Hypoxemia in the absence of blood loss upregulates iNOS expression and activity in macrophages

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Hypoxemia in the absence of blood loss upregulates iNOS expression and activity in macrophages. Am. J. Physiol. 276 (Cell Physiol. 45): C285–C290, 1999.—Regional hypoxia, associated with hemorrhage, is thought to induce a variety of alterations in immune cell function, including upregulation of macrophage-inducible nitric oxide synthase (iNOS) expression and activity (NO production). Furthermore, NO may cause immune cell dysfunction similar to that associated with hemorrhagic shock. However, it remains unknown whether hypoxia per se in the absence of any blood loss is a sufficient stimulus to cause iNOS expression and NO production by macrophages. To study this, male Sprague-Dawley rats (275–325 g) were placed in a plastic box flushed with a gas mixture containing 5% O2-95% N2 for 60 min. Peritoneal and splenic macrophages were isolated 0–5.5 h thereafter, and blood samples were obtained. Nitrite and nitrate (stable degradation products of NO) production by macrophages is of iNOS origin. Elevated NO production has been reported under these pathological conditions (6, 11, 25, 34). Additionally, increased iNOS expression has been implicated in both the pathophysiology and the mortality associated with traumatic and thermal injury (12, 28, 36). Thus, after injury, tissue hypoxia may in itself be a sufficient stimulus to induce activation of macrophage proinflammatory cytokine synthesis, which in turn contributes to the systemic inflammatory response.

In addition to proinflammatory cytokines, macrophages are also a major cellular source of nitric oxide (NO) (18). These cells synthesize the free radical NO from the guanidino group of L-arginine via the inducible form of nitric oxide synthase (iNOS) that is upregulated by proinflammatory stimuli such as TNF-α, interferon-γ (IFN-γ), or lipopolysaccharide (LPS). Recent studies indicate that plasma nitrite and nitrate (NO2-/NO3-; the stable end products of NO) are significantly increased following adverse circulatory conditions, such as trauma-hemorrhage, sepsis, and thermal injury (6, 11, 25, 31, 34). Additionally, increased iNOS expression has been reported under these pathological conditions (16), suggesting that the increased production of NO is of iNOS origin. Elevated NO production has been implicated in cellular and organ dysfunction seen under these conditions (6, 11, 25, 27, 34). However, it remains unknown whether hypoxemia encountered during and after hemorrhagic shock causes the induction of iNOS expression and activity. Albina et al. (1) demonstrated that macrophages exposed to an in vitro anoxic environment display increased iNOS expression and activity. In view of this, it is possible that the regional hypoxemia during hemorrhagic shock (in vivo) may be a sufficient stimulus to induce iNOS expression and activity in macrophages. The aim of the present study, therefore, was to determine whether hypoxemia per se increases NO production by macrophages in vivo and, if so, whether this increase was due to elevated iNOS expression.

STUDIES INDICATE THAT trauma and hemorrhagic shock lead to profound depression of both immunoresponsiveness and organ function (9, 38). Additionally, it has been demonstrated that decreased tissue perfusion during hemorrhagic shock significantly reduces tissue oxygenation, leading to decreased intracellular ATP levels under such conditions (20). Furthermore, there is evidence that regional hypoxia is responsible for initiating the cascade of events leading to the observed alterations in cellular and organ metabolism following trauma-hemorrhage (9). In this regard, hypoxemia in the absence of blood loss or significant hypotension has been demonstrated to induce release of proinflammatory cytokines [tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6] by macrophages (13). Increased synthesis of these proinflammatory cytokines has been implicated in both the pathophysiology and the mortality associated with traumatic and thermal injury (12, 28, 36). Thus, after injury, tissue hypoxia may in itself be a sufficient stimulus to induce activation of macrophage proinflammatory cytokine synthesis, which in turn contributes to the systemic inflammatory response.

MATERIALS AND METHODS

Animals. In these studies, male Sprague-Dawley rats (260–325 g; Charles River Laboratories, Wilmington, MA) were utilized. All animals were fasted for 12 h before the experiment but were allowed water ad libitum. All procedures were carried out in accordance with the guidelines set forth in the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the project was approved by the Institutional Animal Care and
Use Committee of Rhode Island Hospital and Brown University.

Hypoxemia model. Hypoxemia was induced in the rats according to the procedure previously described by Ertel et al. (13), with some modifications. In brief, animals were placed in a plastic box (22 × 23 × 30 cm) with an inlet and an outlet through which the hypoxic gas mixture or room air flowed. Hypoxemia was induced by flushing the plastic box with a gas mixture of 5%O2-95%N2 bubbled through distilled water at a flow rate of −10 l/min via a bottle containing distilled water. The animals were exposed to this hypoxic environment for 1 h, after which they were returned to room air. Previous studies from our laboratory demonstrated that this model of systemic hypoxemia results in a drop of the arterial PaO2 to 40 mmHg after 10 min of hypoxemia (13). The arterial PaO2 remained between 30 and 40 mmHg until hypoxemia was stopped, and it returned to baseline values within 10 min after the hypoxemia period (13). The blood pressure slowly decreased by 14 mmHg, and values returned to normal immediately after hypoxemia. No mortality was observed in the group of animals subjected to hypoxemia (13). Sham animals underwent the same procedure; however, the plastic box was flushed with room air. The animals (6 or 7 animals/group of animals subjected to hypoxemia (13). Sham animals underwent the same procedure; however, the plastic box was flushed with room air. The animals (6 or 7 animals/group underwent the same procedure; however, the plastic box was flushed with room air. The animals (6 or 7 animals/group underwent the same procedure; however, the plastic box was flushed with room air. The animals (6 or 7 animals/group underwent the same procedure; however, the plastic box was flushed with room air. The animals (6 or 7 animals/group underwent the same procedure; however, the plastic box was flushed with room air. The animals (6 or 7 animals/group underwent the same procedure; however, the plastic box was flushed with room air.

Preparation of peritoneal lavage with PBS, and monolayers were established as previously described (4, 41). The spleens were removed aseptically and placed in separate petri dishes containing cold (4°C) PBS. The spleens were dissociated by grinding, suspended in medium, and used to establish peritoneal macrophages. Peritoneal macrophages were harvested from rats by peritoneal lavage with PBS, and monolayers were established as previously described (4, 41). The spleens were removed aseptically and placed in separate petri dishes containing cold (4°C) PBS. The spleens were dissociated by grinding, suspended in medium, and used to establish peritoneal macrophages. Peritoneal macrophages were harvested from rats by peritoneal lavage with PBS, and monolayers were established as previously described (4, 41). The spleens were removed aseptically and placed in separate petri dishes containing cold (4°C) PBS. The spleens were dissociated by grinding, suspended in medium, and used to establish peritoneal macrophages. Peritoneal macrophages were harvested from rats by peritoneal lavage with PBS, and monolayers were established as previously described (4, 41). The spleens were removed aseptically and placed in separate petri dishes containing cold (4°C) PBS. The spleens were dissociated by grinding, suspended in medium, and used to establish peritoneal macrophages. Peritoneal macrophages were harvested from rats by peritoneal lavage with PBS, and monolayers were established as previously described (4, 41). The spleens were removed aseptically and placed in separate petri dishes containing cold (4°C) PBS. The spleens were dissociated by grinding, suspended in medium, and used to establish peritoneal macrophages.

Plasma collection and storage. Whole blood was obtained by cardiac puncture and placed in microcentrifuge tubes (Microtainer, Becton Dickinson, Rutherford, NJ). The tubes were then centrifuged at 16,000 g for 15 min at 4°C. Plasma was separated, placed in pyrogen-free microcentrifuge tubes, immediately frozen, and stored at −80°C until assayed.

Preparation of peritoneal and splenic macrophages. Resident peritoneal macrophages were obtained from rats by peritoneal lavage with PBS, and monolayers were established as previously described (4, 41). The spleens were removed aseptically and placed in separate petri dishes containing cold (4°C) PBS. The spleens were dissociated by grinding, suspended in medium, and used to establish a macrophage culture as previously described (41). The macrophage monolayers were cultured in Click's medium containing 10% FCS for 48 h (at 37°C, 5% CO2, and 90% humidity) without LPS and in the presence of LPS (10 µg/ml). Click's medium contains 1,800 µM L-arginine, compared with 225 µM L-arginine in rat plasma (2). The availability of L-arginine is therefore not limited in the culture medium in vitro. Cell-free culture supernatants were collected, aliquoted, and stored at −80°C until assayed for NO2/NO3 levels.

Determination of NO production. Macrophage NO production was determined by measuring the concentration of the stable NO degradation products NO2/NO3 in the supernatants using a colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI) as previously described (31). Briefly, supernatant samples were thawed, and nitrate in the samples was converted to nitrite by the addition of nitrite reductase. Nitrite concentration was then detected by the addition of Griess reagent and quantitatively measured by analysis with a spectrophotometer (Bio-Tek ELK 311 microplate autoreader, Bio-Tek, Winooski, VT) at 550 nm.

RNA isolation and Northern blot analysis. Total RNA from peritoneal macrophages was isolated using a single-step liquid phase separation by TriPure isolation reagent (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's recommendations. In brief, peritoneal macrophages were washed after 2 h of incubation, 1 ml of TriPure reagent per 5 × 106 cells was added to the tissue culture well, and the adherent cells were removed by scraping them from the well into the reagent to lyse them. The cellular lysate was homogenized by passing it through a pipette several times, transferred into a polypropylene tube, and stored at −70°C until the isolation process was continued. RNA was isolated from the thawed samples by the addition of chloroform. All RNA samples had a ratio of absorbance at 260 nm to absorbance at 280 nm that was >1.5, and RNA content in all samples was found to be similar by electrophoresis of 2 µg of total RNA in an agarose gel and staining with ethidium bromide.

For Northern blot analysis, 15 µg of total RNA were electrophoresed in 1% agarose gels containing 3% formaldehyde. The gels were blot transferred onto GeneScreen membranes (DuPont NEN, Boston, MA) and ultraviolet cross-linked. Prehybridization was carried out for 18 h at 43°C in 50% deionized formamide, 0.25 M NaHPO4, 0.025 M NaCl, 1 mM EDTA, and 7% SDS, with denatured salmon sperm DNA. DNA probes were labeled with γ32P (DuPont NEN) using the random primer method. After hybridization, the membranes were washed at 53°C in 0.25 M SSC (1× SSC is 0.15 M sodium citrate, pH 7.0), 0.1% SDS, 25 mM NaHPO4, and 1 mM EDTA and finally in 25 mM NaHPO4, 1 mM EDTA, and 10% SDS (all reagents from Sigma Chemicals, St. Louis, MO). The membranes were visualized using Instant Imager autoradiography (Packard Instrument, Meriden, CT).

Cell line maintenance. The macrophage cell-line RAW 264.7 was obtained from the American Type Culture Collection and maintained according to their recommendations.

Determination of plasma IFN-γ levels. IFN-γ levels in plasma were determined using a specific bioassay (23). Serial dilutions of plasma were added to RAW 264.7 cells and incubated at 37°C in 5% CO2 for 24 h. Cell-free supernatants were collected, and nitrite concentration was determined by the Griess reaction. The light absorbance was measured at 550 nm with an automated microplate reader (EL-311, BioTek). The IFN-γ concentration in the samples was determined from a standard curve generated with recombinant IFN-γ (Genzyme, Cambridge, MA).

Statistical analysis. Data are presented as means ± SE of six or seven animals per group unless otherwise indicated. One-way ANOVA followed by the Student-Newman-Keuls test as a post hoc test for multiple comparisons was used to determine the significance of the differences between experimental means. P < 0.05 was considered statistically significant.

RESULTS

Hypoxemia induces NO production by macrophages. The NO2/NO3 concentrations in the supernatants of nonstimulated peritoneal macrophages (Fig. 1A) harvested at the end of hypoxia (0 h) and 1.5 h after the end of hypoxia were comparable to those of sham animals. However, peritoneal macrophages harvested 3 or 5.5 h after the end of hypoxia produced significantly greater levels of NO2/NO3 than macrophages from sham animals. NO2/NO3 concentrations in the supernatants of macrophages harvested from rats 3 h after the end of hypoxia were increased 1.25-fold compared with shams (P < 0.05), whereas supernatants from macrophages harvested 5.5 h posthypoxia displayed a 1.6-fold increase in NO2/NO3 concentration (P < 0.05 compared with shams). Although there was a trend toward a higher concentration of NO2/NO3 in the supernatants...
of macrophages harvested 5.5 h posthypoxia compared with 3 h posthypoxia, the values were not significantly different.

Stimulation of peritoneal macrophages with LPS (Fig. 1B) induced NO production in all groups; however, the levels were not significantly different between any of the groups. Furthermore, LPS stimulation induced greater levels of NO production in all groups compared with nonstimulated cells (Fig. 1A). However, this increase in NO$_2$/NO$_3$ concentration with LPS stimulation was significant only (~3-fold) in macrophage cultures obtained from sham-treated rats and animals killed 0 or 1.5 h after the end of hypoxia (P < 0.05).

The effect of hypoxia on NO production by splenic macrophages was also assessed. As was the case for peritoneal macrophages, NO$_2$/NO$_3$ concentrations in the supernatants of nonstimulated splenic macrophages harvested from animals at the end of hypoxia or 1.5 h after the end of hypoxia were not different from those of sham-treated animals (Fig. 2A). However, splenic macrophages harvested 3 or 5.5 h after the end of hypoxia showed significantly elevated levels of NO$_2$/NO$_3$ in the culture supernatants compared with shams (P < 0.05). Unlike peritoneal macrophages, splenic macrophages did not display a trend toward higher NO production at 5.5 h posthypoxia than at 3 h posthypoxia.

Stimulation of splenic macrophages with LPS significantly increased NO$_2$/NO$_3$ levels in the cultures from sham-treated rats, rats killed at the end of hypoxia, or rats killed 1.5 h after the end of hypoxia (5.5-fold increase in shams; 4.6-fold increase at the end of hypoxia; and 2.5-fold increase 1.5 h after the end of hypoxia, P < 0.05). However, like peritoneal macrophages, splenic macrophages obtained from rats 3 or 5.5 h after the end of hypoxia did not display a significant increase in NO production when stimulated with LPS (Fig. 2B).

Macrophage iNOS mRNA is expressed following hypoxemia. The levels of iNOS mRNA in nonstimulated peritoneal macrophages was determined by Northern blot analysis. mRNA for iNOS was not expressed in macrophages from sham-treated rats or macrophages harvested immediately after the end of hypoxia (Fig. 3). However, peritoneal macrophages harvested 1.5 or 3 h after the end of hypoxia showed increased expression of mRNA for iNOS at 130.5 kb (comparable to the positive control).

Hypoxia induces increased levels of circulating IFN-γ. Plasma levels of IFN-γ (Fig. 4) were significantly increased at the end of the hypoxic period (0 h) compared with sham animals (+202.7%, P < 0.05), which remained significantly elevated 1.5 h after the end of hypoxia (P < 0.05 compared with shams). By 5.5 h after
METHODS

After in vivo hypoxia, leading to increased NO production of iNOS mRNA in macrophages was detectable. Activity is upregulated following hemorrhagic shock. A potential mechanism by which iNOS expression and activity in macrophages and therefore represents a hallmark of hemorrhagic shock, we postulated that severe hypoxemia in vivo, in the absence of blood loss, induces iNOS activity (1) and since regional hypoxia is a hallmark of hemorrhagic shock, we postulated that hypoxemia in vivo, in the absence of blood loss, induces iNOS activity in macrophages and therefore represents a potential mechanism by which iNOS expression and activity is upregulated following hemorrhagic shock.

The results of the present study indicate that expression of iNOS mRNA in macrophages was detectable after in vivo hypoxia, leading to increased NO production in vitro. Furthermore, our findings also indicate that hypoxemia caused an increase in circulating IFN-γ soon after the insult and before the expression of iNOS mRNA, thus suggesting a causal relationship. All animals in the present study were subjected to severe hypoxia for 1 h; therefore, it remains unknown what degree or duration of hypoxia is required to induce iNOS expression and increase macrophage NO production.

Several in vivo studies have examined the effect of hypoxemia on iNOS expression by macrophages (1, 22). These in vivo studies show that hypoxemia alone does not induce iNOS expression and indicate that reoxygenation is required, along with a secondary stimulus such as IFN-γ or LPS, for the induction of iNOS. The results of the study presented here are consistent with these in vitro findings, since iNOS mRNA expression was not observed in macrophages isolated immediately following hypoxemia but was present 1.5 h posthypoxemia. This would suggest that reoxygenation is necessary for the induction of iNOS mRNA expression. Additionally, the elevated IFN-γ levels in the plasma during the early posthypoxic period may provide the secondary stimuli required for the induction of iNOS, as indicated by in vitro experiments (22).

Recent studies of Melillo et al. (21) have shown that the iNOS gene promoter sequence contains a hypoxia response element that in combination with IFN-γ leads to the activation of iNOS gene transcription. In this regard, hypoxia in combination with IFN-γ may have induced iNOS transcription and mRNA expression in the present study. These results further support the hypothesis that the increased plasma IFN-γ levels observed early posthypoxemia may contribute to the induction of iNOS expression and activity in the present study. Furthermore, the identification of a functional hypoxia-responsive sequence in the iNOS promoter suggests that iNOS is a hypoxia-inducible gene (21).

The nuclear transcription factor NF-κB is also likely to be important to the induction of iNOS expression following hypoxemia, as it has been shown to be required for activation of iNOS gene expression in macrophages (40) and is also activated under hypoxic conditions (24). Reactive oxygen species that are generated during hypoxemia can activate NF-κB translocation to the nucleus by inducing the disassociation of the inhibitory subunit I-κB by a tyrosine kinase-dependent process (3, 33, 39). Furthermore, recent studies have demonstrated that hemorrhagic shock also induces the activation of NF-κB in immune cells (15, 26, 29). Although the present study did not examine NF-κB, it...
is likely that it plays an important role in the induction of iNOS expression following hypoxemia. Recent studies indicate that NO inactivates NF-κB expression in endothelial cells, which might reflect an adaptive negative feedback mechanism following cell activation (32). A similar control mechanism has not yet been identified in macrophages. Nonetheless, complete inhibition of NO might be detrimental for the host following hypoxic conditions. This, however, remains to be determined.

In vitro stimulation of macrophages with LPS induced similar levels of NO production in all groups, the results presented here suggest that the capacity of macrophages to produce NO in response to LPS is not altered by hypoxemia. Furthermore, the observation that the levels of NO production 3 and 5.5 h after hypoxemia, in the absence of stimulation, were similar to LPS-stimulated levels of production suggests that hypoxia is a sufficient stimulus to induce maximal NO release. In contrast, Arya and García (8) demonstrated both increased constitutive and LPS-stimulated NO production by peritoneal macrophages from animals that were subjected to hypoxia and reoxygenation 1 day after an endotoxin challenge. However, the present observations that the capacity of macrophages to produce NO is not altered by hypoxia suggests that the effect is only at the level of iNOS expression rather than involving altered enzyme kinetics. It remains unknown whether endotoxin priming alters the kinetic parameters of iNOS or whether hypoxia (in the absence of endotoxin) alters the capacity of macrophages to produce NO at later times postinsult than those examined in this study. Although previous studies have shown that hypoxia increases constitutive NO synthase (cNOS)-derived NO production (7), macrophages do not express cNOS, and thus the increased NO₂/NO₃ levels in the supernatants following hypoxia or LPS stimulation are likely to be due to iNOS activity.

Melillo et al. proposed that hypoxia in combination with IFN-γ provides signaling for the induction of iNOS gene expression (21), whereas hypoxia alone is not sufficient to activate iNOS gene expression. The present study supports these findings, as plasma IFN-γ levels were elevated early posthypoxemia, before iNOS expression. However, the cellular source of the increased plasma IFN-γ is unknown. Studies by Klokker et al. (17) have demonstrated that natural killer (NK) cell numbers and activity are increased following hypoxia. NK cells are a major non-T lymphocyte source of IFN-γ (17). Studies to date have not addressed the effect of hypoxemia on IFN-γ production by T lymphocytes. It is, however, possible that the increased levels of IFN-γ observed in the present study might be of NK cell origin as previously demonstrated by Klokker et al. (17). Nonetheless, it remains unknown whether decreased PO₂ levels during hypoxemia directly increase IFN-γ release, leading to the induction of iNOS expression in macrophages, or whether other factors are important. For example, decreased intracellular ATP levels following hemorrhagic shock (20) probably caused by regional hypoxia (9) may trigger a cascade of events leading to iNOS expression. Thus the effect of hypoxemia on iNOS expression might be mediated via decreased intracellular ATP levels. Additionally, activation of other second messenger systems (i.e., cyclic nucleotides, protein kinase C, Ca²⁺) may also be important, as they all have been shown to regulate iNOS activity (18). Further experimental analysis is required to precisely identify the mechanism(s) of iNOS induction following hypoxemia.

Recent studies from our laboratory indicate that PGE₂ levels are increased after 1 h of severe hypoxia (37). PGE₂ is known to be a potent vasodilator (37). The release of PGE₂ therefore might be part of an adaptive response to hypoxemia, allowing more blood flow and oxygen delivery to the ischemic tissues. The release of cNOS-derived NO production also appears to be involved in the regulation of blood flow (5), whereas iNOS-derived NO production has been implicated in producing cellular and organ dysfunction (6, 11, 25, 27, 34). Because specific iNOS blockers are available, e.g., L-NAME, administration of these agents might prevent the deleterious effects of iNOS-derived NO production without blocking the beneficial vasodilatory effects of cNOS-derived NO.

In summary, the results of the present study indicate that hypoxia per se in the absence of blood loss caused increased production of iNOS-derived NO by macrophages. Thus regional hypoxia during hemorrhagic shock may be responsible for the induction of iNOS under such conditions. Because elevated NO production has been implicated in producing cellular and organ dysfunction (6, 11, 25, 27, 34), therapeutic interventions that specifically depress iNOS activity following trauma and hemorrhagic shock may be helpful in decreasing morbidity and mortality after such conditions.

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