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Biosynthesis of nitric oxide activates iron regulatory factor in macrophages

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Biosynthesis of nitric oxide (NO) from L-arginine modulates activity of iron-dependent enzymes, including mitochondrial aconitase, an [Fe-S] protein. We examined the effect of NO on the activity of iron regulatory factor (IRF), a cytoplasmic protein which modulates both ferritin mRNA translation and transferrin receptor mRNA stability by binding to specific mRNA sequences called iron responsive elements (IREs). Murine macrophages were activated with interferon-γ and lipopolysaccharide to induce NO synthase activity and cultured in the presence or absence of N^G-substituted analogues of L-arginine which served as selective inhibitors of NO synthesis. Measurement of the nitrite concentration in the culture medium was taken as an index of NO production. Mitochondria-free cytosols were then prepared and aconitase activity as well as IRE binding activity assessed in parallel. Inhibition of enzymatic activity and induction of IRE binding activity were correlated and depended on NO synthesis after IFN-γ and/or LPS stimulation. Authentic NO gas as well as the NO-generating compound 3-morpholinosydnonimine (SIN-1) also conversely modulated aconitase and IRE binding activities of purified recombinant IRF. These results provide evidence that endogenously produced NO may modulate the post-transcriptional regulation of genes involved in iron homeostasis and support the hypothesis that the [Fe-S] cluster of IRF mediates iron-dependent regulation.

Key words: interferon-γ/iron regulatory factor/macrophages/metalloenzyme/nitric oxide

Introduction

Macrophages activated by lymphokines such as interferon-γ (IFN-γ) or microbial components such as lipopolysaccharide (LPS) participate in non-specific resistance against pathogens (Adams and Hamilton, 1984). According to recent data, the biostatic and cytostatic activities of rodent macrophages depend on the formation of nitric oxide (NO) which is generated by enzymatic oxidation of the guanidino group of L-arginine (Hibbs *et al.*, 1990; Green *et al.*, 1991; Nathan and Hibbs, 1991). NO is also synthesized in other mammalian cells including vascular cells, hepatocytes and neurons, and is implicated in the regulation of vascular tone and neurotransmission (reviewed by Moncada *et al.*, 1991). Several isoforms of NO synthase have been characterized (Stuehr and Griffith, 1992), and the cDNA of three types of NO synthases have been cloned (Nathan, 1992). Macrophage-type NO synthase is inducible and generates large amounts of NO in response to stimulation by cytokines or bacterial immunomodulators (Stuehr and Marletta, 1987; Drapier *et al.*, 1988; Ding *et al.*, 1988).

NO is a free radical with strong affinity for iron (discussed by Traylor and Sharma, 1992) and has been proposed to activate soluble guanylate cyclase, by binding to the iron atom in its haem (Ignarro, 1990). Furthermore, several iron-dependent enzymes including the Krebs cycle aconitase, are inhibited in NO-producing cells (Hibbs *et al.*, 1987; Drapier and Hibbs, 1988; Stadler *et al.*, 1991). Mitochondrial aconitase is an iron-sulfur protein which catalyses the interconversion of citrate and isocitrate. As documented by Beinert and co-workers, the [Fe-S] cluster of aconitase has a catalytic role: in the active enzyme, one iron atom (Fe_a) of the [4Fe-4S] cluster is not bound to cysteine but coordinates to a hydroxyl and a carboxyl group of the substrate (Kennedy *et al.*, 1987; Beinert, 1990).

We and others have shown that NO synthesized by macrophages forms EPR-detectable nitrosyl-iron complexes, both in macrophages and adjacent target cells (Lancaster and Hibbs, 1990; Pellat *et al.*, 1990; Drapier *et al.*, 1991) which appear concomitantly to the loss of mitochondrial aconitase activity. Signals given by NO-producing cells are similar to those of chemically nitrosylated ferredoxins, a class of typical [Fe-S] cluster proteins. This strongly suggested that NO targets the iron-sulfur centre of mitochondrial aconitase and thereby prevents substrate binding (Drapier *et al.*, 1991).

Recent results indicate that a cytosolic *trans*-acting regulator which binds to highly conserved RNA stem-loops called iron responsive elements (IREs) (Casey *et al.*, 1988; Leibold and Munro, 1988) in the 5′ untranslated region of ferritin mRNA and the 3′ untranslated region of transferrin receptor mRNA, is also an aconitase (Kaptain *et al.*, 1991; Haile *et al.*, 1992a). This regulator, referred to as iron regulatory factor (IRF) (Müllner *et al.*, 1989) in this paper, is also known as IRE binding protein (IRE-BP) (Rouault *et al.*, 1988), ferritin repressor protein (FRP) (Walden *et al.*, 1989) or P90 (Harrell *et al.*, 1991). IRF has been characterized by several groups, and this has shed light on the molecular mechanism by which intracellular iron load controls in a coordinate manner both ferritin RNA translation and transferrin receptor mRNA stability (Aziz and Munro, 1987; Müllner and Kühn; 1988; reviewed by Kühn and Hentze, 1992). Based on the iron-regulated bifunctionality of IRF, both as an RNA binding protein and as an aconitase, it was proposed that an iron-sulfur cluster senses iron starvation in the cell and controls IRF-associated activities (Kaptain *et al.*, 1991; Rouault *et al.*, 1991; reviewed by Klausner *et al.*, 1993). This prompted us to investigate whether NO could modulate IRF activity. Data presented

in this paper show that either the induction of NO synthase in living cells after IFN-γ or LPS stimulation or NO as authentic gas or generated from a chemical, reciprocally alter the aconitase and RNA binding activities of IRF.

Results

Activation of macrophages by IFN-γ and LPS alters aconitase activity and IRE binding activity reciprocally

In preliminary assays, we observed that cytosolic aconitase was fully active in extracts and did not require activation by incubation with Fe^{2+} and a thiol as required for its mitochondrial counterpart (Gawron *et al.*, 1974). In a first series of experiments, the effect of IFN-γ and LPS, which are potent macrophage activators, was analysed. Cytosolic extracts were tested in parallel for both aconitase and IRE binding activities. As shown in Figure 1, cytosol of RAW 264.7 macrophages contained a spontaneous low IRE binding activity which was strongly enhanced by treatment with 2% mercaptoethanol (2-ME), known to fully activate IRF (Hentze *et al.*, 1989). This IRE binding activity increased dose dependently in response to an overnight stimulation of RAW 264.7 macrophages by IFN-γ or LPS without a rise in total IRF activity as tested by *in vitro* addition of 2-ME. The activation also correlated with NO synthesis as mirrored by accumulation in the culture medium of nitrite, one of the end-products of the L-arginine−NO pathway, and by the loss of aconitase activity. It is well known that IFN-γ and LPS, when used at sub-optimal doses, synergize to activate macrophages and to induce NO synthesis (Stuehr and Marletta, 1987). As shown in Figure 1, this cooperation was also conspicuous regarding IRE binding activity. Data from densitometric analysis revealed that the value of relative IRF−IRE complexes found in macrophages stimulated by 10 U/ml IFN-γ plus 10 ng/ml LPS was 2-fold higher than the sum of those obtained with IFN-γ or LPS added separately at the same doses.

When cells were stimulated under optimal conditions (see lane 10 for example), we noticed that the level of total IRF activity as assessed by 2-ME treatment of the cytosolic extract, decreased consistently. It is possible that a fraction of active IRF bound to ferritin and transferrin receptor mRNA was therefore no longer accessible to the radiolabelled probe.

Taking advantage of the combination of IFN-γ and LPS, we determined the time-course of changes in aconitase and IRE binding activities (Figure 2). Induction of NO synthase was assessed by determination of nitrite in the culture medium. The kinetics showed a significant change for both the enzymatic activity (panel A) and IRE binding activity (panel B) by 5 h, corresponding to a threshold of 5−10 μM nitrite produced. A complete loss of aconitase activity occurred within 8 h when the level of nitrite reached ~25 μM. Maximal RNA binding activity (reaching up to 50% as compared with total activation after treatment by 2-ME) was observed later (~12 h) when accumulation of nitrite was close to 50 μM. In non-stimulated cells, aconitase activity did not change with time (not shown) and IRF activity was as low after 12 h as at time 0 (Figure 2B).

These results indicate that IRE binding activity in macrophages is under the control of physiological immunomodulators and correlates with NO synthesis.

Increase of IRE binding activity and loss of aconitase activity correlate and are dependent on NO synthesis

To test further if NO biosynthesis is responsible for IRF activation, experiments were performed with RAW 264.7

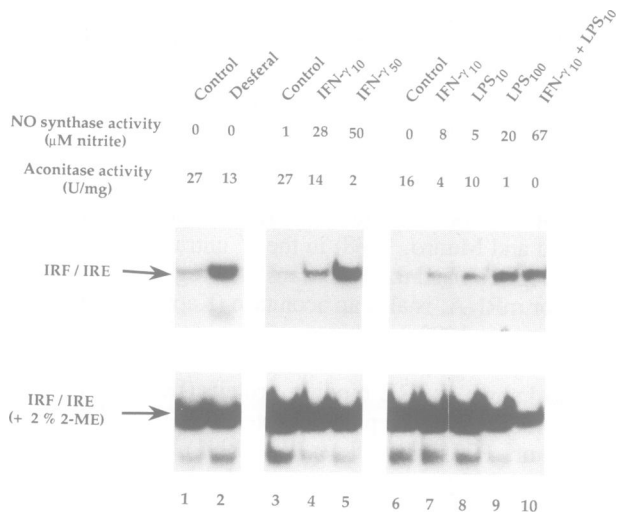

Fig. 1. IFN-γ and LPS alter the aconitase and IRE binding activities of IRF in a dose dependent manner. RAW 264.7 macrophages were cultured for 20 h in the presence of various doses of IFN-γ (in U/ml) or LPS (in ng/ml), used alone (lanes 4, 5, 7, 8 and 9) or in combination (lane 10). In a parallel sample, 100 μM desferrioxamine (Desferal) was added for the same length of time (lane 2). Nitrite was determined in the culture medium to witness induction of NO synthase, and both aconitase and IRE binding activities were assessed in cytosolic extracts, in the absence or presence of 2% 2-mercaptoethanol (2-ME) for the latter as described in Materials and methods (representative experiments out of at least three).

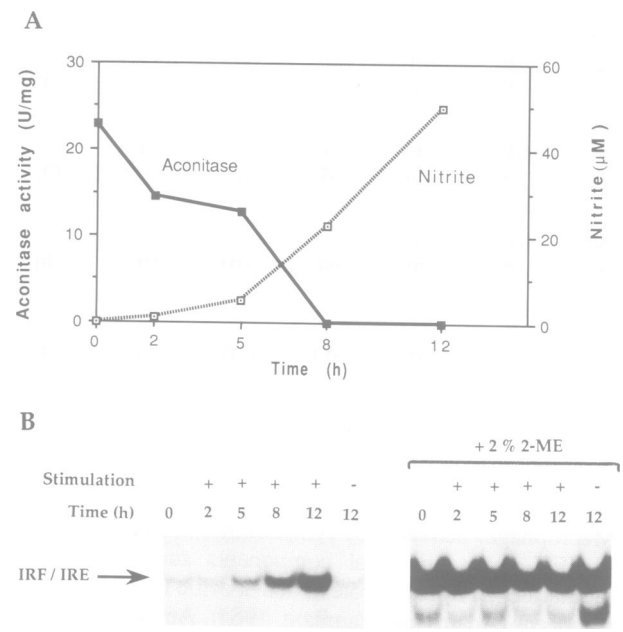

Fig. 2. Time-course of nitrite production, aconitase activity and IRE binding activity in immunostimulated RAW 264.7 macrophages. Cells were stimulated with 100 U/ml IFN-γ and 10 ng/ml LPS. At the time indicated, the concentration of nitrite was determined in culture medium, and cytosolic extracts were analysed both spectrophotometrically for aconitase activity (**A**) and for IRE binding activity by a gel retardation assay using a ^{32}P-labelled ferritin IRE probe (**B**) (similar data were obtained twice).

macrophages stimulated either in culture medium depleted of L-arginine, the substrate of NO synthase, or in complete medium supplemented with N^G-mono-methyl-L-arginine (L-MMA) or N^G-nitro-L-arginine (L-NA), two selective and stereospecific inhibitors of NO synthesis. As expected, in both sets of experiments, NO production was strongly reduced (Figure 3). Interestingly, the presence of NO synthase inhibitors (lanes 3, 6, 9 versus 2, 5, 8, respectively), or depletion of L-arginine in the culture medium (lane 12 versus 11) prevented the loss of cytosolic aconitase activity and, completely, the stimulation of IRE binding. Indeed, scanning of autoradiograms revealed that samples from cells stimulated in the presence of NO synthase inhibitors showed no significant change in IRE binding as compared with controls (lanes 1, 4 and 7). Relative to total activity as revealed by 2-ME treatment, IRE binding was $4.6 \pm 2.4\%$ ($n = 21$) in control cells and $4.6 \pm 2.9\%$ ($n = 10$) in cells stimulated in the presence of inhibitors. These data indicate that NO synthesis is directly responsible for the increase of IRF binding to its target sequence. In control experiments, addition to the culture medium of 1 mM citrulline, nitrite or nitrate, the other end-products of the L-arginine–NO pathway, did not result in inhibition of aconitase activity or in activation of IRE binding activity (not shown).

Similar results were obtained using primary macrophages, explanted from mouse peritoneum and cultured in the presence of NO synthase inducers, i.e. 10^{-12} M IFN-γ with or without LPS. As shown in Figure 4, control cells exhibited a basal NO synthase activity which was strongly increased upon stimulation. Cytosolic extracts of stimulated macrophages exhibited high IRE binding activity and low aconitase activity. Again, L-NA prevented these alterations to a large extent.

It was previously shown that the P388D1 macrophage cell line does not exhibit NO synthase activity in response to IFN-γ or LPS (Stuehr and Marletta, 1987). As expected, these cells did not produce nitrite in response to stimuli (Figure 4, right panel). Interestingly, aconitase activity in cytosolic extracts was not significantly altered after stimulation and IRE binding was as low in cytosolic extracts of stimulated P388D1 cells as in those of control cells cultured without the stimulus. Low activity of IRF was not a consequence of protein degradation or of inhibition of protein synthesis since the activity was restored after incubation of extracts with 2% 2-ME.

These two sets of experiments demonstrate that activation of IRF in response to immunological stimuli occurs in both primary macrophages and macrophage cell lines and is dependent on the synthesis of NO from L-arginine.

Modulation by NO synthesis of IRF activities in non-macrophage cells

To see whether NO synthase-dependent IRF modulation also occurs in non-macrophage cells, we compared IRF activities in EMT-6 adenocarcinoma cells which in response to LPS and IFN-γ produce large amounts of NO (Amber *et al.*, 1988) and in L1210 leukaemia cells which do not. As shown in Figure 5, cytosols of EMT-6 cells previously stimulated with IFN-γ and LPS also exhibited loss of aconitase activity and increased IRE binding activity. Again, inhibition of NO synthase activity largely prevented these effects. In contrast, L1210 leukaemia cells exhibited no change in either cytosolic aconitase activity or IRF activity (right panel).

Effect of NO gas and SIN-1 on recombinant IRF activities in vitro

To test the *in vitro* effect of NO on IRF activities, purified recombinant IRF was incubated with dilutions of authentic NO gas-saturated solution for 15 min at room temperature in oxygen-free atmosphere, or for 1 h at 37°C with SIN-1,

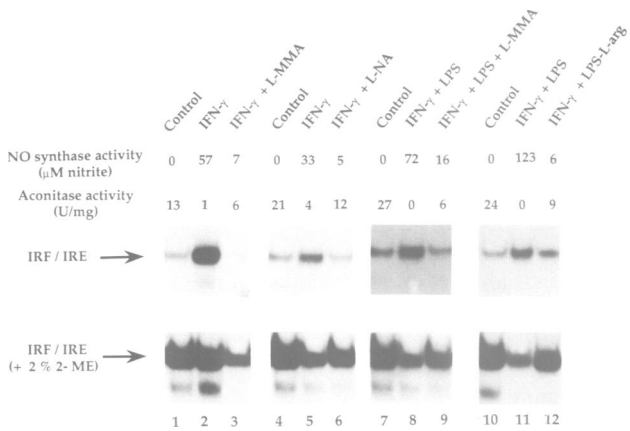

Fig. 3. Inhibition of NO synthesis abrogates both the loss of aconitase activity and the high affinity binding of IRF to an IRE motif. In a first set of experiments, RAW 264.7 macrophages were activated by 100 U/ml IFN-γ alone (lanes 2 and 5), with 0.5 mM N^G-mono-methyl-L-arginine (L-MMA) (lane 3) or with 0.5 mM N^G-nitro-L-arginine (L-NA) (lane 6). In other experiments, 100 U/ml IFN-γ and 10 ng/ml LPS were used in combination (lanes 8, 9, 11 and 12) with 0.5 mM L-MMA (lane 9) or without L-arginine (lane 12). After 20 h, nitrite was determined in the culture medium, as well as aconitase and IRE binding activities in cytosolic extracts as described in Materials and methods. The fact that labelling in lanes 3, 6 and 9 (cells stimulated in the presence of NO synthase inhibitors) is less intense than in lanes 1, 4 and 7 (controls) is due to somewhat less material in the gel retardation assay (compare with 2-ME samples below). One out of at least three experiments is shown.

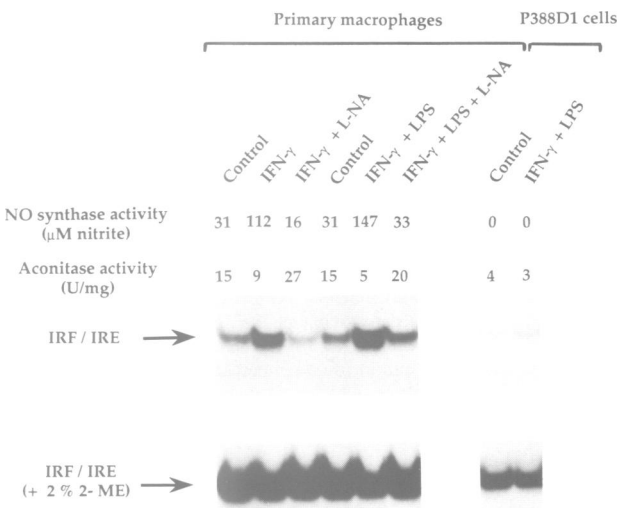

Fig. 4. Aconitase activity and IRF–IRE complex formation in cytosols of mouse peritoneal macrophages and P388D1 macrophages. Cells were incubated for 20 h with 100 U/ml IFN-γ with or without 10 ng/ml LPS, in the presence or absence of 0.5 mM N^G-nitro-L-arginine (L-NA), an inhibitor of NO synthesis. Nitrite was determined in the culture medium, as well as aconitase and IRE binding activities in cytosolic extracts as described in Materials and methods. (Data were obtained twice.)

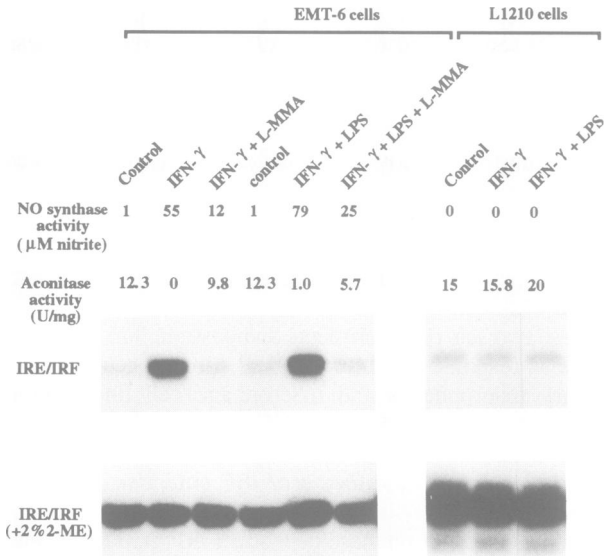

Fig. 5. Aconitase activity and IRF−IRE complex formation in cytosols of non-macrophage cells. Cells were incubated for 20 h with 100 U/ml IFN-γ with or without 10 ng/ml LPS, in the presence or absence of 0.5 mM N^G-mono-methyl-L-arginine (L-MMA), an inhibitor of NO synthesis. Cytosolic extracts were prepared and treated as described in Materials and methods. (Data were obtained twice.)

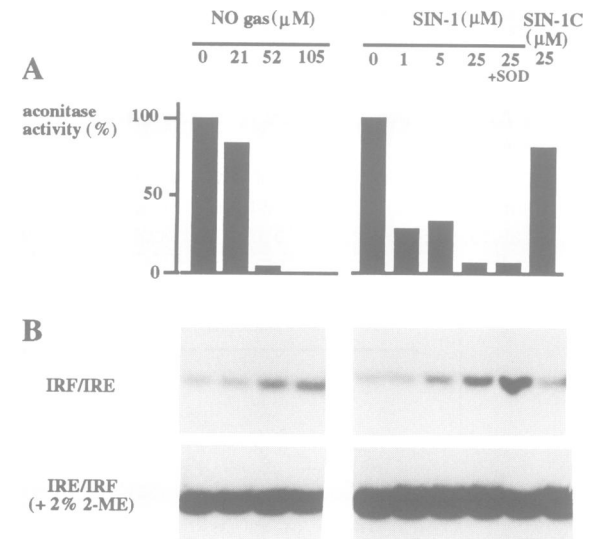

Fig. 6. Effect of SIN-1 and authentic NO gas on aconitase and IRE binding activities of purified recombinant IRF. Sixty nanograms of recombinant IRF were incubated with the indicated concentrations of authentic NO gas for 15 min at room temperature or with SIN-1 and SIN-1C, the other end-product of SIN-1 decomposition, for 30 min at 37°C as described in Materials and methods. Where indicated, excess of superoxide dismutase (SOD) (13 U/μl) was added to prevent any effect of superoxide anion produced during SIN-1 decomposition. Two nanograms protein were then tested for RNA−protein complex formation in the absence or presence of 2% 2-mercaptoethanol (2-ME) with a radiolabelled ferritin H chain IRE probe and 30 ng were assayed for aconitase activity by a spectrophotometric method. (Representative experiments out of at least three.)

a nitrovasodilator which spontaneously liberates NO (Feelisch *et al.*, 1989). As shown in Figure 6, both the NO-saturated buffer and the NO-donor dose dependently inhibited aconitase activity (figure 6A) and significantly increased IRE binding activity (Figure 6B) of IRF. The dose-effect allowed the conclusion that aconitase was lost at ~1−10 μM SIN-1. At similar doses an increase of IRE binding was observed. Incubations with higher concentrations of SIN-1 (i.e. up to 10 mM) activated IRE binding to ~20% of the level reached with 2% 2-ME (not shown). Taken as a control, SIN-1C, a metabolite of SIN-1 without the N-NO group, had no effect on either IRF activity. Addition of superoxide dismutase (SOD) to SIN-1, slightly increased IRF−IRE binding. This observation is consistent with the fact that besides producing NO, SIN-1 also releases superoxide anion which acts as an NO scavenger. SOD by itself had no effect on IRE binding (not shown). Sodium nitroprusside (SNP), another NO donor, also activated IRE binding activity (not shown) but was not found suitable in these experiments since ferricyanide, the by-product of SNP decomposition, is a strong oxidant that dissociates the [Fe−S] cluster by itself (Haile *et al.*, 1992b).

Discussion

The avid binding of NO to transition metals such as iron (Henry *et al.*, 1991) has for a long time designated this gas as a poison. The recent acknowledgement that NO is also a physiological product of mammalian cells has given it a higher status and allowed the discovery that NO is a messenger and effector implicated in metabolic changes, including iron release (Hibbs *et al.*, 1984; Drapier and Hibbs, 1988), modulation of iron-dependent enzyme activities (Hibbs *et al.*, 1987; Drapier *et al.*, 1988; Lepoivre *et al.*, 1990), mobilization of iron from ferritin (Reif and Simmons, 1990) and formation of coordination complexes with iron-carrying macromolecules (Lancaster and Hibbs, 1990; Pellat *et al.*, 1990).

Here, we provide evidence for the first time that NO could also modulate the post-transcriptional control of genes coding for proteins that maintain cellular iron homeostasis. Indeed, activity of IRF, the bifunctional regulator of ferritin and transferrin receptor mRNA expression, is sensitive to NO production. This was shown by inducing NO synthase in living cells stimulated by 10^{-12} M IFN-γ and/or LPS. Furthermore, the effect of authentic NO gas and of the NO-donor SIN-1 on recombinant IRF *in vitro* points directly to NO being the active effector.

In previous studies, we and others have shown that mitochondrial aconitase is inhibited in NO-generating cells (Drapier and Hibbs, 1986, 1988; Stadler *et al.*, 1991) as well as in cell extracts treated with authentic NO gas (Hibbs *et al.*, 1990; Stadler *et al.*, 1991). In this manuscript, we report that endogenously produced NO alters in the cytosol both aconitase activity and IRE binding activity of IRF in an opposite manner, supporting the idea that a common target of NO is responsible for both functions. The iron−sulfur cluster of IRF is a good candidate in this respect since (i) such a cluster controls enzymatic activity in mitochondrial aconitase (Kennedy *et al.*, 1983; Beinert, 1990) and (ii) NO is known to form adducts readily with haem and non-haem iron proteins (Nelson, 1987; Rousseau *et al.*, 1988; Petrouleas and Diner 1990) including iron−sulfur enzymes (Meyer, 1981; Michalski and Nicholas, 1987), and with synthetic iron−sulfur compounds (Butler *et al.*, 1988). In mitochondrial aconitase, ligation of citrate or isocitrate to an iron atom (Fe_a) of the [4Fe−4S] cluster allows catalysis by dehydration−rehydration of the substrate (Beinert, 1990).

It is now acknowledged that IRF is the cytosolic aconitase and also possesses such a dynamic [Fe−S] cluster (Kaptain *et al.*, 1991; Constable *et al.*, 1992; Haile *et al.*, 1992a; Kennedy *et al.*, 1992; Emery-Goodman *et al.*, 1993). Furthermore, it was recently shown that only apoprotein is able to bind to IRE motifs (Haile *et al.*, 1992b; Emery-Goodman *et al.*, 1993). Therefore, loss of one iron atom (Fe_a) from the [4Fe−4S] cluster would be sufficient to prevent aconitase activity whereas absence of the entire cluster may be required for high affinity IRE binding. This is in line with our demonstration that the dose needed for substantial activation of IRE binding is higher than the threshold of NO required to inhibit aconitase activity. Consequently, it seems attractive to propose that NO at low doses would prevent substrate binding by coordinating to the crucial iron atom (Fe_a), and at higher doses by disrupting the [Fe−S] cluster. The conclusion that the cluster is a likely target of NO action is supported by preliminary *in vitro* experiments with a recombinant IRF mutant: when cysteine 503 involved in the attachment of the [4Fe−4S] cluster was changed to serine, IRF could not be activated by incubation with SIN-1 (H.Hirling *et al.*, unpublished data). In turn, the conformational change resulting from cluster disruption may extend to the IRE binding site, rendering it more accessible. This proposal is in keeping with the recent demonstration that reaction of NO with the [4Fe−4S] cluster of *Azotobacter vinelandii* nitrogenase alters the nucleotide binding domain of the protein, located 20 Å farther away (Hyman *et al.*, 1992).

Alternatively, it is also possible that NO reacts with a free sulfhydryl group in the RNA binding domain. Indeed, it has been reported that NO is able to form stable *S*-nitroso complexes with thiols (Stamler *et al.*, 1992), and several studies stressed the fact that thiolate groups in the DNA binding domains of *trans*-activators play an important part in their ability to bind DNA (Toledano and Leonard, 1991; McBride *et al.*, 1992). It is thus possible that NO modulates binding activity of IRF by combining with a crucial sulfhydryl group(s) or by changing their redox state, known to be important for binding of IRF to an IRE motif (Hentze *et al.*, 1989).

Besides that, it has previously been shown that NO synthesis is responsible for the loss of iron from NO-producing macrophages (Hibbs *et al.*, 1984; Drapier and Hibbs, 1988; Wharton *et al.*, 1988). It is thus conceivable that cells adapt to NO-induced iron depletion in the same way they would compensate for iron loss after chelation.

Further studies are required to appreciate fully the biological significance of our observation but it is likely that modulation of IRF can occur not only in cells able to produce high amounts of NO under adequate conditions (macrophages, hepatocytes, vascular cells) but also in cells which are unable to produce NO and which come into the close vicinity of NO-producing cells. Indeed, we have previously demonstrated that NO derived from activated macrophages can yield coordination complexes with iron in an adjacent target cell that does not synthesize NO (Drapier *et al.*, 1991).

Regulation of iron metabolism is essential for all mammalian cells and particularly for macrophages since they ingest senescent erythrocytes and catabolize haem. The iron load of macrophages is thus high, and they adapt by initiating or repressing ferritin mRNA translation (Testa *et al.*, 1991). An accurate tuning is thus required to maintain a balance between iron acquisition, storage and biosynthetic pathways. IFN-γ has been reported either to decrease (Hamilton *et al.*, 1984; Byrd and Horwitz, 1989; Bourgeade *et al.*, 1992) or increase (Taetle and Honeysett, 1988) expression of transferrin receptors on monocytes/macrophages. This apparent puzzling situation may be due to the fact that NO production was not controlled in these experiments. Indeed, the conditions under which human monocytes synthesize NO have not yet been established, and NO synthase activity may be induced in these cells only in pathological states (Hunt and Goldin, 1992).

Whether sensitivity of IRF to NO production in response to immunological stimuli leads to a decrease of ferritin content and/or to higher expression of transferrin receptor in macrophages cannot be answered by our data. Preliminary experiments in immunostimulated RAW 264.7 cells showed no increase in transferrin receptor mRNA levels. However, this finding is not conclusive, since IFN-γ, besides activating NO synthesis, also inhibited cell proliferation. These are circumstances that decrease transferrin receptor expression which may counteract a possible induction by NO. Concerning ferritin expression, recent results of Weiss *et al.* (1993) indicate that the J774 macrophage cell line, when producing NO, exhibits increased IRE binding activity and synthesizes less ferritin.

In conclusion, our results have far-reaching implications as they disclose that endogenously produced NO, besides altering the activity of iron-dependent enzymes post-translationally, can also modulate gene expression. This idea is supported by recent findings indicating that NO, as gas or released from NO donors, modulates cytokine and oncogene mRNA levels in a myelomonocytic cell line (Magrinat *et al.*, 1992) and activates the transciption factor NF-$\varkappa$B in human monocytes (Lander *et al.*, 1993). At this juncture, it is tempting to speculate that other *trans*-regulators that may contain iron or sulfhydryl groups, essential for binding activity, represent a potential target for endogenous NO.

Materials and methods

Media and reagents

Dulbecco's Modified Eagle's Medium (DMEM) and low endotoxin fetal calf serum were obtained from Gibco (Paisley, Scotland). Murine recombinant interferon-γ (2×10^7 U/mg) was produced by Genentech and provided by Dr G.R.Adolf (Boehringer Ingelheim, Vienna, Austria). N^G-mono-methyl-L-arginine was from Calbiochem (La Jolla, CA). All other chemicals and *Escherichia coli* LPS (serotype 0111: B4) were from Sigma (Saint Louis, MO). The sydnonimines SIN-1 and SIN-1C were synthesized by Cassella AG (Frankfurt, Germany) and kindly provided by Mrs Winicki, Laboratoires Hoechst, France. Desferal (desferrioxamine) was a gift from Ciba-Geigy (Basel, Switzerland).

Primary macrophages and macrophage cell lines

Eight to ten week old C_3H/HeN mice were injected i.p. with 2 ml thioglycolate broth 4−5 days before sacrifice. Peritoneal macrophages were purified by adherence and cultured in 80 mm Petri dishes (Nunc, Denmark) in a humidified 5% CO_2, 95% air incubator. The purified population was composed of >90% macrophages as determined by neutral red uptake and latex bead phagocytosis. Cell viability, assessed by Trypan blue exclusion, was >95%. The macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection. The macrophage cell line P388D1 was kindly provided by Dr B.Dugas (Institut Henri Beaufour, Les Ulis, France). L1210 leukaemia cells and EMT-6 cells, a spontaneous Balb/c mammary adenocarcinoma cell line, were kindly provided by Dr John B.Hibbs, University of Utah, Salt Lake City, UT.

Cell lines were cultured in plastic flasks (Nunc, Denmark) with high glucose-DMEM supplemented with 5% endotoxin-low fetal calf serum, 5 mM glutamine and antibiotics. Cells were passaged every 4 days. For

experiments, macrophages were detached by vigorous pipetting and, after centrifugation, plated in fresh medium containing the various agents. Cell cultures were activated with various concentrations of IFN-γ and LPS, used alone or in combination. N^G-mono-methyl-L-arginine (L-MMA) and N^G-nitro-L-arginine (L-NA) two *N*-guanido-substituted analogues of L-arginine that are inhibitors of NO synthase, were used at 0.5 mM final concentration.

Preparation of cytosolic extracts

Cells (5×10^6/ml) were treated with 0.007% digitonin at 4°C in 0.25 M sucrose, 100 mM HEPES, pH 7.2. Digitonin selectively permeabilizes cholesterol-rich plasma membranes, leaving intact mitochondrial membranes (Fiskum *et al.*, 1980). After centrifugation, at 1500 *g* for 15 min, supernatants were carefully collected and centrifuged at 150 000 *g* for 20 min in a Beckman TL 100 ultracentrifuge to spin down any particulate material. In preliminary experiments, supernatants were found free of mitochondrial material as assessed by the absence of cytochrome *c* oxidase activity (Wharton and Griffith, 1962). Cytosolic extracts were aliquoted, concentrated by vacuum drying or subjected to ultrafiltration on microconcentrators (Microsep Filtron, Northborough, MA). Lyophilized and concentrated samples were kept at −80°C until use.

Preparation of NO gas-saturated buffer

NO-containing stock solution was prepared by bubbling authentic NO gas (AGA, France) for 30 min into physiological salt solution that had been previously deoxygenated. This NO-saturated buffer (2.1 mM) was used at various dilutions.

Measurement of nitrite

Nitrite, one of the end-products of the NO pathway, was determined in the culture medium as previouly described (Hibbs *et al.*, 1987), by using the Griess reagent containing final concentrations of 0.5% sulfanilamide, 0.05% *N*-(1-naphthyl) ethylenediamine hydrochloride in 45% acetic acid.

Determination of aconitase activity

Aconitase activity was measured spectrophotometrically, by following the rate of disappearance of *cis*-aconitate at 240 nm as previously described (Drapier and Hibbs, 1986). Units are nmol substrate consumed/min at 37°C.

Gel retardation assay

IRE−protein interactions were measured as previously described (Müllner *et al.*, 1989) by incubating a molar excess of [^{32}P]CTP-labelled RNA transcript from plasmid pSPT-fer with 1 μg cytoplasmic protein in a 20 μl reaction volume. After a 20 min incubation at room temperature, RNase T_1 (1 U/ml) and heparin (5 mg/ml) were sequentially added for 10 min each. IRE−protein complexes were resolved in 6% non-denaturing polyacrylamide gels as described (Leibold and Munro, 1988). In parallel experiments, samples were treated with 2% 2-mercaptoethanol (2-ME) prior to addition of the RNA probe. Autoradiographs were quantified by scanning densitometry using a Hirschmann Elscript 400 AT scanner.

Preparation of recombinant human IRF

The full-length human IRF cDNA clone in plasmid pGEM-hIRF (Hirling *et al.*, 1992) was subcloned into the bacterial expression vector pGEX-2T (Pharmacia, Uppsala, Sweden) behind the IPTG-inducible *tac* promoter. Upon expression this vector yields in-frame a fusion protein with a 26 kDa glutathione *S*-tranferase domain attached to the N-terminus of human IRF, and the connecting peptide can be cleaved by thrombin (Smith and Johnson, 1988). Expression and purification of the fusion protein were performed as described (Smith and Johnson, 1988) except for the lysis of bacteria, where lysozyme at 2 mg/ml was added instead of Triton X-100. Soluble fusion protein was isolated by binding to glutathione−Sepharose CL4B (Pharmacia) and eluted by digestion with thrombin (Sigma). The yield of recombinant IRF was ~10 μg/100 ml bacterial culture.

Treatment of recombinant IRF by NO gas and SIN-1 in vitro

Purified IRF (60 ng) was incubated with NO gas or SIN-1 in 200 μl of 10 mM HEPES pH 7.9, 40 mM KCl, 3 mM $MgCl_2$, 5% glycerol, 20 μg/ml bovine serum albumin (BSA) for 15 min at room temperature or 1 h at 37°C, respectively, prior to aconitase measurement (30 ng protein) and bandshift analysis (2 ng protein). Where indicated, the incubation was performed in the presence of superoxide dismutase (Sigma) at 13 U/μl. Incubation with the NO gas-saturated solution was performed identically, except that the buffer was previously deoxygenated by bubbling with argon (Carbagas, Switzerland).

Protein determination

The protein content of extracts was determined by using the Bio-Rad protein assay (Bio-Rad, Richmond, CA) with BSA as a standard.

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