Studies on coagulation-induced inflammation in mice
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Hypoxia alters the set point of the coagulation and inflammation balance in mice.

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Submitted
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Abstract

Hypoxia is a major determinant of the outcome of various pathological conditions. The effects of oxygen deprivation are complex and may involve defense mechanisms, like coagulation and inflammation. However, the \textit{in vivo} effects of systemic hypoxia on these mechanisms remain poorly documented. Therefore, we studied the time course of coagulation activation and cytokine production during and after cessation of hypoxia.

At 8% \textit{O}_2 mice did survive but became severely hypothermic, and were unable to eat, drink or move about normally. Within minutes after re-exposure to normoxia, body temperature and mouse behavior normalized. Thrombin-anti-thrombin complexes were determined as a marker of coagulation activation. Plasma thrombin-anti-thrombin complexes were detectable within 4 hours of hypoxia and were cleared within 24-hours of recovery at ambient oxygen levels. Thrombin-anti-thrombin complexes in tissue homogenates could not be detected. Hypoxia induced large alterations in cytokine and chemokine profiles in tissue like brain, heart, lung and kidney, while no plasma cytokines or chemokines could be detected. The locally altered cytokine profile remained for at least 10 days after cessation of hypoxia.

In conclusion, the \textit{in vivo} effects of hypoxia involve a transient systemic induction of coagulation, together with a local, massive and persisting cytokine and chemokine response.

Introduction

Although multiple physiological factors contribute to the outcome of pathological conditions like cardiovascular and pulmonary disease, oxygen deprivation is a common denominator. The consequences of oxygen deprivation on cellular functions are quite complex, and involve a series of metabolic and biosynthetic events associated with adaptations to a hypoxic environment. With respect to coagulation, hypoxia enhances the expression of tissue factor (TF)\textsuperscript{1,2} and plasminogen activator inhibitor-1 (PAI-1),\textsuperscript{3} whereas it diminishes thrombomodulin activity \textsuperscript{4} and plasminogen activator expression.\textsuperscript{3} Consequently, hypoxia engenders a prothrombotic phenotype.\textsuperscript{5} Hypobaric hypoxia, as it occurs at high altitude, has often been suggested to be a risk factor for the development of venous thrombosis.\textsuperscript{6,7}

In addition to its role in coagulation, hypoxia influences cytokine production. This assertion is mainly based on a large body of \textit{in vitro} data. For instance, interleukin (IL)-6 and IL-8 expression levels are increased in hypoxic human pulmonary vascular smooth muscle cells.\textsuperscript{8} Human endothelial cells (HUVECs) respond to low oxygen levels by increasing the production of IL-1,\textsuperscript{9} IL-6,\textsuperscript{10} and IL-8,\textsuperscript{11} whereas human intestinal epithelial cells express increased levels of tumor necrosis factor-\textit{\alpha} (TNF-\textit{\alpha}) and interferon-\textit{\gamma} (INF-\textit{\gamma}).\textsuperscript{12}

The \textit{in vivo} inflammatory effects of hypoxia are less well examined. Exposing male C3H/HeN mice to 5% \textit{O}_2 for one hour results in increased activity of TNF-\textit{\alpha}
and IL-6 in plasma, whereas cytokine activities in pro-estrus females remain unaltered. The increase in plasma cytokines is caused by enhanced synthesis and release of these cytokines from both peritoneal macrophages and Kupffer cells. Exposing mice to 8-10% of oxygen increases IL-1α levels in both plasma and lung homogenates with a maximal increase after 8 hours of hypoxia, whereas IL-6 levels are upregulated in the pulmonary vasculature. The influx of mononuclear phagocytes and neutrophils in lung tissue of mice exposed to 6% O2 further illustrates the role of hypoxia in inflammation.

It is well established that inflammatory mediators influence the coagulant response and vice versa. Insights in the cross-talk between inflammatory mediators and coagulation activation stem mainly from endotoxemia models. In these models, endotoxin is administered to humans, baboons or mice resulting in the induction of inflammatory mediators and the activation of the clotting cascade. Inhibition of TNF-α by anti-TNF-α antibodies in these models not only diminishes the inflammatory response but also inhibits coagulation activation. Increasing the level of pro-inflammatory cytokines in endotoxemia models induces activation of the coagulation cascade. Furthermore, in a rat model of inferior vena caval thrombosis, administration of the anti-inflammatory cytokine IL-10 decreases both inflammation and thrombus weight. Enhanced coagulation is also often accompanied by inflammation. Ex vivo coagulation of human whole blood results in enhanced IL-1β, IL-8 and IL-6 production. Thrombin is believed to be one of the key-players in the link between coagulation and inflammation. Coagulation-induced cytokine production is, indeed, attenuated by inhibitors of thrombin generation (e.g. Tissue Factor Pathway Inhibitor) or activity (e.g. hirudin). The protein C anticoagulant pathway may also provide a link between coagulation and inflammation. LPS-studies in rats have demonstrated that activated protein C (APC) modulates the effects of cytokines such as TNF-α and blocks neutrophil activation. The role of APC in inflammation is further supported by the finding that the upregulation of IL-6 and IL-8 in human endothelial cells is enhanced by protein S.

Although it is evident that hypoxia influences the expression level of several coagulation factors and numerous inflammatory mediators, the in vivo consequences of systemic hypoxia remains poorly understood. Therefore, to increase our understanding concerning the interplay between hypoxia, inflammation and coagulation, we studied the time course of coagulation activation and cytokine production during and after cessation of hypoxia.

Methods

Animals

C57BL/6 wild-type mice were purchased from Charles River (Zeist, The Netherlands). All mice were housed in the same temperature-controlled room with alternating 12h light/dark cycles, and were allowed to equilibrate for at least 5 days before the study. Animals were provided regular mice chow (SRM-A; Hope Farms, Woerden, The Netherlands) and water ad libitum. Mice were used at 8-10
weeks of age. The experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center, Amsterdam, The Netherlands.

**Exposure of mice to hypoxia**

Mice were exposed to normobaric hypoxia using a custom-made hypoxia-chamber containing an oxygen sensor (Marin Assist, Hazerswoude, The Netherlands). The lowest non-lethal oxygen level was estimated by placing the mice in the hypoxia-chamber and gradually lowering the oxygen concentration by introducing nitrogen gas until the first mice died. Next, 64 mice were placed in the hypoxia chamber. The oxygen level was lowered to 8% within one hour. After 1, 4, 16 or 24 hours of 8% O₂ the mice were sacrificed or subjected to a 24-hour-recovery period at normoxia (n=4 females and 4 males per time point). In addition, animals exposed to 8% O₂ for 24 hours were sacrificed after a 24-, 72- or 240-hour recovery period. Animals (n=8) maintained under normal oxygen conditions were used as normoxic controls. Animals were sacrificed by bleeding from the vena cava inferior after being anesthetized by intraperitoneal injection of FFM (1:1:2 hypnorm (Janssen Pharmaceutica, Beerse, Belgium), dormicum (Roche, Mijdrecht, The Netherlands), H₂O (sterile water for injection, Braun Melsungen AG, Melsungen, Germany); 0.1 mL per 10 grams body weight).

Immediately before the mice were sacrificed, body temperature (intrarectal) and weight were measured, and they were injected with 400U of heparin (i.v.) to prevent post mortem coagulation. Blood and organs (brain, heart, kidney, liver, lung and spleen) were processed for cytokine measurements and histological analysis.

In addition, pO₂, pCO₂, HCO₃⁻, hemoglobin and hematocrit levels of mice exposed to 8% O₂ during 16 hours were analyzed. Blood was sampled by heart puncture via a lateral approach and analyzed using an ABL 505 blood gas analyzer and an OSM3 oximeter (Radiometer, Copenhagen, Denmark).

**Cytokine measurements**

To obtain tissue homogenates, organs were placed in 9 volumes (w/v) of Greenburger lysis buffer (pH 7.4, 150 mmol/L NaCl, 15 mmol/L TrisHCl, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, 1% Triton (v/v), 10 pmol/L pepstatin A, 10 pmol/L leupeptin and 10 pmol/L aprotinin), homogenized and centrifuged twice (1780g and 20,800g, respectively). ELISAs were performed on the supernatant of these organ homogenates and on plasma by using commercially available kits (IL-1β, IL-12p40, INF-γ, macrophage inflammatory protein (MIP-2) and TNF-α: R&D Diagnostics, Minneapolis, USA; IL-6, IL-10: PharMingen, Heidelberg, Germany). Detection limits were 31 pg/mL.
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**TNF-α bioactivity assay**

To investigate whether TNF-α detected in tissue homogenates is biologically active, TNF-α levels were measured using the WEHI 164 clone 13 fibroblast cytotoxicity assay.\(^{26-28}\) Briefly, cells were resuspended at 5\(\times\)10⁵ cells/mL in Dulbecco’s modified essential medium (DMEM, Gibco) supplemented with 5% fetal calf serum (FCS), 1% penicillin (10,000 U/mL) / streptomycin (10,000 μg/mL), 1% L-glutamine, dispersed in 96-well cell culture plates, and incubated overnight in a humidified environment at 37°C/5% CO₂. Samples and 0.25 μg/mL actinomycin D were added to the cells. With each assay a titration of mouse rTNF-α (Pharmingen) was included as a standard. Samples were then incubated for 24 h, after which 0.25 μg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to the wells and the samples were then incubated for an additional 24 h at 37°C. After adding 3% SDS in 0.01 M HCl, the plates were read in a micro-ELISA reader at 550 nm.

**Histology**

Shortly after sacrificing the mice, brain, heart, kidney, liver, lung, and spleen were removed, fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. 5-μm-thick sections were stained with hematoxylin and eosin according to standard protocols. All slides were coded and scored for the presence or absence of blood clots, and for the degree of inflammation. Inflammation was characterized by the influx of granulocytes and by the presence of endothelialitis (i.e. sticking of leukocytes to the vessel wall).

**Activation of coagulation cascade**

Thrombin-anti-thrombin (TAT) complexes were measured in plasma and in tissue homogenates with a mouse-specific (rabbit anti-mouse antibodies), ELISA-based method.\(^{29}\) In short, rabbits were immunized with mouse thrombin or rat antithrombin. Antithrombin antibodies were used as capture antibody, digoxigenin-conjugate anti-antithrombin antibodies were used as detection antibodies and dilutions of mouse serum were used for the standard curve, yielding a lower detection limit of 0.25 ng/mL.

**Statistics**

Results are presented as mean ± SEM. Statistical significance of differences between two groups (hypoxic vs. normoxic or recovered vs. not recovered) was determined by use of the unpaired Student’s t-test. A probability (P) of < 0.05 was considered statistically significant.
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Results

Exposure of mice to hypoxia

In order to establish the lowest non-lethal oxygen dose, mice were subjected to decreasing concentrations of oxygen. In contrast to previous observations, the threshold of non-lethal hypoxia was 8% O₂ in our hands. The previously reported level of 5 or 6% O₂, which was claimed not to interfere with normal mouse behavior, caused immediate death in our animals. At the observed threshold of 8% O₂, the mice became severely hypothermic within one hour (figure 1). Furthermore, the hypoxic mice showed signs of dyspnea and did not eat, drink (resulting in reversible weight loss of 14.43% ± 0.17 (mean ± SEM) after 24 hours of hypoxia) or move about normally. Within minutes after re-exposure to ambient oxygen levels, body temperature (figure 1) and behavior normalized.

![Figure 1. Body temperature of mice before, during and after hypoxia (n=8).](image)

Evidence that lowering ambient oxygen levels reduces systemic oxygen levels was obtained from blood gas measurements. As shown in table 1, arterial pO₂ decreased 2.6-fold from 90 mm Hg to 35 mm Hg, as a consequence of lowering ambient O₂ levels from 21 to 8% (2.6-fold). Venous oxygen levels were 12 mm Hg during hypoxia. In addition, pCO₂ and HCO₃⁻ were severely reduced, suggesting a metabolic acidosis.

Table 1: Blood gas values of hypoxic and normoxic arterial blood (mean±SEM, n=8 per group)

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>pCO₂ (mm Hg)</th>
<th>pO₂ (mm Hg)</th>
<th>HCO₃⁻ (μmol/l)</th>
<th>Hemoglobin (g/dl)</th>
<th>Hematocrit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxic</td>
<td>7.35±0.03</td>
<td>42±2</td>
<td>90±3.5</td>
<td>26±0.4</td>
<td>11±0.2</td>
<td>42±0.6</td>
</tr>
<tr>
<td>Hypoxic</td>
<td>7.29±0.01</td>
<td>22±0.5</td>
<td>35±1.1</td>
<td>10±0.1</td>
<td>13±0.2</td>
<td>41±0.5</td>
</tr>
</tbody>
</table>
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Cytokine measurements

To study hypoxia-induced inflammation, wildtype mice were exposed to a normobaric oxygen level of 8% during 16 hours after which plasma and organs were collected for cytokine measurements. To assess the pro-inflammatory effects of hypoxia, we measured IL-1β, IL-6 and TNF-α. IL-10 was evaluated as a measurement of the anti-inflammatory potential. To address latent leukocyte influx MIP-2 was measured. Finally, IL-12 and INF-γ were measured to gain insights in the T-helper cell response.

Plasma cytokine levels were below the detection limit of 31 pg/mL in either hypoxic or normoxic mice. Tissue cytokine levels were detectable in all organs tested and were significantly different between both groups of mice. Hypoxia resulted in elevated levels of IL-6, IL-10 and TNF-α, whereas IL-1β, MIP-2 and IL-12 levels were reduced. INF-γ levels remained unaltered. Figure 2 displays the data for lung homogenates; measurements in brain, heart, kidney liver and spleen gave comparable results (not shown).

In order to further delineate the relationship between hypoxia and cytokine production, we performed a time course study in which we measured TNF-α levels in brain, lung and kidney homogenates after 1, 4, 16 or 24 hours of hypoxia. As shown in figure 3, TNF-α levels rose within one hour and remained elevated during prolonged hypoxia. When mice were allowed to recover for 24
hours at normoxic conditions, TNF-α levels remained elevated despite the fact that the mice behaved normally and showed no obvious signs of disease. It is well conceivable that the mice will recover after long-term exposure to normoxia and therefore mice were allowed to recover for 3- or 10-days. Moreover, it is possible that the TNF-α detected in the homogenates is not biologically active. However, even after a 10-day recovery, TNF-α levels were still as high as immediately after hypoxia and appeared to be cytotoxic when analyzed using the WEHI 164 clone 13 cytotoxicity assay (figure 4). This suggests that systemic hypoxia rapidly induces a long-lasting local inflammatory state, without signs of a systemic inflammatory response.

**Figure 4.** TNF-α levels in lung homogenates after different time spans of recovery at normoxia, measured using a bioactivity assay. Values depicted at 0 days of recovery are measured immediately after 24 hours of 8% O2

**Activation of blood coagulation**

To investigate whether hypoxia activates blood coagulation, thrombin-antithrombin (TAT) complexes were determined in plasma and in tissue homogenates. As shown in figure 5, plasma TAT complexes were first detected after a hypoxic period of four hours. Formation of TAT complexes was an ongoing process during prolonged exposure to hypoxia. After re-exposure to normoxia, TAT complexes were cleared from the circulation within 24 hours. Remarkably, during a 24-hour recovery period subsequent to one or four hours of
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Hypoxia, TAT complexes were still formed. In tissue homogenates, TAT complexes could not be detected at any of the time points studied.

**Histology**

To confirm that systemic hypoxia induces coagulation and inflammation, tissue slides were screened for the presence of blood clots and for inflammatory mediators. As shown in figure 6, no signs of hypoxia-induced coagulation or leukocyte influx could be detected in these, H&E stained, tissues.

![Figure 6. Representative histology (H&E staining) of lung (A, B, C) and brain (D, E, F) slides of mice exposed to hypoxia. (A, D) control mouse, (B, E) mouse exposed to 24 hours of 8% O₂, (C, F) mouse exposed to 24 hours of 8% O₂ followed by a 10-day recovery period. Original magnification for each photograph: 40x](image)

**Discussion**

In the present study we analyzed the interplay between hypoxia, coagulation and inflammation by studying the time course of coagulation activation and cytokine production during and after cessation of hypoxia. Exposing mice to a hypoxic environment increased the level of TAT-complexes in plasma but not in tissues, suggesting that hypoxia provokes systemic activation of the coagulation cascade. This increased coagulation normalized after cessation of hypoxia, and paralleled general physiological and behavioral parameters like body temperature and food intake. The cytokine response was very different. Hypoxia increased cytokine levels only in tissues and this local inflammatory response remained elevated after a 10-day-recovery period. Taken together these data suggest that coagulation and cytokine production take place in separate compartments of the body: hypoxia-induced activation of the coagulation cascade is restricted to the circulation, whereas cytokine production takes place locally in tissues only.
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Our study in C57Bl/6 mice reveals several intriguing novel insights. The combination of transient, systemic activation of the coagulation cascade with enduring localized production of cytokines suggests the absence of a direct cross-talk between hypoxia-induced coagulation and inflammation. To the best of our knowledge, hypoxia is the first example of a physiological trigger inducing coagulation and inflammation at the same time, but not in the same compartment. Previous studies on interactions between inflammation and coagulation responses to experimental endotoxemia in human volunteers and baboons consistently showed intricate associations of these mechanisms within the same blood compartment. In these studies the liberation of pro-inflammatory cytokines including IL-6 and TNF-α after endotoxin stimulation led to a procoagulant response in blood. In the present study the increment in the same cytokines in tissues did not result in the formation of visible local fibrin. Furthermore, increased systemic IL-10 production is known to inhibit endotoxin-induced tissue factor production. In our model, however, tissue IL-10 apparently does not influence systemic coagulation as plasma TAT-complexes were continuously formed when IL-10 levels were at their maximum. Future studies aiming at specific inhibition of distinct cytokines or coagulation factors during hypoxia should answer the question if coagulation and inflammation are indeed uncoupled processes during hypoxia.

Another interesting observation is that environmental hypoxia results in a local inflammatory response without overt signs of systemic inflammation. A possible explanation might be that environmental hypoxia leads to local hypoxia, resulting in local activation of transcription factors like HIF-1α and NF-IL-6, which might lead to local production of cytokines. Alternatively, one might suggest that leukocytes activated by environmental hypoxia migrate from the vasculature into the surrounding tissue. Consequently, activated leukocytes would be removed from the blood compartment explaining the absence of a measurable systemic response. However, analysis of H&E stained tissue slides did not reveal leukocyte migration into the tissues, thereby eliminating this potential explanation. Noteworthy in this respect is Knöferl’s report stating that in male but not in female C3H/HeN mice, TNF-α and IL-6 are systemically increased after a short period of hypoxia. Whether this systemic alteration in cytokine profiles, which was not observed in our study employing C57Bl/6 mice, reflects strain differences remains speculative. It is however important to realize that the systemic cytokine levels reported by Knöferl are rather low and fall below the detection limit of our ELISAs (31 pg/mL).

The discrepancy between the presence of cytokines in tissue homogenates and the absence of inflammatory signs in histological sections is quite confusing. However, the animals seem to be recovered from the lack of oxygen within minutes after re-exposure to normoxia. Consequently, we expect that severe systemic hypoxia does not result in permanent tissue damage despite grossly altered cytokine levels. One might argue that anti-inflammatory effectors, e.g. IL-10, counteracted the effects of pro-inflammatory cytokines, like IL-6 and TNF-α. On the other hand, it might be that the cytokines detected by ELISA are not biologically active and therefore do not induce tissue inflammation. This possibility is negated by our observation that TNF-α detected in lung
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Homogenates proved to be cytotoxic and thus biologically active. Alternatively, in whole tissue homogenates, intracellular, extracellular and membrane-bound fractions of the cytokines were measured, whereas for obvious reasons the intracellular pool does not contribute to tissue inflammation. The extracellular part of the membrane-bound fraction probably only contributes to inflammation via direct cell-cell interactions\(^{34}\) thereby reducing its activity compared to secreted cytokines. Overall, protein levels detected in tissue homogenates by ELISA might be an over-estimation of bioactive cytokine levels thereby explaining the absence of histological signs of inflammation.

Careful inspection of the alterations of specific cytokines showed an increase in tissue IL-6 and TNF-\(\alpha\) whereas the level of the pro-inflammatory cytokine IL-1\(\beta\) was decreased. IL-6, TNF-\(\alpha\) and IL-1\(\beta\) are all secreted by activated monocytes, endothelial cells and granulocytes.\(^{34}\) IL-1\(\beta\) is synthesized as precursor (pro-IL-1\(\beta\)) protein, which is proteolytically activated by proteases like caspase-1.\(^{35}\) Hypoxia-induced diminished activity of caspase-1 is therefore an attractive explanation for the different expression profiles of IL-1\(\beta\) and IL-6/TNF-\(\alpha\).

MIP-2 is a key protein involved in chemotaxis\(^{34}\) and high MIP-2 levels are associated with leukocyte recruitment. In agreement with the absence of leukocyte infiltrates in hypoxic tissues, MIP-2 levels were not increased and even decreased in these tissue homogenates. The physiological relevance of decreased MIP-2 levels is subject of ongoing experiments.

The active isoform of IL-12 (IL-12p40) was down regulated by hypoxia whereas INF-\(\gamma\) levels remained unaltered. This suggests that the involvement of T-cells in the adaptation to hypoxia is unlikely.\(^{34}\)

It should be stressed that some of the in vivo alterations in cytokine profiles as a consequence of ambient hypoxia do not resemble in vitro data. For example, IL-1\(\beta\), which was down regulated in vivo, is reported to be upregulated in HUVEC cells.\(^9\) This discrepancy stresses that experiments focusing on triggers influencing multiple physiological parameters, like hypoxia, should ideally be performed in animal models.

Short-term ambient hypoxia (1 hour) resulted in severe hypothermia. Intriguingly, within minutes after re-exposure to normoxia the body temperature normalized. A contributing factor to the observed hypothermia is certainly the solitary behavior of the hypoxic animals resulting in diminished body heat from keeping each other warm. However, the drop in temperature is so impressive that other regulators should also play a role. Both IL-1\(\beta\) and IL-1 converting enzyme have been reported to be involved in temperature regulation and more profoundly in the induction of fever.\(^{35,36}\) Lowered IL-1\(\beta\) levels might therefore be involved in hypoxia-induced hypothermia. The rapid normalization of body temperature seems to exclude altered protein levels causing this phenomenon. Therefore, it is tempting to assume that adaptation to lack of oxygen parallels the poorly understood mechanism(s) behind hibernation or torpor.\(^{37,38}\)

A final intriguing observation is the difference in oxygen levels used by different research groups for hypoxia-studies in mice. Levels reported in various mouse strains vary from 5% up to 12% O\(_2\).\(^{10,13,14,39}\) In C57Bl/6 mice, 6% O\(_2\),\(^{40}\) 8% O\(_2\),\(^{41}\) and 10% O\(_2\)\(^{42}\) have been reported not to interfere with normal mouse behavior. However, exposure to 6% O\(_2\) caused immediate death in our animals (unpublished
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results). 8% O₂ was the lowest level our mice could endure, but still resulted in severe hypothermia and altered behavior. Explanations for these differences in lethal oxygen levels are hard to offer but may be related to the specific sub-strains of mice used or the method of applying hypoxia and/or measuring ambient oxygen levels. If either of these explanations holds true can only be determined with comparative studies employing similar mouse strains.

In summary, the current study adds important in vivo data concerning the interplay between hypoxia, coagulation and inflammation. Environmental hypoxia transiently induced coagulant activity in the circulation, which was not accompanied by inflammation. In contrast, on the tissue level hypoxia caused endured enhanced cytokine production in the absence of activation of the coagulation cascade.

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