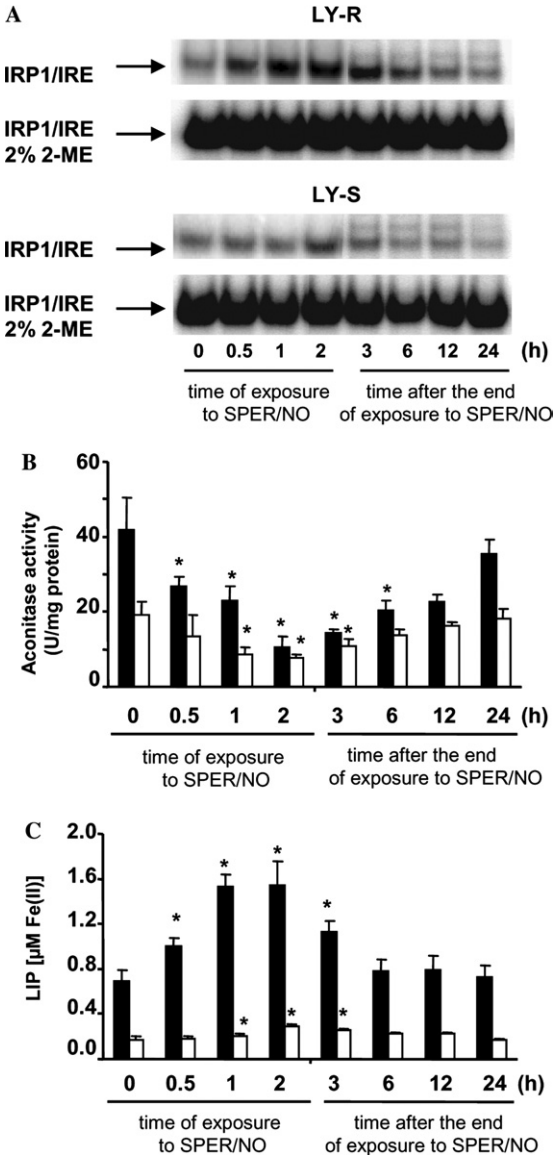

Fig. 1. Changes in IRP1 activities and LIP levels in LY cells during and after exposure to SPER/NO. LY cells were incubated for the indicated times with 25 μM SPER/NO. After the end of 2-h exposure of LY cells to NO, LY cells were extensively washed and further cultured in a fresh medium for the indicated times. (A) Cytosolic extracts (2 μg) were analyzed for IRP1 RNA-binding activity by electrophoretic mobility shift assay with an excess of ^{32}P-labelled IRE in the presence or absence of 2% 2-ME as described in Materials and methods. The results are typical of three independent experiments. (B) Sixty micrograms protein from the respective LY-R (solid bars) and LY-S (open bars) cytosolic extracts used in the parallel band-shift assay was analyzed for aconitase activity as previously described [13]. (C) LIP levels in LY-R (solid bars) and LY-S cells (open bars) were measured using a fluorescent probe, calcein, as previously described by Epsztejn et al. [16]. Data shown in (B,C) are means ± SD for three independent experiments. *Significant difference from the controls, $P < 0.05$. For all tested parameters, control values (time 0) were obtained from LY cells cultured for 2 h in the absence of SPER/NO.

activation of IRP1 RNA-binding activity (Fig. 3A), inhibition of aconitase activity (Fig. 3B), and increase in LIP levels (Fig. 3C). However, LIP levels upon NO

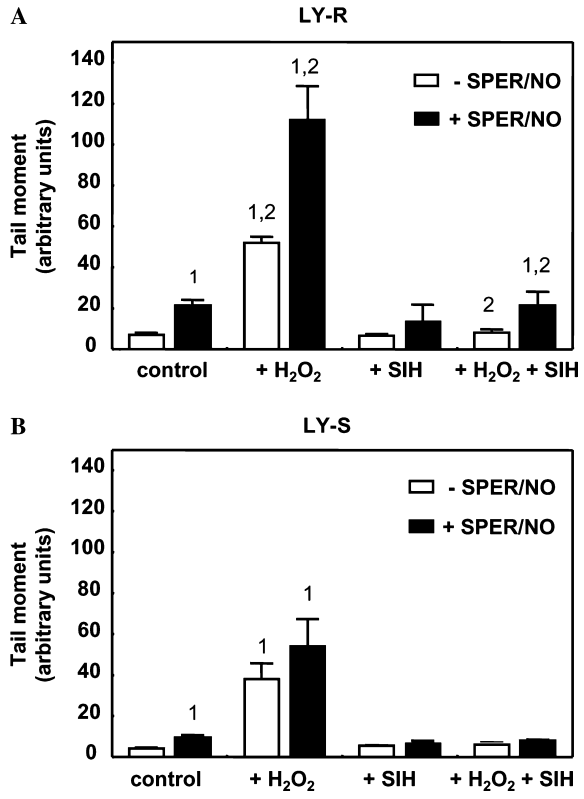

Fig. 2. DNA damage in LY cells exposed to NO. LY cells were exposed for 2 h to 25 μM SPER/NO as described in Materials and methods. Subsequently cells were treated with SIH (100 μM, 30 min, 37 °C) and/or H_2O_2 (25 μM, 5 min, 4 °C), where indicated. Data shown are means ± SD for three independent experiments. [1]Significant difference from the control, $P < 0.05$. [2]Significant difference between NO-treated and untreated cells, $P < 0.05$. Solid bars represent cells exposed to NO, whereas open bars represent untreated cells.

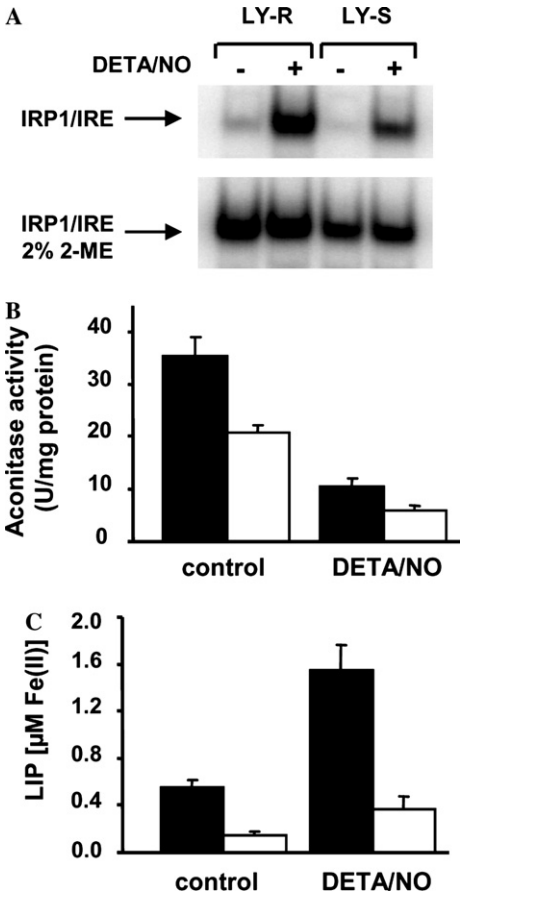

Fig. 3. IRP1 activities and LIP levels in LY cells exposed to DETA/NO. LY cells were incubated for 16 h with 100 μM DETA/NO. For all tested parameters, control values were obtained from LY cells cultured for 16 h in the absence of DETA/NO. (A) IRE-binding activity of IRP1, (B) its aconitase activity, and (C) LIP levels in LY cells were determined as described in the legend to Fig. 1. The results shown in (A) are typical of three independent experiments. Data shown in (B,C) are means ± SD for three independent experiments.

exposure were consistently higher in LY-R than in LY-S cells.

In order to assess the possible influence of changes in Ft and TfR on the substantial increase in LIP levels in LY cells seen in response to both burst and chronic exposure to NO, we examined Ft subunit proteins and TfR mRNA levels (Fig. 4, left-hand and middle panels). Considering the well-characterized contribution of HO-1-mediated heme degradation to the supply of iron to LIP [20], we also determined the mRNA level of this inducible protein (Fig. 4, right-hand panel). Samples for protein and RNA isolation were taken at the time when LIP levels in LY cells had increased following treatment with SPER/NO or DETA/NO. Results shown in Fig. 4A demonstrate that after 2-h treatment with SPER/NO the expression of neither of the Ft subunits nor TfR mRNA stability was influenced by NO. In contrast, after 16 h treatment with DETA/NO a classical, NO-regulated expression pattern was observed, i.e., down-regulation of Ft subunit levels and up-regulation of TfR mRNA (Fig. 4B). We also found that the abundance of HO-1 mRNA in NO-treated LY cells also depends on the NO exposure conditions. Thus, in SPER/NO-treated LY cells the increase in HO-1 mRNA levels was small as opposed to the strong up-regulation observed in the two cell lines exposed to DETA/NO (Fig. 4, right-hand panel).

Discussion

The pair of L5178Y mouse lymphoma lines has long been considered a suitable model for exploring cellular mechanisms of sensitivity/resistance to oxidative stress [21]. Previously, we highlighted the importance of distinct patterns of constitutive regulation of intracellular iron metabolism in LY-R and LY-S cells for their differential sensitivity to H_2O_2 [8]. As cellular iron content has recently been shown to determine several biological effects of NO [22], we took advantage of the different steady-state LIP and IRP1 statuses in the two LY lines to investigate the relationship between NO, IRP1, and LIP level. We show for the first time that NO-mediated

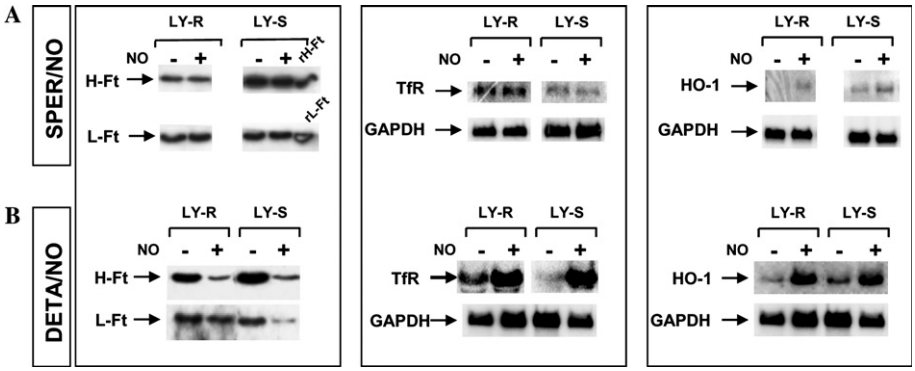

Fig. 4. Distinct regulation of Ft subunit, TfR, and HO-1 expression in LY cells upon burst and chronic exposure to NO. These analyses were performed using LY cells incubated for 2 h with 25 μM SPER/NO (A) or for 16 h with 100 μM DETA/NO (B). Left-hand panel, 50 μg of total cell protein extracts was analyzed by Western blotting to assess H- and L-Ft levels. Middle panel, 10 μg of total RNA was analyzed by Northern blotting to assess TfR mRNA expression. Right-hand panel, 10 μg of total RNA was analyzed by Northern blotting to assess HO-1 mRNA levels. Control cells were cultured in the absence of NO donors for the same time periods. Representative results from three independent experiments are shown.

activation of IRP1 RNA-binding activity and inhibition of its aconitase activity are accompanied by an increase in LIP level. Importantly, this connection between the modulation of IRP1 activities and increased levels of LIP was observed in LY-R, iron-rich as well as in LY-S iron-poor lymphoblasts, exposed to NO from chemical donors differing in their rate of NO release (Figs. 1 and 3). The elevation in LIP levels observed in mouse LY cells exposed to DETA/NO is in line with a recent study showing an increase in the free iron pool in bovine endothelial cells in response to chronic exposure to NO [9]. On the other hand, endogenously produced NO has been shown to decrease the LIP level in murine BV-2 microglial cells [23]. Interestingly, after removing the source of NO (SPER/NO) from the LY cells, we observed a converse relationship between recovery of IRP1 function and LIP fluctuation, i.e., the progressive loss of IRP1 RNA binding and recovery of aconitase activity were paralleled by the gradual return of up-regulated LIP to normal steady-state levels. Taken together, these results provide evidence that, in contrast to a previous assessment [7], NO-mediated activation of IRP1 is not due to LIP depletion and the resulting impairment of de novo IRP1 [4Fe–4S] cluster assembly. We found that of the two factors antagonistically modulating IRP1 function, i.e., NO and high LIP level, the effect of the former largely prevailed. Thus, we support the proposal that IRP1 is a primary target of NO-like chemical messengers [24].

The question arises as to the biological consequences of such severe disturbance of LIP homeostasis in LY cells exposed to NO. Keeping in mind the capacity of chelatable cellular iron to promote the formation of the highly toxic hydroxyl radical via the Fenton reaction [25], it seems reasonable to hypothesize that NO-dependent up-regulation of LIP levels in LY cells may promote the establishment of a prooxidant status in cells, thereby increasing the likelihood of oxidative injury. Since DNA damage is the most well-characterized hallmark of differential, LIP-dependent sensitivity/resistance of LY cells to H_2O_2-mediated oxidative stress [21], we attempted to establish the relationship between NO-induced elevation of LIP level and the occurrence of iron-catalyzed oxidative DNA lesions. DNA damage in cells exposed to SPER/NO was slightly elevated as compared to untreated controls. This effect was more pronounced in iron-rich LY-R cells. This is in agreement with the results of Stopper et al. [26], who found little DNA damage in SPER/NO-treated LY cells. Interestingly, in both cell lines, we observed that NO released by SPER/NO potentiated DNA damage caused by subsequent exogenous H_2O_2 challenge (Fig. 2). This genotoxic effect was largely abolished when the two cell lines were treated with SIH, a highly permeant iron chelator. Furthermore, we clearly show that NO sensitizes LY cells to subsequent oxidative stress-mediated genotoxicity, depending on their iron status.

The cellular chelatable iron pool is not restricted to the cytosol but can also be detected in other cellular compartments including the nucleus [27]. It is now well established that H_2O_2-dependent DNA damage is due to hydroxyl radicals generated in the presence of iron in close vicinity to DNA [25]. The question of whether the cytosolic LIP level may reflect that of nuclear LIP has not yet been definitely resolved, although available data point to a very close correlation between cytosolic LIP level and nuclear iron content [28] as well as between the amount of iron in the cytosol and the yield of oxidative DNA damage [29]. Taken together, our results allow establishing a direct relationship between the amount of iron in cytosolic LIP and the yield of DNA damage in mammalian cells exposed to H_2O_2.

Intracellular paths of iron traffic and their association with labile iron pools are poorly understood. The identification of subcellular and molecular sources of iron released to the cytosolic LIP in response to NO is therefore of great interest. Our results clearly show that

NO induces a rise in LIP level irrespective of NO exposure conditions, i.e., burst and chronic exposure corresponding to high and low NO flux rates, respectively. Despite such a uniform response we hypothesized that the mechanisms underlying LIP elevation may be different. Indeed, several NO-mediated processes have been shown to depend upon the NO flux rate [19,30]. Since Ft and TfR are key molecules influencing LIP levels in mammalian cells [1] and are controlled by NO via an IRP1-dependent regulatory mechanism [4,5], we determined their expression in LY cells exposed to the two examined NO donors. We demonstrated that the rapid increase in LIP levels in LY cells incubated with SPER/NO is not associated with any changes in levels of either H- and L-Ft subunits or TfR transcript abundance (Fig. 4A). In contrast, the delayed increase in LIP levels observed in DETA/NO-treated cells was very likely associated with down-regulation of Ft subunit expression, and up-regulation of TfR mRNA (Fig. 4B), i.e., decreased intracellular iron sequestration and increased cellular iron uptake, respectively. In numerous studies, NO-induced heme oxygenase 1 (HO-1) has been shown to be responsible for an increase in catalytic iron in the cytosol as a result of heme degradation [20,31]. However, the small HO-1 mRNA induction in LY cells exposed to SPER/NO is thought unlikely to be responsible for the 3–4-fold rise in LIP levels. Conversely, the expression pattern of HO-1 mRNA in the two cell lines after 16-h exposure to DETA/NO suggests some contribution of HO-1 to the increase in LIP levels through enhanced heme degradation. Other mechanisms responsible for NO-induced increase in LIP levels in LY cell lines may be also involved. These include, for example, release of iron from [Fe–S] proteins, i.e., cytosolic aconitase [32,33]. Interestingly, in a recent report, Watts et al. suggest that NO intercepts a transit iron pool and mediates indirect mobilization of iron from Ft [34].

In summary, in this study we have provided a new set of data demonstrating that NO can modulate IRP1 activities independently of fluctuations of LIP, a fraction of cellular iron thought to be a sensor for the IRP1/IRE regulatory mechanism. We also delineate for the first time a comprehensive IRP1-mediated pattern of changes in the iron metabolism of mammalian cells exposed to sustained NO action: induction of IRP1 IRE-binding activity, with subsequent down-regulation of Ft and up-regulation of TfR expression, resulting in the up-regulation of LIP level and its involvement in cellular genotoxicity.

Acknowledgments

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