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IFN-γ-ACTIVATED MACROPHAGES: DETECTION BY ELECTRON PARAMAGNETIC RESONANCE OF COMPLEXES BETWEEN L-ARGININE-DERIVED NITRIC OXIDE AND NON-HEME IRON PROTEINS

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Interferon-γ induces the L-Arginine-dependent pathway that leads to the formation of nitrogen oxides in murine macrophages with subsequent inhibition of mitochondrial non-heme iron-dependent enzymes. To evaluate a possible role of nitric oxide through binding to enzymes containing iron-prosthetic groups, we used Electron Paramagnetic Resonance spectroscopy. In IFN-γ-activated macrophages, we observed the appearance of a signal in the g=2.04 region which is consistent with that given by nitrosyl-iron-sulfur complexes. Appearance of this signal was dependent on the presence of L-Arginine in the culture medium. Furthermore, we detected a virtually identical signal in macrophages non stimulated by IFN-γ, following exposure to nitric oxide (after addition of an excess of nitrite in the presence of ascorbate). These data suggest that L-Arginine-derived nitric oxide may alter the configuration of the catalytic site of certain mitochondrial enzymes by coordinating to iron at their iron-sulfur cluster(s).

Cytotoxic activated macrophages and endothelial cells are capable of producing nitrogen oxides derived from the guanido group of L-Arginine through a recently discovered metabolic pathway (1-3). IFN-γ, alone or in combination with LPS or TNF, is a potent inducer of this pathway in murine macrophages (4-6). Oxidation of L-Arginine into nitrogen oxides by cytotoxic activated macrophages is a prerequisite for metabolic dysfunction including growth arrest of target cells (7) and inhibition of mitochondrial non heme iron-sulfur enzymes both in target cells and in activated macrophages themselves (7,8); these enzymes are aconitase of the citric acid cycle and NADH-Ubiquinone oxidoreductase (complex I) and Succinate-Ubiquinone oxidoreductase (Complex II) of the respiratory chain. Nitric oxide (NO·) was recently identified as one of the products of L-Arginine oxidation both in endothelial cells (3) and in activated macrophages (9-11) and was proposed as an activated macrophage effector molecule (10). Indeed, gaseous NO· is able to induce loss of activity of aconitase, complex I and complex II as well as growth inhibition in L1210 leukemia cells (10,12). To further characterize this phenomenon at a molecular level, we determined whether coordination complexes between L-Arginine-derived NO· and iron-containing proteins could be detected in IFN-γ-activated macrophages by EPR spectroscopy. This report

Abbreviations: DMEM, Dulbecco's Modified Essential Medium ; EPR, Electron Paramagnetic Resonance; IFN-γ, murine recombinant interferon-gamma; LPS, lipopolysaccharide; MO, macrophages; NMMA, N^G-monomethyl-L-Arginine; TNF, Tumor Necrosis Factor.

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points to the existence of a signal of (NO-Fe-S)-type in peritoneal macrophages as well as in the RAW 264.7 macrophage cell line activated by IFN-γ in the presence of L-Arginine.

MATERIALS AND METHODS

Media and Reagents. Dulbecco's Modified Essential Medium (DMEM) and low endotoxin fetal calf serum (0.11 ng/ml) were obtained from Gibco BRL, Cergy Pontoise, France. Murine recombinant interferon gamma (2 x 10^7 U/mg) was produced by Genentech (San Francisco, CA) and provided by Dr. G.R. Adolf (Boehringer Ingelheim, Vienna, Austria). N^G-monomethyl-L-Arginine (NMMA) was obtained from Calbiochem (La Jolla, CA). All other chemicals were obtained from Sigma (St Louis, MO)

Macrophage culture. Eight to 10 week-old C3H/HeN female mice (Charles River, France) were injected i.p. with 1 ml thioglycolate broth (Institut Pasteur Production, Paris, France) prior to being sacrificed 4 days later. Cells were harvested from the peritoneum and allowed to adhere for 2 h at 37°C in 5 % CO_2. After extensive washings, cell monolayers consisted of more than 95 % macrophages as assessed by neutral red uptake and latex beads phagocytosis. This macrophage population will be referred to as "elicited macrophages". The continuous macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Rockville, MD). The P388D1 macrophage cell line was kindly provided by Dr. B. Dugas, Institut Beaufour, les Ulis, France. Both cell lines were maintained in DMEM supplemented with 2mM glutamine, 1 mM pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin. Elicited macrophages and macrophage cell lines were cultured in 100-mm plastic tissue culture dishes (Nunc, Denmark) to a concentration of 1 x 10^6 cells/ml, either in DMEM supplemented as described above or in RPMI 1640 (kit select-amine, Gibco Laboratories) reconstituted with all ingredients, except L-Arginine. Media were added with 5 % or 10 % heat inactivated fetal calf serum for elicited macrophages or for cell lines, respectively.

Spectrophotometric measurements of Nitrite (NO_2^-) and aconitase activity. Release of NO_2^- in culture medium was measured spectrophotometrically as described (8) by using the Griess reagent. Aconitase activity was measured as previously described (13) with a spectrophotometer (Ultrospec II, LKB, Sweden) by following the initial rate of cis-aconitate disappearance at 240 nm.

EPR spectroscopy. Eight to 10 x 10^7 cells were collected with a rubber policeman in 0.25 M sucrose, 50 mM Hepes pH 7.2 and permeabilized with 0.007 % digitonin. After centrifugation, cells were resuspended in the sucrose/Hepes buffer and directly transferred into EPR tubes and packed by centrifugation (15 min. x 2000g). Samples were rapidly frozen in liquid nitrogen and stored at 77 K until needed. EPR spectra were obtained at 77K using a Varian E 109 spectrometer. The spectrometer was coupled to a Digital Minc 11 computer and spectra were stored for subsequent baseline subtraction and difference spectrum performance.

RESULTS AND DISCUSSION

Peritoneal macrophages as well as RAW 264.7 macrophages were stimulated with 200 U/ml IFN-γ for 24 hours. Release of nitrite (NO_2^-), a stable product of NO· found in the culture medium, was measured to monitor the L-Arginine-derived formation of nitrogen oxides. Aconitase activity was followed in parallel to NO_2^- synthesis and EPR performance (Table I). Prior to performing aconitase measurements and EPR spectra, macrophages were treated with digitonin that selectively permeabilizes plasmic membranes. This treatment was aimed at eliminating cytoplasmic material extracellularly in order to concentrate mitochondria.

The EPR spectra at 77 K of the digitonin-permeabilized macrophages are shown in Figure 1. In unstimulated macrophages two symmetrical narrow signals at g_1=2.005 and g_2=2.030 with peak-to-peak line-width of 16 gauss, were always observed (Figure 1A). As shown in Figure 1 (lower panels) these signals did not saturate. They could either be produced by two independent paramagnetic species, or represent the anisotropic $g_{//}$ and $g_{\perp}$ values of a single paramagnetic

TABLE I. NO_2^- synthesis and inhibition of aconitase activity in macrophages exposed to various treaments

Macrophages	Treatment	NO_2^- (nmol./ml)	Aconitase activity (% of control)
Thioglycolate-elicited	None	3.3 ± 3.0	100
	IFN-γ	73.0 ± 24.3	11
	IFN-γ with no L-Arg	4.0 ± 2.9	75
	IFN-γ with L-Arg and NMMA	26.8 ± 8.4	nd*
RAW 264.7	None	2.2 ± 0.6	100
	IFN-γ	40.1 ± 2.6	22
P388D1	None	2.7 ± 1.2	100
	IFN-γ	1.5 ± 0.5	109

Macrophages (1.2 x 10^6/ml) were incubated for 24 hours with 200 U/ml IFN-γ in RPMI with or without 1.15 mM L-Arginine (elicited macrophages) or in complete medium (macrophage cell lines). Supernatants (150ml) were collected for NO_2^- determinations. Values are the mean ± SD for 3 to 7 separate experiments. Aconitase activity was measured in lysates of digitonin-permeabilized cells. Values are the mean for 3 to 6 separate experiments.* not determined.

center, of unknown origin in either case. A typical EPR spectrum of IFN-γ-activated macrophages is shown in Figure 1B; in addition to the g_1=2.005, the much broader g_2=2.030 signal (24 gauss) consisted of a high-field trough at g_3=2.016 and an additional overlapping signal at g_4=2.042. A typical computerized difference spectrum shown in Figure 1C allows to clearly distinguish the g_3 and g_4 signals and points out the contribution of the IFN-γ effect. The microwave power saturation behavior of the different signals at 77K, shown in Figure 1 (lower right panel), allows also to differentiate the g_3 and g_4 signals from the intrinsic g_1 and g_2 signals. While the g_1 and g_2 signals did not saturate or did so only slightly, the g_3 and g_4 signals saturated markedly above 10 mW in a similar way. The difference spectrum and the saturation behaviour strongly suggest that g_3 and g_4 actually arise from the same paramagnetic center and may represent respectively the $g_{//}$ and $g_{\perp}$ values of a single anisotropic signal. For convenience, this signal will be referred to as the "g=2.04 signal" in order to distinguish it from the g_2=2.030 intrinsic signal.

To determine whether the appearance of the g=2.04 signal was a consequence of the oxidation of L-Arginine into nitrogen oxides, we performed the EPR spectra of IFN-γ-activated macrophages from a media depleted of L-Arginine or containing both L-Arginine and N-G-monomethyl-L-Arginine (NMMA), a competitive inhibitor of the oxidation of L-Arginine into

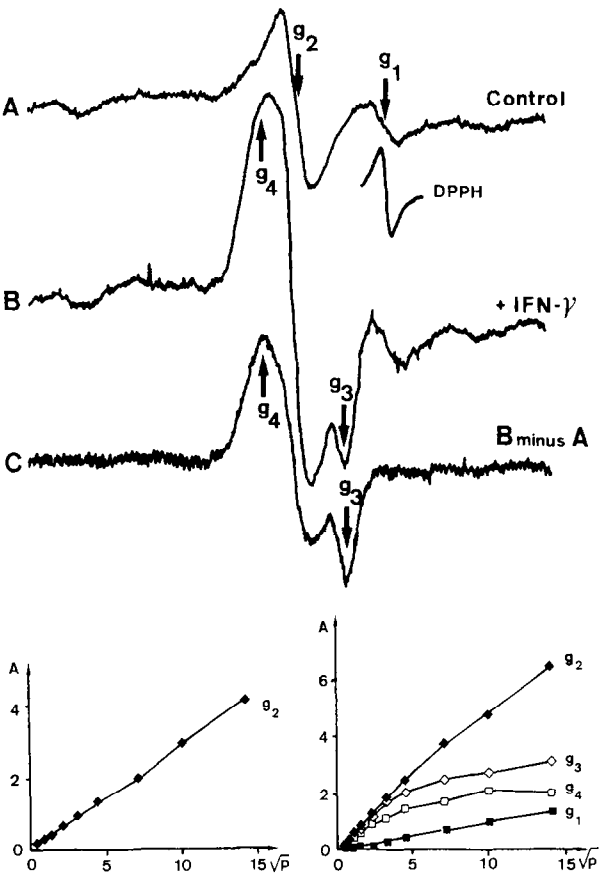

Figure 1. Typical EPR spectra from digitonin-permeabilized elicited macrophages incubated for 24 hours in complete RPMI medium alone (control, A) or added with 200 U/ml IFN-γ (B). C: computer difference between EPR spectra from IFN-γ activated macrophages and from control macrophages. Spectra were recorded at 77 K with microwave power of 10 mW, amplitude modulation,10 gauss; time constant, 0.5 s; klystron frequency, 9.18 GHz. DPPH (1,1-diphenyl-2-picryl-hydrazyl) stable radical was used as a marker with g=2.0036.
Lower panels: microwave saturation behaviour of the EPR signals in control macrophages (left panel) and in IFN-γ-activated macrophages (right panel). Amplitudes, expressed in arbitrary units, were measured at the amplitude extrema of g_1=2.005, g_2=2.030, g_3=2.015 and g_4=2.042, and were plotted against the square root of the microwave power P in mW.

nitrogen oxides (7). Under these conditions, EPR spectra of IFN-γ-activated macrophages (Figure 2C and D) were identical to that exhibited by control macrophages (the EPR spectrum of control macrophages was not modified in absence of L-Arginine in the medium). These results show that appearance of the g=2.04 signal in IFN-γ-activated macrophages was dependent on the synthesis of nitrogen oxides from L-Arginine. This observation is reminiscent of previous findings from several laboratories which attributed a signal at $g \simeq 2.037$ to nitrosyl-iron sulfur complexes in proteins or peptides (14-18).

As shown in Figure 3B, the g=2.04 signal also appeared in the continuous murine cell line RAW 264.7 exposed to IFN-γ. Furthermore, a spectrum nearly identical to that exhibited by IFN-γ-stimulated macrophages was observed in control macrophages treated with an excess of $NaNO_2$ and ascorbate after helium deoxygenation, either in RAW 264.7 cells (Figure 3C) or in elicited

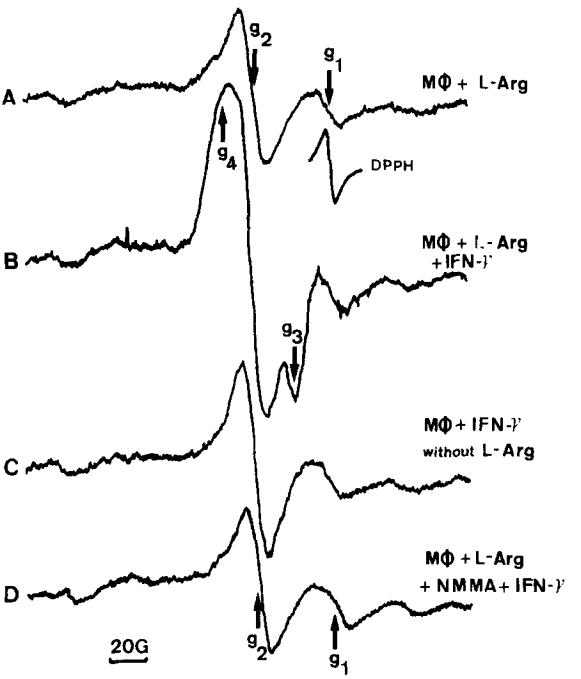

Figure 2. Representative EPR spectra from digitonin-permeabilized elicited macrophages (MØ), incubated for 24 hours in RPMI medium added with 1.15mM L-Arginine (A), with 1.15 mM L-Arginine plus 200 U/ml IFN-γ (B), with 200 U/ml IFN-γ (C) and with L-Arginine plus 0.5mM NMMA and 200 U/ml IFN-γ (D). Instrument settings as noted in the legend to Figure 1.

macrophages (not shown). This treatment was previously shown to generate NO· in situ and to create in cell homogenates an EPR detectable signal at g=2.035 corresponding to nitrosyl-iron sulfur complexes (17,19).

P388D1 cells cannot be induced by IFN-γ or LPS to oxidize L-Arginine into nitrogen oxides (20). As expected, only minute amounts of NO_2^- were found in the culture medium after IFN-γ treatment in the presence of L-Arginine and we showed that aconitase activity was not affected (Table I) Figure 3E shows an EPR spectrum of IFN-γ-stimulated P388D1 cells. This spectrum was similar to the spectra of non-stimulated macrophages.

Taken as a whole, these results demonstrate that paramagnetic nitrosyl-iron complexes appear in macrophages under the action of IFN-γ. Since there was no evidence of the hyperfine structure characteristic of NO-heme protein in our preparations, only non-heme iron seems to be involved. Furthermore our data underline the correlation existing between the L-Arginine-NO pathway, the inhibition of aconitase activity and the formation of the nitrosyl-iron complexes. Activities of aconitase, Complex I and Complex II are dependent on the integrity of one or several (4Fe-4S) cluster(s) (19,20). As previously observed for bacterial iron-sulfur enzymes, the possibility exists that NO· (or NO_2^-) which has strong affinity for iron, can coordinate to such a cluster with resulting loss of enzyme activity (19,23,24). It is thus tempting to propose that activated macrophage-derived NO·, a neutral molecule that can cross cell membranes, is responsible for a change of the catalytic site configuration of certain mitochondrial iron-dependent enzymes. This could explain, at the molecular level, the L-Arginine-dependent inhibition of aconitase, Complex

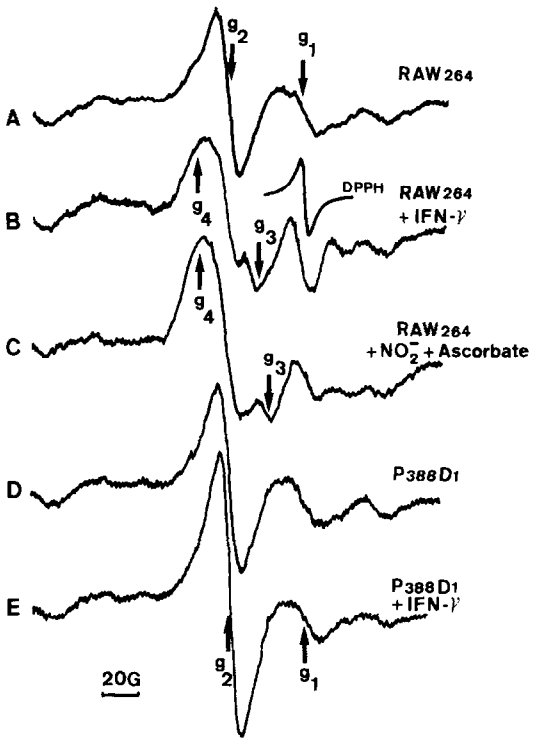

Figure 3. Representative EPR spectra from digitonin-permeabilized macrophage cell lines. RAW 264.7 and P388D1 cells were incubated for 24 hours in DMEM alone (A, C and D) or added with 200 U/ml IFN-γ (B and E). An aliquot of digitonin-permeabilized control macrophages was flushed with helium and treated at room temperature for 30 min. with 5mM $NaNO_2$ plus 5mM ascorbate (pH 6) prior to being processed for EPR spectroscopy as described in material and methods (trace C). Instrument settings as noted in the legend to Figure 1.

I and Complex II observed in both activated macrophages and target cells cocultivated in close contact.

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