Iron regulatory protein-1 (IRP-1) is a bifunctional [4Fe-4S] protein that functions as a cytosolic aconitase or as a trans-regulatory factor controlling iron homeostasis at a post-transcriptional level. Because IRP-1 is a sensitive target protein for nitric oxide (NO), we investigated whether this protein is nitrated in inflammatory macrophages and whether this post-transcriptional modification changes its activities. RAW 264.7 macrophages were first stimulated with interferon-γ and lipopolysaccharide (IFN-γ/LPS) and then triggered by phorbol 12-myristate 13-acetate (PMA) in order to promote co-generation of NO and O2. IRP-1 was isolated by immunoprecipitation and analyzed for protein-bound nitrotyrosine by Western blotting. We show that nitration of endogenous IRP-1 in NO-producing macrophages boosted to produce O2 was accompanied by aconitase inhibition and impairment of its capacity to bind the iron-responsive element (IRE) of ferritin mRNA. Lost IRE-binding activity was not recovered by exposure of IRP-1 to 2% 2-mercaptoethanol and was not due to protein degradation. Inclusion of cis-aconitate with cell extract to stabilize the [4Fe-4S] cluster of holo-IRP-1 rendered protein insensitive to nitration by peroxynitrite, suggesting that loss of [Fe-S] cluster and subsequent change of conformation are prerequisites for tyrosine nitration. IRP-1 nitration was strongly reduced when IRP-1 was isolated by synthetic peroxynitrite or by SIN-1, an inducer of NO. Interestingly, recent biochemical and resonance Raman studies have allowed the detection of tyrosine nitration on pure recombinant IRP-1 and consequently supported the trans-regulatory activity of IRP-1 and consequently disturbed iron homeostasis in various cellular systems exposed to inflammatory conditions. More specifically, NO converts IRP-1 from an aconitase to a trans-regulatory factor by directly targeting its [Fe-S] cluster leading to its complete disassembly. In this way, the aconitate generated by NO, in a reducing environment, tightly binds to IRE(s) and exerts its iron-regulatory function. In the early 1990s, it was reported that simultaneous production of NO and superoxide (O2) by activated macrophages led to intracellular formation of peroxynitrite. Since NO can turn into a powerful oxidizing and nitrating agent such as peroxynitrite in vivo, its reactivity toward key enzymes of cellular metabolism has become an active area of investigation. Regarding IRP-1, in vitro studies demonstrated that peroxynitrite causes the direct inhibition of its aconitase activity by promoting complete disruption of the [4Fe-4S] cluster but, unlike NO, without stimulating the IRE-binding activity of IRP-1. These data suggest that peroxynitrite might generate additional post-translational modifications of IRP-1 in comparison with NO. Interestingly, recent biochemical and resonance Raman studies have allowed the detection of tyrosine nitration on pure recombinant IRP-1 by synthetic peroxynitrite or by SIN-1, an NO/O2 donor. However, in vivo studies of IRP-1 nitration have never been undertaken. Currently, the physiological mechanism of protein nitration in vivo is much debated, and whether peroxynitrite is really the reactive precursor of nitrating species in biological systems has been questioned. Indeed, alternative mechanisms, such as the one mediated by the nitrite/H2O2/myeloperoxidase pathway, have now been identified, particularly in activated neutrophils and certain macrophages. In mammalian cells, iron regulatory proteins (IRP-1 and -2) orchestrate iron trafficking, storage, and availability by modulating ferritin and transferrin receptor expression at a post-transcriptional level. They operate by interacting with one or several specific stem-loop RNA structures called iron-responsive elements (IREs), which are located in untranslated regions (UTR) of several mRNAs. At low intracellular iron concentration, IRPs bind to the IRE of ferritin mRNA at its 5'-UTR and block translation, whereas they stabilize transferrin receptor mRNA through direct interactions with several IRE motifs in the 3'-UTR. One critical discrepancy between the two IRPs is that only IRP-1 functions as a cytosolic aconitase when holding a [4Fe-4S] cluster, which masks the IRE-binding domain. In this case, holo-IRP-1 catalyzes the interconversion of citrate into isocitrate via the intermediate formation of cis-aconitate, as does its mitochondrial counterpart in the Krebs cycle. It is well documented that nitric oxide (NO) also stimulates the trans-regulatory activity of IRP-1 and consequently disturbs iron homeostasis in various cellular systems exposed to inflammatory conditions. More specifically, NO converts IRP-1 from an aconitase to a trans-regulatory factor by directly targeting its [Fe-S] cluster leading to its complete disassembly. In this way, the apoprotein generated by NO, in a reducing environment, tightly binds to IRE(s) and exerts its iron-regulatory function. In the early 1990s, it was reported that simultaneous production of NO and superoxide (O2) by activated macrophages led to intracellular formation of peroxynitrite. Since NO can turn into a powerful oxidizing and nitrating agent such as peroxynitrite in vivo, its reactivity toward key enzymes of cellular metabolism has become an active area of investigation. Regarding IRP-1, in vitro studies demonstrated that peroxynitrite causes the direct inhibition of its aconitase activity by promoting complete disruption of the [4Fe-4S] cluster but, unlike NO, without stimulating the IRE-binding activity of IRP-1. These data suggest that peroxynitrite might generate additional post-translational modifications of IRP-1 in comparison with NO. Interestingly, recent biochemical and resonance Raman studies have allowed the detection of tyrosine nitration on pure recombinant IRP-1 by synthetic peroxynitrite or by SIN-1, an NO/O2 donor. However, in vivo studies of IRP-1 nitration have never been undertaken. Currently, the physiological mechanism of protein nitration in vivo is much debated, and whether peroxynitrite is really the reactive precursor of nitrating species in biological systems has been questioned. Indeed, alternative mechanisms, such as the one mediated by the nitrite/H2O2/myeloperoxidase pathway, have now been identified, particularly in activated neutrophils and certain macrophages. In this study, we have therefore explored whether nitration of IRP-1 could occur in a whole-cell (physiological) context. To address this issue, we used the murine RAW 264.7 macro-
Endogenous IRP-1 Nitration in Activated Macrophages

phages and favored the generation of endogenous nitrating species by stimulating them with inflammatory and/or pharmacological stimuli, thus seeking IRP-1 tyrosine nitration. We then investigated the biological significance of this endogenous nitration by measuring both IRP-1 functions in parallel, in the presence or absence of cis-acantone, a substrate that protects the holo-form of IRP-1. Finally, we also attempted to identify the nitrating pathway that mediates IRP-1 nitration in activated macrophages.

**EXPERIMENTAL PROCEDURES**

**Products**—Murine recombinant interferon-γ (IFN-γ; specific activity, 2 × 10^5 units/mg) was provided by R&D Systems. Escherichia coli lipopolysaccharide (LPS; serotype 111:14); phorbol 12-myristate 13-acetate (PMA), 4-aminoazobenzoic acid hydrazide (BAAB), salicylhydroxamic acid (SHA), p-hydroxybenzoic acid hydrazide (pHBAH), epicatechin, and cis-acantone were from Sigma. Selenomethionine was from Calbiochem (VWR International). α-Monothymyl-γ-arginine (L-NMA) and S-ethylisothiourea (EIT) were from Cayman Chemical (Ann Arbor, MI). Peroxynitrite was synthesized according to the method of Uppu and Pryor (16) and concentrated by freezing. Residual hydrogen peroxi- de in the final solution was removed by treating peroxyamine solution over solid granular manganese dioxide (WWR International). The macrophage RAW 264.7 cell line was cultured in high glucose Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (Eurobio, Les Ulis, France) at 37 °C in a 5% CO2 atmosphere. Cells (5 × 10^6) were treated with 20 units/ml IFN-γ and 50 mM LPS for 18 h to induce endogenous NO` production. To stimulate the production of O2·− in the presence of NO`, a second stimulation by 50 mM PMA was applied to IFN-γ-LPS (pre)-stimulated cells for additional times as indicated in each figure. To analyze the signaling pathway of nitrating species, cells were also treated with IFN-γ-LPS or IFN-γ-LPS/PMA as described above, in the presence of NO synthase inhibitors (1 mM t-NMA, 100 mM EIT), peroxynitrite scavengers (100 mM epicatechin, 30 mM selenomethionine), or myeloperoxidase inhibitors (50 mM ABAH, 50 mM SHA, 50 mM pHBAH).

**Nitrite Measurement**—The nitrite content in cell culture supernates was determined using the Griess reagent containing 0.5% sulfanilamide and 0.05% N-(1-naphthyl)ethylenediamine hydrochloride in 45% acetic acid. Nitrite reacts with Griess color reagent to give a red-violet diazo-dye, which is measured spectrophotometrically at 543 nm; nitrite concentration is calculated from a sodium nitrite standard curve.

**Citoplasmonic Extracts**—After each treatment, cells were harvested in 25% trichloroacetic acid containing 100 mM sodium bicarbonate to favor formation of the nitrosoperoxocarboxy- late intermediate, which causes more nitration and less sulhydryl oxidation (17). In some experiments, cytosols were pretreated with increasing concentrations of cis-acantone prior to incubation with a bolus of peroxynitrite. Concentration of peroxynitrite stock solution was determined at 302 nm (ε = 1670 M^-1 cm^-1) in 0.1 M NaOH solution before use. The pH of each reaction mixture was carefully checked after peroxynitrite addition and was maintained at 7.5 in all experiments.

**Aconitate Activity**—Aconitate activity was measured spectrophotometrically by following the disappearance of the cis-acantone at 240 nm (18). Fifty micrograms of cytosolic extracts were preheated at 37 °C in 0.1 mM Tris-HCl buffer at pH 7.4 before starting the enzymatic reaction with the aconitate substrate. Units correspond to nmol of substrate consumed/min, and ε240 nm = 3.6 M^-1 cm^-1. 

**Electrophoretic Mobility Shift Assay**—IRP-1/IRE interactions were visualized as described previously (19). Briefly, 2 μg of protein was incubated with an excess of 32P-labeled H-chain ferritin in 20 μl of 10 mM Hepes, pH 7.6; 40 mM KCl, 3 mM MgCl2, and 5% glycerol (buffer A). After parallel experiments, samples were treated with 2% SDS and transferred to nitrocellulose membranes. The membranes were then blocked in 3% milk, phosphate-buffered saline for 20 min at room temperature with constant agitation and then incubated with polyclonal anti-nitrotyrosine antibody (diluted 1:5000; Cayman Chemical) in blocking buffer overnight at 4 °C. After several washings, the blots were incubated with a peroxidase-conjugated goat-antirabbit secondary antibody (Dako, Trappes, France) for 1 h at room temperature. The immunoreactive bands were detected by using the enhanced chemiluminescence Western blotting detection system (Amer sham Biosciences).

**Enzyme-linked Immunosorbent Assay for 3-Nitrotyrosine**—Recombinantly purified human IRP-1 was incubated with 5 mM cis-acantone for 10 min at room temperature before adding 500 μM SIN-1 for an additional 30 min at 37 °C in phosphate-buffered saline, pH 7.6. No pH variation was detected at any time during incubation. 3-Nitrotyrosine-modified IRP-1 was quantified in 96-well plates using 0.5 μg IRP-1. Hybridization was carried out with the mouse monoclonal nitrotyrosine antibody (Cayman Chemical) for 2 h at 37 °C followed by several washings with phosphate-buffered saline, 0.05% Tween 20. Sequential incubations were then performed with horseradish peroxidase-conjugated anti-mouse IgG (1:2000) for 1 h at 37 °C. Absorbance was measured at 490 nm after color development was initiated by substrate addition.

**Electronic Paramagnetic Resonance (EPR) Spectroscopy and Spin Trapping**—RAW 264.7 macrophages were stimulated with 100 μM LPS and then treated with 0.007% digitonin on ice. After 10 min, cell lysate was ultracentri- fuged at 100,000 × g for 1 h at 4 °C. The cytosolic fraction (supernatant) was tested for protein concentration, and aconitate activity was measured before storage at −80 °C.

**In Vitro Treatment with a Bolas of Peroxynitrite**—Cytosols (100 μg) of control cells were treated with increasing concentrations of peroxynitrite added as a bolus in 50 mM phosphate buffer, pH 7.5, containing 100 mM sodium bicarbonate to favor formation of the nitrosoperoxocarboxy- late intermediate, which causes more nitration and less sulhydryl oxidation (17). In some experiments, cytosols were pretreated with increasing concentrations of cis-acantone prior to incubation with a bolus of peroxynitrite. Concentration of peroxynitrite stock solution was determined at 302 nm (ε = 1670 M^-1 cm^-1) in 0.1 M NaOH solution before use. The pH of each reaction mixture was carefully checked after peroxynitrite addition and was maintained at 7.5 in all experiments.

**RESULTS**

**Tyrosine Nitration of Cytosolic IRP-1 by Peroxynitrite**—Our previous work concerning the mechanism of action of peroxynitrite on IRP-1 has shown that the molsidomine metabolite SIN-1, which spontaneously decomposes to yield both NO` and O2·− concomitantly, thus forming a steady flux of peroxynitrite, was able to nitrate pure recombinant human IRP-1 in vitro (6). To address whether cellular endogenous IRP-1 might also be nitrated in response to nitrogen-derived species, we first ex- posed the cytosolic fractin of resting RAW 264.7 macrophages to increasing concentrations of synthetic peroxynitrite. IRP-1 was then immunoprecipitated from cytosols, and tyrosine ni- tration on IRP-1 was detected by Western blot analysis using a polyclonal nitrotyrosine antibody. As shown in Fig. 1, IRP-1 from untreated cytosol exhibited high aconitate activity (50 units/mg defined as 100% activity in this assay) and non- significant nitration. In contrast, the addition of peroxynitrite to resting cell cytosol led to IRP-1 nitration, which was rapidly detectable in the low micromolar range (Fig. 1, B and C). It is noteworthy that the appearance of nitration after a short ex- posure of 10 μM peroxynitrite was accompanied by a significant decrease in cytosolic aconitase activity (76% of the control value) (Fig. 1A). Increasing concentrations of peroxynitrite, from 25 to 50 μM, progressively rendered IRP-1 nitration more intense and cytosolic aconitate less active (Fig. 1, A and B). In parallel, we also observed that IRP-1 IRE-binding activity in
Endogenous IRP-1 Nitration in Activated Macrophages

Fig. 1. IRP-1 tyrosine nitration by increasing concentration of peroxynitrite. Cytosols (100 μg) from control cells were exposed to increasing concentrations of peroxynitrite (ONOO\(^{−}\)) in 50 mM phosphate buffer containing 100 mM sodium bicarbonate at pH 7.5. A, after peroxynitrite treatment, cytosolic aconitase activity was measured in each sample. B, in parallel, cytosols were immunoprecipitated with anti-IRP-1 antibody followed by Western blot analysis using a specific anti-nitrotyrosine antibody. Recombinant human IRP-1 (rhIRP1) was treated with 500 μM peroxynitrite and used as the molecular weight positive nitrated control. C, the level of tyrosine-nitrated IRP-1 was quantified by densitometry using Bio-Rad Analyzer Gel Doc 2000. The experiments were performed at least three times, and a representative result is shown. A.U., arbitrary units.

Fig. 2. IRP-1 tyrosine nitration by macrophages activated with IFN-γ/LPS or IFN-γ/LPS/PMA. A, scheme showing treatment of RAW 264.7 macrophages with 20 units/ml IFN-γ, 50 ng/ml LPS, either in the presence or absence of 50 nM PMA. Two sets of parallel cell culture flasks were treated with IFN-γ/LPS. After 18 h of incubation, cells from one set of flasks were stimulated with PMA for an additional 3 and 6 h, whereas cells in the second set of flasks were incubated for an additional 6 h without any other stimulation. B, nitrite was determined in the cell culture medium. Cells were then harvested, and cytosolic fractions were isolated as described under “Experimental Procedures,” and aconitase activity was measured. IRP-1 was immunoprecipitated from cytosols, and its nitration was revealed by Western blot analysis with a polyclonal anti-nitrotyrosine antibody. The experiments were performed at least three times, and a representative result is shown.

Endogenous Nitration of IRP-1 in IFN-γ/LPS- and IFN-γ/LPS/PMA-activated Macrophages—To address the question of whether endogenous IRP-1 might be nitrated in a whole-cell physiological context, murine RAW 264.7 macrophages were incubated with IFN-γ and LPS for 18 h to stimulate NO\(^{−}\) production through NO synthase-2 induction (Fig. 2A). Then, activated cells were further incubated with or without 50 nM PMA for an additional 3 or 6 h to stimulate O\(^{2−}\) production (20). We checked that during this time frame nitrite accumulated linearly in the cell culture medium (65, 80, and 95 μM at times 0, 3, and 6 h after PMA addition, respectively) and that stimulated cells produced NO\(^{−}\) using an EPR spin-trapping assay (data not shown). Then we harvested the cells and extracted their cytosols. IRP-1 was isolated from cytosols by immunoprecipitation, and its nitration was detected by Western blot analysis using a polyclonal nitrotyrosine antibody. As shown in Fig. 2B, constitutively expressed IRP-1 was not nitrated in intact resting macrophages, whereas its cytosolic aconitase activity was high and nitrite production was insignificant. However, after 24 h of IFN-γ/LPS stimulation, the protein exhibited tyrosine nitration. Interestingly, the intensity of IRP-1 nitration increased further in a time-dependent manner when IFN-γ/LPS-treated macrophages were co-stimulated with PMA. Our data also showed that IRP-1 nitration in activated macrophages was accompanied by complete inhibition of cytosolic aconitase activity of IRP-1 (Fig. 2B). In another set of experiments, we investigated the direct involvement of the NO-signaling pathway in endogenous nitration of IRP-1. RAW 264.7 macrophages were stimulated for 24 h with IFN-γ/LPS or IFN-γ/LPS/PMA, as described in Fig. 2, in the presence of L-NMA and EIT, two inhibitors of inducible NO synthase. We showed in Fig. 3 (A and B) that the L-NMA/EIT combination prevented both nitrite production and inhibition of aconitase activity in stimulated cells. Importantly, these two events were correlated with a significant decline in IRP-1 tyrosine nitration (Fig. 3C, compare lanes 5 and 6 with lanes 2 and 3). We also noticed that treatment of PMA alone triggers neither nitration of IRP-1 nor inhibition of cytosolic aconitase activity.

Inhibition of IRP-1 Activities after Optimal Tyrosine Nitration—To investigate further the relationship between IRP-1 nitration and its functions, in parallel experiments we measured IRP-1 IRE-binding activity in control and activated macrophages. As expected from earlier studies, binding of IRP-1 to the IRE of ferritin mRNA was stimulated in IFN-γ/LPS-treated macrophages (Fig. 4A). However, when PMA was added along with IFN-γ/LPS, IRP-1 was far less potent in binding IRE (Fig. 4A, upper panel, compare lane 2 with lane 3). Remarkably, this impairment of IRP-1 in binding the IRE motif correlated with the strongest IRP-1 nitration (compare Fig. 3C with Fig. 4A). We also performed the binding assay in the presence of 2% 2-ME prior the IRE probe addition (Fig. 4A, lower panel). It is well known that this treatment allows full expression of IRP-1 IRE-binding activity (21). Under these conditions, the IRP-1 of control cells was totally converted into its IRE-binding form (Fig. 4A, lower panel, lane 1). As previously shown, full IRP-1 IRE-binding capacity of IFN-γ/LPS-
Endogenous IRP-1 Nitration in Activated Macrophages

...treated cells measured in the presence of 2% 2-ME was half that of control (Fig. 4A, lower panel, compare lanes 1 and 2, and Fig. 4B). This was previously explained by a 50% decrease in IRP-1 protein level in response to NO (22) and illustrated here in Fig. 4C (compare lanes 1 and 2). Remarkably, when IFN-γ/LPS-treated cells were co-incubated with PMA, the full IRE-binding activity of IRP-1 (in the presence of 2% 2-ME) was further reduced (Fig. 4A, lower panel, compare lanes 1, 2, and 3, and Fig. 4B) to a residual value of 30% of control. This 2-ME-resistant reduction of IRP-1 IRE-binding activity was not due to IRP-1 protein loss, because IRP-1 level was not further reduced during co-stimulation of IFN-γ/LPS-treated cells with PMA (Fig. 4C, compare lanes 2 and 3). These results show that PMA-induced loss of IRE-binding capacity of IFN-γ/LPS-stimulated cells results from a post-translational modification of IRP-1.

We then asked whether IRP-1 nitration precedes or follows [Fe-S] cluster disruption. To solve this issue, we preincubated the cytosols of untreated cells with increasing concentrations of cis-aconitate before adding 25 μM peroxynitrite, a concentration that significantly enhanced nitration of IRP-1 in vitro and considerably reduced aconitase activity (Fig. 1). In this set of experiments, we used cis-aconitate because this aconitase substrate is known to protect IRP-1 through stabilization of the [4Fe-4S] cluster of IRP-1 (12, 23). After cis-aconitate/peroxynitrite treatment, aconitase activity was determined in cytosols, and nitration of IRP-1 was sought after immunoprecipitation and Western blot analysis. As shown in Fig. 5, A and B, untreated cytosols exhibited high aconitase activity of IRP-1 and no significant IRP-1 nitration. As expected, peroxynitrite alone led to a 93% inhibition of aconitase activity, which was accompanied by pronounced tyrosine nitration on IRP-1. Notably, IRP-1 nitration by peroxynitrite was progressively reduced to the basal level in cytosols preincubated with increasing concentrations of cis-aconitate. This reduction was accompanied by progressive protection of IRP-1 aconitase activity (Fig. 5B). Parallel control studies demonstrated that 1 mM cis-aconitate did not inhibit nitration of bovine serum albumin induced by 25 μM peroxynitrite. We also observed the same protective effect of cis-aconitate on purified recombinant human IRP-1 as regards both nitration and aconitase inhibition by SIN-1, which spontaneously released a low, steady flux of peroxynitrite at physiological pH (data not shown). These data thus show that the [4Fe-4S] aconitase form of IRP-1, when protected by cis-aconitate, is not sensitive to in vitro nitration by peroxynitrite.

Involvement of a Nitrate/H₂O₂/Myeloperoxidase Pathway in IRP-1 Tyrosine Nitration—In biological systems, endogenous production of peroxynitrite, which results from the simultaneous production of O₂⁻ and NO⁻, requires very specific conditions to nitrate proteins efficiently (24). Alternative mechanisms have recently been identified in endogenous tyrosine nitration (14). In particular, it has been reported that nitrite, the primary metabolic end-product of NO⁻, can be oxidized by my-
Cytosolic extracts were treated with 0, 0.1, 0.5, and 1 mM cis-aconitate prior to exposure to 25 μM peroxynitrite (ONOO⁻) for 15 min at room temperature. A, treated cytosols were then immunoprecipitated with anti-IRP-1 antibody and analyzed by Western blotting using a specific anti-nitrotyrosine antibody. B, in parallel, aconitase activity was determined in control and treated cytosols. The experiments were performed three times, and a representative result is shown.

eloperoxidase in the presence of H₂O₂ to yield NO₂⁻, the main contributor to tyrosine nitration (25). Moreover, involvement of this pathway has been outlined in RAW 264.7 cells (15). We therefore investigated the molecular mechanism of intracellular IRP-1 nitration by incubating IFN-γ/LPS- or IFN-γ/LPS/PMA-treated RAW 264.7 macrophages with the potent and selective myeloperoxidase inhibitors ABAH, pHBAH, and SHA (14, 26, 27) or the peroxynitrite scavengers epicatechin and selenomethionine (28, 29). After 24 h, nitrite production was measured in cell culture medium, and aconitase activity, as well as nitration of IRP-1, was determined in cell cytosols. We first report, in Figs. 6A and 7A, that peroxynitrite scavengers and myeloperoxidase inhibitors did not prevent inducible NO synthase expression and activity in macrophages stimulated by IFN-γ/LPS in the presence or not of PMA, as indicated by unaffected nitrite production. As expected, IRP-1 aconitase activity was fully inhibited in all cases except for control and PMA-treated cells, which did not produce NO²⁻. In parallel, we showed that incubation of activated cells with either epicatechin, selenomethionine, or the three myeloperoxidase inhibitors completely abrogated IRP-1 tyrosine nitration in IFN-γ/LPS- and IFN-γ/LPS/PMA-activated macrophages (Figs. 6B and 7B). We also considered the possibility that nitrite, as a myeloperoxidase substrate, could induce nitration of IRP-1 in RAW 264.7 cells cultured in absence of stimulation. These cells did not display alteration of the IRP1 [Fe-S] cluster as testified by high aconitase activity, and no IRP1 nitration was detectable by immunoblotting (data not shown).

We then investigated whether impairment of IRE binding by IRP-1 in IFN-γ/LPS/PMA-activated macrophages, shown in Fig. 4A, was relieved by 2% 2-ME when nitration was prevented. As shown in Fig. 8, in the presence of SHA or pHBAH, full IRE-binding activity of IRP-1 regained the same level as in macrophages activated with IFN-γ/LPS without co-stimulation with PMA (compare lanes 4 and 5 with lanes 2 and 3).

**DISCUSSION**

IRP-1, the most abundant pool of potential IRE-binding activity in mammalian tissues, is equipped with a redox-sensitive [Fe-S] cluster and is therefore liable to respond to NO⁻ and congeners (30). Recently, we have shown that IRP-1 is also a potential protein target of nitration by peroxynitrite in vitro (13). However, important questions still remain regarding the physiological relevance of IRP-1 nitration and how this process is triggered in vivo. In this study, we investigated IRP-1 tyro-
Endogenous IRP-1 Nitration in Activated Macrophages

Fig. 8. Effect of salicylhydroxamic acid and p-hydroxybenzoic acid hydrazide on IRP-1 IRE-binding in IFN-γ/LPS/PMA-stimulated macrophages. Cells were activated with 20 units/ml IFN-γ and 50 ng/ml LPS with or without 50 nM PMA as described for Fig. 2. In parallel, stimulated macrophages were also incubated with 50 μM SHA or pBAH. After 24 h, cytosols were prepared, and IRE-binding activity was determined by electrophoretic mobility shift assay in the presence of 2% 2-ME. The experiments were performed three times, and a representative result is shown.

Nitrogen dioxide (NO₂), which is the major nitrating species, can be formed by rapid decomposition of peroxynitrite at physiological pH or by the oxidation of nitrite (the stable end-product of NO metabolism) by peroxides in the presence of H₂O₂ (31). Nonetheless, the question of which of these two proposed mechanisms promotes peroxynitrite-mediated nitration has been fiercely debated. IRP-1 nitration by IFN-γ/LPS- or IFN-γ/LPS/PMA-treated macrophages was fully prevented by both peroxynitrite scavengers and myeloperoxidase inhibitors, suggesting that peroxynitrite may operate along with the nitrite/H₂O₂/peroxidase activities to nitrate IRP-1. However, as prevention was complete with both types of compounds, this scenario is unlikely. We therefore wondered about the actual specificity of the peroxynitrite scavengers. First of all, it is worth recalling that most peroxynitrite scavengers also react with O₂⁻, which is the precursor of H₂O₂, the substrate of myeloperoxidase. Accordingly, both pathways might be affected by O₂⁻ scavengers. Moreover, recent findings have shown that epicatechin, at concentrations used in this study, inhibits myeloperoxidase-mediated nitration in vitro (32). Finally, it has been proposed that peroxides may catalyze peroxynitrite-dependent nitration via a two-electron oxidation reaction (33–35). Altogether, these issues may explain why IRP-1 nitration was prevented both by the two presumed peroxynitrite scavengers and by myeloperoxidase inhibitors, casting doubt on the participation of the peroxynitrite pathway in peroxidase-independent IRP-1 endogenous nitration. Taken together, these data and considerations are in favor of the nitrite/H₂O₂/peroxidase pathway as the major IRP-1 nitrating pathway in physiologically activated macrophages.

IRP-1 is a bifunctional metalloprotein in which the aconitate and IRE-binding activities are mutually exclusive, depending on the presence or absence of its [4Fe-4S] cluster (1). In the present study, we show that endogenous nitration of IRP-1 in activated macrophages boosted with PMA is associated with impairment of both IRP-1 functions. We found that lack of IRP-1 binding to IRE in cells co-generating NO and O₂⁻ was not due to IRP-1 protein degradation but rather to a post-translational modification that was not reversible by high 2-ME concentrations. Importantly, the recovery of IRP-1 IRE binding under those reducing conditions occurred only when nitration was prevented by myeloperoxidase inhibitors. A number of reports have indicated that the bulky nitro group can prevent protein activity by steric hindrance (36, 37). Therefore, we postulate that nitration, a fairly stable modification, arose at a strategic location(s) in the backbone of the IRP-1 apo-protein, blocking its binding to the IRE motif. Similarly, it has been reported that endogenous nitration of peroxisome proliferator-activated receptor-γ inhibited its ligand-dependent translocation from cytosol to nucleus in LPS-stimulated RAW 264.7 macrophages (38).

To determine whether nitration of IRP-1 follows or precedes aconitase loss, we exposed IRP-1-containing cytosols to a concentration of peroxynitrite that is efficient for nitration in the presence of cis-aconitase. We took advantage of the capacity of cis-aconitase to interact directly with [4Fe-4S] clusters of aconitases, which results in holo-IRP-1 stabilization (23). We observed that cis-aconitase dose-dependently prevented IRP-1 nitration by peroxynitrite or SIN-1, as well as inhibiting its aconitase activity. These results point to nitration as an event downstream of [Fe-S] disruption and therefore of aconitase loss. Accordingly, nitrate, a substrate for myeloperoxidase, which can trigger global nitration in RAW 264.7 macrophages (15), led neither to inactivation of cytosolic aconitate nor to subsequent endogenous IRP-1 nitration. Since the aconitase form of IRP-1 was insensitive to nitration, we can presume that potentially reactive tyrosines of holo-IRP-1 is not accessible to the nitrating agent. It is held that IRP-1, devoid of its cluster, gains a more relaxed conformation than the [4Fe-4S] cluster-containing IRP-1, rendering its regulatory binding site accessible to IRE (39, 40). As peroxynitrite and NO⁺ disrupt the [Fe-S] cluster of IRP-1 in vitro (13), we propose that complete loss of [Fe-S] cluster is a prerequisite to allow one or several tyrosine residues initially buried in holo-IRP-1 to become accessible to nitrating species.

In our cellular model, it is worth noting that moderate nitration of IRP-1 mediated by IFN-γ/LPS stimulation was not associated with any loss of IRP-1 IRE-binding activity. Under these conditions, nitration of IRP-1 would modify tyrosines not critical for adequate binding to IRE. In contrast, endogenous (without 2-ME) and full (with 2-ME) IRE-binding activities were reduced when IFN-γ/LPS-stimulated cells were also exposed to PMA. Generation of an oxidative burst upon PMA would therefore contribute to nitration of specific tyrosines close to the IRE-binding domain. This may simply result from strengthened overall IRP-1 nitration. Alternatively, based on a previous report (41), it was tempting to consider that PMA-boosted production of reactive oxygen species, associated with iron knocked out from an IRP-1-disrupted [Fe-S] cluster, would favor local nitration through Fenton chemistry. Indeed, in vitro studies previously showed that redox metals, particularly iron, can catalyze protein tyrosine nitration promoted by peroxynitrite (42) or by nitrite/H₂O₂ (41, 43). However, our results showing that myeloperoxidase inhibitors completely abrogated nitration strongly suggest that “autocatalytic” nitration of IRP-1 by its own iron was not relevant under these conditions. In IRP-1, seven conserved tyrosines are located in one part of the IRE-binding domain, including the [Fe-S] cluster-proximal tyrosine 501 (44), and are thus good candidates for nitration. Future studies, including mass spectrometry experiments, are
required to localize nitrated tyrosines, but we have already demonstrated that endogenous tyrosine nitration can persistently hamper IRP-1 functioning.

The biological significance of these findings remains debatable, but some clues may be foreseen in the particular metabolism of macrophages, which are prone to produce both reactive oxygen species and nitrogen-derived species. In pathophysiological situations such as inflammatory diseases, target cells adjacent to NO-producing macrophages undergo NO/IRP-1 regulation (45). Upon IRP-1 activation, ferritin is repressed and transferrin receptor is up-regulated, resulting in a toxic iron overload (46). Moreover, recent evidence indicates that nitration of tyrosine-bound proteins is a dynamic process sensitive to oxygen tension (47). Accordingly, we speculate that prevention of IRP-1 activation by nitration in macrophages during inflammatory processes might be an intrinsic protective mechanism against the noxious effect of iron through Fenton-like reactions.

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Endogenous IRP-1 Nitration in Activated Macrophages 43351