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

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Angele, Martin K., Martin G. Schwacha, Nadia Smail, Robert A. Catania, Alfred Ayala, William G. Cioffi, and Irshad H. Chaudry. 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 splenic and peritoneal macrophages cultured for 48 h was significantly increased 3 and 5.5 h after hypoxemia. The increase in NO production by macrophages was preceded by elevated expression of iNOS mRNA at 1.5 h after hypoxemia. Additionally, interferon-γ (IFN-γ) levels in plasma from rats subjected to hypoxemia were significantly elevated soon after the insult (0–1.5 h posthypoxemia), suggesting a causal relationship between IFN-γ production and upregulation of iNOS activity. We propose that a hypoxemia-induced increase in macrophage iNOS activity following hemorrhage may in part be responsible for the observed immune dysfunction. Thus attempts to suppress macrophage iNOS activity after this form of trauma may be helpful in improving immune function under those conditions.

inducible nitric oxide synthase; nitric oxide; interferon; hemorrhagic shock; splenic macrophages; peritoneal macrophages

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.

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 pathophysiological 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.

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

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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% O_2-95% N_2 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 P_o_2 to 40 mmHg after 10 min of hypoxemia (13). The arterial P_o_2 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/time point) were killed at the end of hypoxia (0 h), 1.5, 3, or 5.5 h after the end of hypoxia produced significantly greater NO_2^-/NO_3^- concentrations in the supernatants of macrophages from rats 5.5 h after the end of hypoxia harvested 5.5 h posthypoxia displayed a 1.6-fold increase in NO_2^-/NO_3^- concentrations in the supernatants of macrophages from rats 3 h after the end of hypoxia compared with shams (P < 0.05), whereas supernatants from macrophages harvested from rats 5.5 h posthypoxia displayed a 1.6-fold increase in NO_2^-/NO_3^- concentration (P < 0.05 compared with shams). Although there was a trend toward a higher concentration of NO_2^-/NO_3^- in the supernatants
of macrophages harvested 5.5 h posthypoxyia 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 NO₂/NO₃ 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, NO₂/NO₃ 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 NO₂/NO₃ 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 NO₂/NO₃ 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 expression and activity following hemorrhagic shock. In light of the observations that macrophages exposed to hypoxic conditions in vitro express iNOS, since iNOS expression has been shown in a model of prolonged hemorrhagic shock 5 h after the beginning of the hypotensive period (16). However, it remains unknown which factor(s) mediate the induction of iNOS expression and activity following hemorrhagic shock. In light of the observations that macrophages exposed to hypoxic conditions in vitro express iNOS activity (1) and since regional hypoxia is a hallmark of hemorrhagic shock, we postulated that hypoxia 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.

DISCUSSION

The experiments reported here were conducted to investigate the role of severe hypoxemia in vivo in modulating macrophage iNOS expression and activity. Hypoxemia has been shown to induce the expression and release of proinflammatory cytokines favoring the adaptation of the organism to hypoxic conditions (13, 14). A similar elevation of circulatory proinflammatory cytokines has been reported following hemorrhagic shock (10), elective surgery (30), severe thermal injury (19), or traumatic injury (35). Moreover, recent studies have shown that after hemorrhagic shock there are increased plasma NO2/NO3 levels (31). This increase in plasma NO2/NO3 appears to be due to induction of iNOS; since iNOS expression has been shown in a model of prolonged hemorrhagic shock 5 h after the beginning of hypoxemia (31). However, it remains unknown which factor(s) mediate the induction of iNOS expression and activity following hemorrhagic shock. In light of the observations that macrophages exposed to hypoxic conditions in vitro express iNOS activity (1) and since regional hypoxia is a hallmark of hemorrhagic shock, we postulated that hypoxia 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 vitro studies have examined the effect of hypoxemia on iNOS expression by macrophages (1, 22). These in vitro 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 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 NO$_2$/NO$_3$ 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 hypoxia 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 Po$_2$ 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, Ca$^{2+}$) 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 PGE$_2$ levels are increased after 1 h of severe hypoxia (37). PGE$_2$ is known to be a potent vasodilator (37). The release of PGE$_2$ 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-N$^{6}$-(1-iminoethyl)lysine, 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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REFERENCES


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