EPR Detection of Heme and Nonheme Iron-containing Protein Nitrosylation by Nitric Oxide during Rejection of Rat Heart Allograft*

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The paramagnetic molecule nitric oxide (NO), produced from l-arginine by a specific enzyme (NO synthase), has been shown to be involved in a surprising variety of mammalian cellular responses, including the regulation of T cell immunity to alloantigens in vitro. In cytotoxic activated macrophages, NO production results in a characteristic pattern of alteration of iron-containing enzyme function that is mimicked by exposure to NO. Electron paramagnetic resonance (EPR) studies have shown the formation of iron-nitrosyl species during macrophage activation and also during sepsis, indicating that alteration of iron-containing protein function may be the result of the well-documented tendency of NO to bind to metal ions. We have recently shown that the NO synthesis induced during alloantigenic activation of rat splenocytes inhibits lymphocyte proliferation and cytotoxic T-lymphocyte generation. This report demonstrates that iron-nitrosyl EPR signals similar to those observed in macrophages and during sepsis are present in the blood and in the grafted tissue of rats during the rejection of allogeneic (but not syngeneic) heart grafts. These signals are found in the blood and at the site of allograft rejection, but are not found in other tissues (such as spleen and lung), and are obliterated by administration of the immunosuppressant FK506. These results directly demonstrate the formation of iron-nitrosyl complexes during vascularized allograft rejection and suggest that subsequent destruction of iron-containing protein function plays an important role in the rejection response.

Recently, nitric oxide (NO) has been shown to be produced by many mammalian cell types and to be involved in a variety of important cellular processes, including regulation of vascular tone, platelet aggregation, neurotransmission, and immune activation (1–5). NO is synthesized by the oxidative deamination of a guanidino nitrogen of l-arginine by at least two different types of flavin-containing enzyme (1), termed NO synthase (NOS). The enzymes from brain (6, 7) and macrophages (8, 9), representing these two types, have recently been purified, and the gene for the neural enzyme has been cloned (10).

Many of the cellular actions of NO can be attributed to the well-documented tendency of this radical molecule to bind to and thus modify the activities of iron-containing enzymes, forming iron-nitrosyl complexes. Thus, when functioning as an intracellular messenger, NO binds to heme and thus activates soluble guanylyl cyclase (3, 4, 6, 7). When functioning as a cytotoxic effector, destruction of several iron-containing enzymatic activities occurs, including aconitase and mitochondrial electron transfer (1, 5, 11), as well as ribonucleotide reductase (12). Since NO has been used extensively as a spectroscopic probe for iron-containing proteins for over 50 years, an extensive background of information exists for the spectroscopic examination of the molecular actions of NO in mammalian cells. Because of the characteristic paramagnetic properties of iron-nitrosyl complexes, EPR spectroscopy has been utilized extensively in these studies. This technique, because of its ability to detect such complexes against a large background of diamagnetic species in whole cells and tissues, has recently been applied to the study of the actions of NO when produced from l-arginine upon immune activation. Thus, EPR-observable iron-nitrosyl signals have been detected in blood during septic and hemorrhagic shock (13), in cytotoxic activated macrophages (14, 15) and their tumor cell targets (16), and in the destruction of pancreatic islet β-cells by the cytokine interleukin-1, a model for insulin-dependent diabetes mellitus (17).

We have recently provided evidence that l-arginine-dependent NO synthesis is involved in another system of immune activation, alloantigen-specific activation of rat splenocytes, as judged by the detection of nitrite/nitrate (stable end products of the reaction of NO with oxygen) and of citrulline (the product of l-arginine metabolism by this pathway) and by the effects of Nω-monomethyl arginine, a specific inhibitor of NOS (18–20).1 NO plays a negative regulatory role in this system through inhibition of lymphocyte proliferation and the generation of cytotoxic T cells. NO production is detected during the in vivo rejection of a sponge matrix allograft (19, 20), and serum nitrite/nitrate levels are elevated in the course of the rejection reaction of vascularized organ allografts.1 Similar conclusions have been reached by other workers (22, 23). There have, however, been no reports of the direct observation of NO production and its intracellular effects in samples from recipient animals during the in vivo rejection of vascularized allograft.

It is thus of interest to determine whether EPR spectroscopy could provide such direct evidence in allograft rejection for a biochemical mechanism of NO action that involves the destruction of intracellular iron-containing protein function. Utilizing the well-described heterotopic heart graft model in the rat we report here that, indeed, such EPR-detectable complexes are formed, observable both in the blood and in the graft tissue.

**EXPERIMENTAL PROCEDURES**

Heterotopic heart transplantation was performed in the Brown-Norway (RT1<sup>+</sup>) to Lewis (RT1<sup>+</sup>) combination according to the method of Ono and Lindsay (24). As previously described, the allograft rejection is first observed clinically on postoperative day (POD)* 5–7 and is completed (complete cessation of the graft heartbeat) between POD 11–13 (25). Packed erythrocytes or minced tissue (grafted heart, liver, lung, and spleen) samples were obtained from the sacrificed animals on POD 3 or 5 and frozen. The minced tissue samples were thawed and homogenized with an equal volume of 0.1 M HEPES buffer, pH 7.4 in a Brinkmann Polytron homogenizer (model PCU-2) at a power setting of 3–4, for 30 s. The homogenate was immediately transferred to a quartz EPR tube and frozen at 77 K. The blood samples were thawed, likewise transferred to EPR tubes, and frozen. For treatment with gaseous NO, 3.3 M sodium HEPES buffer, pH 7.4, was added to 10% of the sample volume prior to anaerobic NO exposure (total volume = 3 ml). The pH changed slightly from 7.4 to 7.1. Samples were kept on ice, and processing times were less than 20 min during which time we have noticed no decline in signal intensity. The signals are stable indefinitely when frozen. For testing the effects of FK506, Lewis recipients of Brown-Norway heart grafts were treated with FK506 (suspended in 0.9% NaCl) IM, at a dose of 1 mg/kg body weight/day, from day 0 to 2, and blood and tissue samples were collected on POD 5. EPR spectra were performed at 77 K on a Varian E-109 spectrometer, at 8 gauss modulation amplitude, 100 kHz modulation frequency, and 9.032 GHz microwave frequency. The instrument gains and microwave power settings were as designated in the figure legends. The instrument was calibrated periodically by using a sample of methyl viologen radical.

**RESULTS**

**EPR of Blood from Graft Recipients**—Fig. 1 presents EPR spectra, recorded at 77 K, of samples of blood from recipients of allogeneic and syngeneic heart transplants and from naive animals. We have recently shown that during allograft rejection serum nitrite/nitrate levels, an indicator of NO formation within the first clinical signs of rejection is the appearance in the blood of a signal indicative of the formation of nitrosyl hemoglobin (28). The g<sub>2</sub> signal derives from varying amounts of radicals or as an artifact of sample preparation (27). On POD 5 (spectra E–G), also three animals), coincident with the first clinical signs of rejection is the appearance in blood of a signal indicative of the formation of nitrosyl hemoglobin complexes (28–30). This spectrum displays an upward feature at g = 2.078 (denoted by the circles), a nitrogen hyperfine triplet with splitting of 17.5 gauss centered at g = 2.012 (the arrows denote g = 2.00), and a downward feature at g = 1.99. Virtually identical heme-nitrosyl signals are observed in the blood of rats subjected to endotoxic or hemorrhagic shock (13).

**Spectrum H** shows a sample of rat blood treated with NO gas. This spectrum is identical to that for fully nitrosylated hemoglobin (29, 30). By comparison with published data for the various forms of nitrosylated hemoglobin (29, 30), the peak at g = 2.039 (denoted by the triangle) is from ß-NO complexes, which are not appreciably present in the allograft samples (E–G). The shoulder at g = 2.078 (denoted by the circle) corresponds to the low field peak in the allograft samples and is predominantly from a-NO complexes. The nitrogen hyperfine triplet, while visible, is comparatively much smaller in magnitude than for the blood samples from allografts (E–G), consistent with previous studies on fully nitrosylated hemoglobin (29, 30).

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1 The abbreviations used are: POD, postoperative day; HEPES, 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid; FK506, an immunosuppressant compound that is a macrolide lactone with a hemiketal-masked α,β-diketoamide incorporated in a 23-member ring (48); mW, milliwatt; J, IM, intramuscular injection.
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Spectra I–L show that blood samples from recipients of syngeneic (Lewis, RT1 to Lewis, RT1') heart grafts display only the small radical signal on both POD 3 (I, J) and POD 5 (K, L), demonstrating that the formation of nitrosyl hemoglobin is a specific result of the activation of NO production during allograft rejection.

EPR of Tissues from Graft Recipients—Fig. 2 shows EPR spectra of heart allograft tissue specimens. Spectra A–C show allograft samples from three rats on POD 3, which contain signals similar to those from naïve heart (D), exhibiting only a radical signal at g = 2.00 (denoted by the arrow). In allograft samples on POD 5 (E and F, two different animals), a complex signal is observed which at first glance resembles that of only nitrosyl hemoglobin. However, spectrum G shows an EPR spectrum of the same sample as spectrum E, observed at lower microwave power (0.06 mW) versus 1 mW (A–F) to discriminate between different species that might be present. This spectrum is most probably attributable to a composite of three signals: a broad heme-nitrosyl (approximated by the dotted line), probably nitrosyl myoglobin which displays no nitrogen hyperfine features (31), plus the additional presence of an axial feature at g = 2.04 (triangle) and g = 2.015 (open circle) and a radical feature of undetermined origin at g = 2.00. On the basis of comparison with small molecular weight complexes (32–35) and of NO-treated nonheme iron complexes of amino acids and proteins (36–41), the axial feature at g = 2.04 is attributable to nonheme iron-dinitrosyl complexes and probably accompanies the destruction of cellular iron-containing proteins by NO (11, 14–17, 42, 43). The relative magnitude of these three signals at 1 mW (spectra E and F) compared to 0.06 mW (G) is due to the lower relaxation rate at higher microwave power of the signals from the nonheme iron-dinitrosyl and radical signals relative to nitrosyl heme. Spectra H–K show that no iron-nitrosyl signals are observed on either POD 3 (H and I) or 5 (J and K) in syngeneic grafts, illustrating the specificity of the rejection reaction on the development of these signals.

EPR examination of lung, spleen, and liver tissue from recipients of either allogeneic or syngeneic heart grafts shows no detectable iron-nitrosyl signals, either on POD 3 or 5 (data not shown), demonstrating that the signal is localized at the site of rejection and in the blood.

Effect of FK506 on Iron-Nitrosyl Formation—We have recently shown that the addition of the immunosuppressive macrolide FK506 to cultures of sponge matrix allograft infiltrating cells plus alloantigen inhibits the usual production of NO in this system (20). This is presumably due to the fact that FK506 is known to inhibit the production of the essential lymphokines that can up-regulate NO synthase in macrophages (20, 44). When FK506 is administered for 3 days to recipients of heart allografts, the functional graft survival is prolonged beyond 30 days. Furthermore, as shown in Fig. 3, FK506 treatment prevents appearance of nitrosyl hemoglobin in the blood (A–C) and of nonheme iron-dinitrosyl complexes in the heart tissue (D–F) on POD 5.

DISCUSSION

EPR spectroscopy of NO-treated metal centers has been used to examine the properties of the metal ligation environ-

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ment in a variety of metalloproteins and small complexes. This extensive background of information can provide a basis on which to interpret EPR signals observed in cells and biological systems when activated to produce NO via NOS, including cytotoxic activated murine macrophages (14, 15) and their tumor targets (16), destruction of pancreatic islet β cells by interleukin-1 (17), as well as in the blood of rats subjected to septic or hemorrhagic shock (13). In these systems, depending on the sample, two distinct types of iron-nitrosyl signals are commonly observed: 1) heme-mononitrosyl in blood with principal g values around 2.08, 1.99, and 2.01 (often with nitrogen hyperfine structure, depending on the quaternary conformation of the hemoglobin to which it is complexed), and 2) an axial signal in cells at g = 2.04 and 2.015, attributable to nonheme iron-dinitrosyl complexes (14-17, 32-41).

We report here the application of this spectroscopic technique to another system involving activation of L-arginine-dependent NO synthesis, namely allograft rejection in the rat, where we have previously presented evidence for a more specific role for NO (18-20). EPR signals attributable to nitrosyl hemoglobin are observed in the blood, and both heme-nitrosyl and nonheme iron-dinitrosyl signals are observed in heart allograft tissue. The signals appear simultaneously with the onset of the allograft rejection, do not appear in syngeneic grafts, and are prevented by administration of the immunosuppressant FK506. In the recipient animal, tissue iron-nitrosyl signals are localized to blood and the site of the allograft response (i.e. not present in liver, lung, or spleen). These results show that formation of the signals is a specific result of immune activation, as opposed to, for example, an artifact from sample preparation. As in EPR studies of other immune-induced activation of NO production (5, 13-17), these results provide evidence for a role for the formation of iron-nitrosyl complexes in the molecular mechanism of NO action in the allograft rejection reaction with consequent loss of iron-containing protein function, although further biochemical studies will be required to establish this including the effects on allograft rejection of inhibition of NO synthesis by NOS inhibitors. It is important to recognize at this point the possibility that the signals observed in the grafted tissue are due to the suppression of immune responses (18-20, 22, 23) or to the rejection reaction per se.

The reactions of hemoglobin with nitrogen oxides, both in vitro and in vivo, have been extensively studied and provide information as to the origin of the EPR signal observed here. Although various ligation and oxidation reduction reactions can occur, the most direct method for the formation of nitrosylferrohemoglobin is either through direct reaction with NO (29, 30) or the reaction of erythrocytes with nitrite (45, 46). In both cases, anaerobic conditions (i.e. deoxyferrohemoglobin) are required. Thus, this species may conceivably be formed either by reaction of deoxyhemoglobin with NO at the site of NO production, or by reaction with circulating nitrite. We have found that the intensity of the nitrosylferrohemoglobin signal is much greater in intensity in rats injected with lipopolysaccharide to induce a septic response than during allograft rejection, even though the circulating nitrite/nitrate levels are similar. This suggests that the formation of this species does not occur through reaction with circulating nitrite but rather occurs by a more specific mechanism, perhaps through direct reaction with NO at the site of its production. This may indicate that EPR examination of blood samples could provide important information in in vivo studies such as the relative accessibility of the circulation to the specific site(s) of NO production under various conditions.

With respect to the origin of the g = 2.04 signal in the graft tissue (typical for nonheme iron-dinitrosyl complexes (14-17, 32-41)), phenotypic analysis of rat heart allograft infiltrating cells reveals that a substantial portion of these cells possess the macrophage antigens ED-1 and ED-2 (47). We have recently demonstrated that rat allograft infiltrating cells produce large amounts of NO in culture and that the adherent macrophages within the graft infiltrating cell population are responsible for the major portion of the NO production (19). It is thus conceivable that the g = 2.04 signal observed is located at least in part within allograft infiltrating macrophages and may indicate alterations of iron-containing protein function. This hypothesis is supported by recent reports demonstrating a g = 2.04 signal in NO-producing unspecific cytotoxic activated macrophages (14, 15). Furthermore, the observation by Drapier et al. (16) that this same signal is also observed in tumor target cells cocultured with activated, NO-producing macrophages provides evidence that this might be at least one mechanism by which NO serves a negative regulator function in an immune response through the inhibition of iron-containing protein function. However, NO may also be playing additional roles, including immune-induced destruction of the allograft tissue.

REFERENCES
19. Deleted in proof
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