Hyperbaric oxygen treatment attenuates cytokine induction after massive hemorrhage

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Yamashita, Masatomo, and Mamoru Yamashita. Hyperbaric oxygen treatment attenuates cytokine induction after massive hemorrhage. Am J Physiol Endocrinol Metab 278: E811–E816, 2000.—We investigated the effect of hyperbaric oxygen treatment (HBO) on cytokine induction after hemorrhage, because hypoxia induces cytokines in vitro.Chronically cannulated conscious rats were subjected to 40 ml/kg of hemorrhage and resuscitated with the shed blood and twice the volume of saline either under room air (room air group) or under 100% oxygen at 3 atmospheres absolute (hyperbaric group). Rats exposed to HBO with no hemorrhage served as controls. Time course changes in plasma endotxin level, arterial ketone body ratio (AKBR), serum tumor necrosis factor (TNF), interleukin-6 (IL-6), and their hepatic mRNA were detected in the three groups. Plasma endotxin levels increased significantly after hemorrhage, and there were no significant differences between the room air group and the hyperbaric group. In the room air group, AKBR dropped rapidly after hemorrhage and became minimal at hour 1, which was associated with significant increases in TNF-α and IL-6 at both mRNA and circulating levels. HBO significantly attenuated decreases in AKBR after hemorrhage with a significant reduction of mortality and cytokine induction. These results indicate that HBO attenuated the cytokine induction after hemorrhage by improving liver ischemia, and they suggest that tissue hypoxia may be responsible, at least in part, for cytokine induction after massive hemorrhage.

hemorrhagic shock; hypoxia; tumor necrosis factor; interleukin-6

Cytokines are small polypeptides or glycoproteins serving in the intercellular signaling pathways. They affect growth and metabolism, acting mainly in a paracrine and/or autocrine manner. In responding to external stimuli, vertebrates set in motion molecular and cellular interactions to facilitate a return to physiological homeostasis, and cytokines are deeply involved in these complex interactions. Tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) are proinflammatory cytokines and have great influence on temporal and spatial patterns of inflammatory responses. Release of these inflammatory cytokines into the local tissue milieu enhances antimicrobial function and helps tissue repair, but their overproduction after hemorrhagic shock may cause tissue injury (2, 29). Therefore, elucidation of the mechanism whereby cytokine induction occurs after massive hemorrhage may provide new prospects in the treatment of hemorrhage-related disorders. Several factors, including hypoxia, neuroendocrine release, and endotoxemia, have been implicated in cytokine induction after hemorrhage, but the precise mechanism is still unknown (6).

During the 1960s, there was widespread enthusiasm for hyperbaric oxygen treatment (HBO) for various diseases, including myocardial infarction, stroke, senility, and cancer. Enthusiasm waned after results of clinical trials showed little benefit for these diseases, and now the overzealous claims about the effectiveness of HBO have a legacy of skepticism among physicians. However, animal studies and clinical trials over the last two decades have produced a set of indications for which HBO may be beneficial (9). Under 100% oxygen at 3 atmospheres absolute (ata), the arterial oxygen tension would be over 2,000 mmHg, or an increase of about 6 vol% of arterial oxygen, which should provide enough oxygen to tissues, even in the total absence of hemoglobin (3). Therefore, HBO may affect cytokine induction after massive hemorrhage, if tissue hypoxia is involved in the induction. In this study, chronically cannulated conscious rats were divided into three groups, and time course changes in plasma endotxin level, arterial ketone body ratio (AKBR), serum TNF and IL-6, and hepatic mRNA of TNF-α and IL-6 were detected. The three groups were the RA group (40 ml/kg hemorrhage over 1 h under room air without HBO), the HB group (40 ml/kg hemorrhage over 1 h with HBO), and the NH group (HBO with no hemorrhage). We compared the changes among the three groups and examined the effect of HBO on the cytokine induction after massive hemorrhage.

Materials and Methods

Animal preparation. Male Wistar rats (Charles River, Tsukuba, Japan) weighing 290–310 g were anesthetized with pentobarbital sodium (50 mg/kg ip), and femoral arterial and venous cannulas were placed using sterile procedures, as previously described (7). After recovery, all rats were housed individually in a temperature-controlled environment with a 12:12-h light-dark cycle and were allowed to move freely. Access to food and water was given ad libitum. On the morning of the fourth day after cannulation, the following three experiments were begun. All the experiments were approved by the Animal Care Committee of the University of Tsukuba.

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EXPERIMENT 1. To detect the changes in mean arterial blood pressure (MABP) after hemorrhage, five conscious rats were subjected to a total of 12 ml (40 ml/kg) hemorrhage under room air (7 ml at time 0, followed by 0.5 ml each at minutes 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60). Rats were bled by drawing blood manually into a plastic syringe containing 30 units of heparin sodium. Then, rats were resuscitated with shed blood and twice the volume of saline at hours 1 and 4. MABP was measured continuously by connecting the femoral arterial cannula to a high-pressure transducer (Gould, Oxnard, CA).

Experiment 2. Thirty chronically cannulated conscious rats were divided into three groups (n = 10 for each group), and survival rate and changes in AKBR and plasma endotoxin level were detected. The RA and HB groups were subjected to 40 ml/kg of hemorrhage and then resuscitated by the same protocol as in experiment 1. The HB and NH groups were exposed to HBO individually in a 15.2-liter hyperbaric chamber for animal experiments (P-5100S; Hanyuda, Tokyo, Japan) by the following protocol: 1) denitrogenation by flushing with 21/min of 100% oxygen from minute -20 to minute -15; 2) pressurization to 3 ata 100% oxygen from minute -15 to time 0; 3) 100% oxygen at 3 ata from time 0 to minute 60, a constant flow of 0.5/min of oxygen being employed to prevent the hematocrit of carbon dioxide; 4) decompression from minute 60 to minute 90. The chamber has a small side hole through which the arterial and venous catheters were exteriorized. The survival rate was determined 24 h after hemorrhage. At time 0 and at hours 1, 2, 4, and 6, 0.5 ml of blood was withdrawn from the arterial catheter, cooled in ice immediately, and centrifuged at 4°C for measurement of AKBR and plasma endotoxin level. An equal volume of saline was replaced every time 0.5 ml of blood was withdrawn for assay. Acetoacetate (AcAc) and β-hydroxybutyrate (BOH) in the plasma were measured enzymatically with a Ketorex kit (Sanwa Kagaku Kenkyusho, Nagoya, Japan) using a semiautomated measuring system (Keto-340; Ibara Electronic Kassai, Japan). AKBR was calculated as AcAc divided by BOH (16). Plasma endotoxin levels were assessed by a colorimetric limulus test using a limulus amoebocyte lysate and a chromogenic substrate (Endotoxin Test D; Seikagaku, Tokyo, Japan). The assay sensitivity was 2 pg/ml. The plasma taken at hour 1 was also used for measurement of corticosterone. B levels.

Data were analyzed by two-way ANOVA in experiment 3 and were analyzed by two-way ANOVA corrected for repeated measures over time in experiments 1 and 2. Differences among the RA, HB, and NH groups at the individual time points were analyzed with the Newman-Keuls test after two-way ANOVA. Survival rate was compared by χ²-test among the three groups. Significant differences are indicated (P < 0.05).

RESULTS

Experiment 1. Forty milliliters per kilogram hemorrhage caused a prompt decrease in MABP under room air (Fig. 1A). MABP was maintained at between 35 and 45 mmHg for the first 60 min and then recovered gradually during fluid resuscitation. MABP stabilized after flushing with 100% oxygen from minute -20 to time 0; therefore, the time 0 rats were not exposed to 3 ata. For hour 1, rats were exposed to 3 ata 100% oxygen from time 0 to minute 30 and then decompressed from minute 30 to minute 60; therefore, the hour 1 rats in the HB and NH groups were exposed to 3 ata for 30 min.

The serum TNF level was determined by cytotoxicity against L929 cells in the presence of 1 μg/ml of actinomycin D, as previously described (20). TNF levels were calculated using recombinant human TNF-α (Asahi-Kasei, Tokyo, Japan) as a standard and expressed as picograms per milliliter. The sensitivity of this bioassay was 10 pg/ml. The serum IL-6 level was determined as hybridoma growth factor using M6H0.BSF2 cells, as previously described (15). Serum IL-6 levels were calculated using recombinant human IL-6 (Fuji-Rebio, Tokyo, Japan) as a standard and expressed as picograms per milliliter. The sensitivity of this bioassay was 20 pg/ml.

Messenger RNA of TNF-α and IL-6 was measured semiquantitatively by reverse transcription and PCR, as previously described (28-30). Briefly, the first strand of cDNA was synthesized by RT (MLMV-RT; Gibco BRL, Rockville, MD), and the resulting cDNA samples were adjusted to PCR buffer conditions. A three-temperature step PCR cycle program was carried out with an Omnigene Temperature Cycler (Hybaid, Middlesex, UK) by 40 s of annealing at 68°C, 1 min of extension at 72°C, and 1 min of denaturation at 94°C. After PCR, the products were quantitated by HPLC, as previously described (28). An endotoxin-treated rat, killed 2 h after 100 μg/kg intravenous endotoxin, was used as a positive control (Escherichia coli lipopolysaccharide, serotype 0111:B4; Sigma, St. Louis, MO). Twenty-five to thirty-five PCR cycles were run to determine the dependency of the amount of PCR products on cycle numbers. Linearity between the amount of amplified cDNA and that of applied RNA was checked in the preplateau exponential phase. The relative multiple increases in TNF-α and IL-6 mRNA at each time point after hemorrhage compared with the level of normal untreated rats were determined after normalization for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The primers were 5′-CACCCACGCTCTTTCTGTCCTGAAGAC-3′ and 5′-CCGGACTGCTGCGTGATGTCTAAGTACT for TNF-α and IL-6; 5′-GACTGATGTTGTTGACGCCACTGCG and 5′-TAGCCACTCTTCTGTGACTCTAACT for IL-6; and 5′-TGATGACATCAAGAGTGGTTGAAAG and 5′-TCCTTGAGGCCATGTAGGCCCAT for GAPDH (28, 30). The lengths of the PCR products were 546, 509, and 240 base pairs for TNF-α, IL-6, and GAPDH, respectively.

Statistics. Data were analyzed by two-way ANOVA in experiment 3 and were analyzed by two-way ANOVA corrected for repeated measures over time in experiments 1 and 2. Differences among the RA, HB, and NH groups at the individual time points were analyzed with the Newman-Keuls test after two-way ANOVA. Differences between the baseline levels and the individual time points were also analyzed with the Newman-Keuls test after ANOVA. Survival rate was compared by χ²-test among the three groups. Significant differences are indicated (P < 0.05).
below the prehemorrhage level after fluid resuscitation.

Experiment 2. All rats survived for 6 h after hemorrhage. In the RA group, 6 out of 10 rats died within 24 h, whereas only one rat died in the HB group, and no rats died in the NH group. The survival rate in the RA group was thus significantly lower than the rates in the HB and NH groups. In the NH group, no significant changes were detected either in plasma endotoxin level or AKBR compared with the baseline levels. In the RA and HB groups, plasma endotoxin levels increased significantly after hemorrhage and peaked at hour 2; there were no significant differences between the RA and HB groups at any time points (Fig. 1B). In the RA group, AKBR dropped rapidly after hemorrhage and became minimal at hour 1. Both AcAc and BOH decreased, but AcAc decreased more profoundly (data not shown). AKBR recovered slowly during fluid resuscitation and then decreased again in the RA group (Fig. 1C). BOH remained low, and the recovery of AKBR was due to an increase in AcAc (data not shown). These decreases in AKBR after hemorrhage were attenuated by HBO, and the difference between the RA and HB groups was significant at hour 1. Plasma B levels at hour 1 were 45 ± 7, 43 ± 8, and 9 ± 4 µg/dl in the RA, HB, and NH groups, respectively. The plasma B level in the NH group was significantly lower than in the other two groups, but the difference between the RA and HB groups was not significant.

Experiment 3. When reverse-transcribed RNA from an endotoxin-treated rat was subjected to an increasing number of PCR cycles, a period of exponential increase in PCR products was followed by a period of saturation between 25 and 35 cycles (data not shown). Good linearity between the amount of reverse-transcribed RNA and that of the resulting PCR product was acquired over a range of 0.0125 to 0.2 µg at 29 and 27 PCR cycles for TNF-α and IL-6, respectively (data not shown). All further PCR experiments were performed using 0.2 µg of reverse-transcribed RNA at these PCR cycles. Hepatic TNF-α mRNA and serum TNF level increased significantly after hemorrhage in the RA group and peaked at hours 2 and 4, respectively (Fig. 2). These increases were attenuated by HBO, and the differences between the RA and HB groups were significant at hours 1 and 2 for TNF-α mRNA and at hour 4 for serum TNF level. Hepatic IL-6 mRNA and serum IL-6 levels also increased significantly after hemorrhage in the RA group (Fig. 3). These increases were attenuated...
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by HBO, and the differences between the RA and HB groups were significant at hours 4 and 6 for IL-6 mRNA and at hour 6 for serum IL-6 levels. In the NH group, no significant changes were detected in either serum TNF or IL-6. TNF-α mRNA and IL-6 mRNA showed small increases after HBO in the NH group, but these changes were not significant compared with the baseline levels.

DISCUSSION

Various endocrine and metabolic responses are induced after hemorrhage to maintain the constancy of the internal environment, and induction of cytokines to hemorrhage is one of these host defense responses. After moderate hemorrhage, restitution of blood volume can be accomplished by movement of fluids in the interstitium or cells to the effective circulating volume (8). After massive hemorrhage, however, cell swelling may occur, and the blood volume restitution may be impaired, resulting in the disturbance of tissue perfusion, which further exacerbates tissue hypoxia. The exact mechanism whereby cell swelling occurs after massive hemorrhage is not yet determined, but the most accepted view is that cell swelling results from a failure of oxygen delivery (23). According to this view, as oxygen delivery fails, cells are unable to generate sufficient quantities of ATP; as a result, the electrogenic Na+ pump, mediated by the hydrolysis of ATP, fails, and cell swelling occurs. Excessive induction of inflammatory cytokines may follow this state and lead to further deterioration of organ functions (2).

In vitro studies have shown that hypoxia increases TNF-α secretion by macrophages (21). It is possible that tissue hypoxia also triggers overproduction of inflammatory cytokines in vivo. If rats are subjected to massive hemorrhage under 100% oxygen at 3 ata, tissue hypoxia after hemorrhage should be attenuated, because fluids in the interstitium and cells contain enough oxygen. This attenuation may block cell swelling and thereby inhibit further deterioration of tissue oxygenation. In this study, HBO significantly attenuated decreases in AKBR after hemorrhage, with a significant reduction in mortality and cytokine induction. Although we could not directly measure oxygen utilization or oxygen content in the liver in this study, the results suggest that HBO attenuated cytokine induction after hemorrhage by improving liver ischemia. These results indicate that tissue hypoxia may be responsible, at least in part, for cytokine induction after massive hemorrhage.

Because the liver plays a crucial role in recovery from shock, it is desirable to know the degree of metabolic disturbances in the liver after hemorrhagic shock, but conventional chemical measurements give little information regarding energy production. AKBR is a ratio of AcAc to BOH in arterial blood and well reflects the hepatic mitochondrial redox state. Many studies have shown that AKBR provides accurate information about the degree of liver ischemia after massive hemorrhage (16). The redox state in mitochondria is normally maintained reduced under conditions of normal oxygen delivery to the mitochondria. However, if oxygen delivery decreases, the mitochondrial redox state becomes highly reduced because of the decreased oxidation of NADH to NAD+. In this state, the entry of pyruvate into the mitochondria and its conversion to acetyl-CoA and the entry of acetyl-CoA into the tricarboxylic acid cycle are inhibited, resulting in severe impairment of energy production. In 1967, Krebs et al. showed that the NAD+/NADH ratio in liver mitochondria parallels the AcAc-to-BOH ratio (25). Although it is difficult to measure the AcAc-to-BOH ratio in the mitochondria, the ratio in the hepatic venous blood is expected to reflect that in the liver mitochondria, because ketone bodies are produced only in the liver and pass freely through the mitochondrial and cell membranes. Moreover, it was shown that the AcAc-to-BOH ratio in the peripheral arterial blood correlates well with that in the hepatic venous blood (26). Therefore, we can assess the redox state and the degree of impairment of energy production during hemorrhagic shock by measuring the AcAc-to-BOH ratio in the peripheral blood, i.e., AKBR. The mitochondrial redox state of NADH is correlated with the cytoplasmic redox state, and the latter is in equilibrium with the pyruvate-to-lactate ratio (25). Although the pyruvate-to-lactate ratio in liver tissue correlates well with the hepatic redox state and the energy charge, the pyruvate-to-lactate ratio in the peripheral blood does not seem to correlate well with them (26). This is due to the fact that glycolysis occurs not only in the liver but also in other organs. Moreover, during shock, lactate produced in the peripheral tissues may not be transported to the liver. Thus the redox state in the liver under hemorrhagic shock correlates better with the arterial AcAc-to-BOH ratio than does the pyruvate-to-lactate ratio, and a reduction in oxygen delivery seems to be mainly responsible for a decrease of AKBR after hemorrhage. A change in pH is also a potent perturbing factor of AKBR, because pH changes the equilibrium constant of the β-hydroxybutyrate dehydrogenase system (25). Although we could not measure pH in the liver, pH may have affected AKBR in this study.

In previous studies, we had performed hemorrhagic shock experiments by fixing MABP at 40 mmHg for 60 min (27, 28), but in this experiment, we adopted a fixed-volume model because we could not measure the blood pressure within a hyperbaric chamber. The blood pressure transducer we used in this study has a closed membrane, and therefore, when it was placed in the chamber, it was damaged as the pressure in the chamber increased. Calibration in the chamber was also impossible. Formerly, we had a chance to detect the changes in blood pressure during 20 ml/kg hemorrhage under HBO (unpublished data). In that experiment, we introduced an anesthetized rabbit into a bigger oxygen chamber and measured the blood pressure directly by the height of the blood in the cannula connected to the femoral artery. In that study, MABP was higher by 5–10 mmHg than that of a rabbit bled under room air. Therefore, the favorable effect of HBO on the circulatory system may also contribute to its efficacy. In this
study, the blood variables returned to near baseline at hour 4. Hemodilution from the infused fluid may have had some effect on this return, because infusion was done between hours 1 and 4. However, after 20 ml/kg hemorrhage without fluid resuscitation, hepatic cytokine mRNA also returned to near-normal level at hour 4 (29), and AKBR recovered within 2 h in reversible shock models of rats (26). Therefore, the return of the blood variables at hour 4 seems to be basically a physiological response rather than a dilution effect.

Factors other than tissue hypoxia, such as neuroendocrine release and endotoxemia, have been implicated in cytokine induction after massive hemorrhage (6). Circulating levels of glucocorticoid and epinephrine increase rapidly after hemorrhage, and a complex communication seems to exist between the neuroendocrine and the immune systems (13). Komaki et al. (10) showed that there is an inverse relationship between IL-6 and B concentrations in hemorrhaged rats (30% of the total blood) by use of adrenalectomy and B replacement, and they suggested the possibility that the neuroendocrine system has some regulatory effect on cytokine induction after hemorrhage. In this study, however, plasma B level at the maximum bleed out point was not significantly different between the RA and HB groups. Pharmacological studies have shown that administration of either glucocorticoid or epinephrine has no effect or even suppresses the induction of inflammatory cytokines (12, 24). Therefore, the neuroendocrine response does not seem to be the main factor that induces inflammatory cytokines after hemorrhage, although its regulatory effect cannot be ruled out. Lactic acid accumulates in tissues where metabolic demand exceeds oxygen supply, and it was shown that addition of lactate to cultured macrophages stimulates the release of an angiogenic factor (19). Reduction of lactate level by HBO may also contribute to its effect on cytokine induction. Endotoxin is a strong inducer of cytokines both in vitro and in vivo. Bogin et al. (4) showed that HBO improves both mortality and morbidity in rabbits treated with Shigella endotoxin. There is a possibility that HBO affects cytokine induction after endotoxin treatment in vivo. In our previous study, treatment with bactericidal/permeability-increasing protein partially inhibited induction of TNF-α mRNA and IL-6 mRNA in rat liver 8 h after hemorrhagic shock but did not attenuate the induction at hours 1 and 6 (27). Endotoxemia seems to play a role, especially in the late-phase induction of inflammatory cytokines after hemorrhagic shock. In this study, the plasma endotoxin levels were not significantly different between the RA and HB groups, whereas an early cytokine induction was significantly attenuated by HBO. These results suggest that tissue hypoxia may be another mechanism by which cytokine induction occurs after massive hemorrhage. On the other hand, a recent study has shown that hyperoxia activates the nuclear transcription factor and increases TNF-α gene expression in mouse pulmonary lymphocytes by producing reactive oxygen intermediates (22). In this study, mRNA of TNF-α and IL-6 showed gradual increases after HBO in the HB and NH groups. Reactive oxygen intermediates may also be involved in cytokine induction after hemorrhage, especially in the reperfusion stage after ischemia. Interestingly, HBO diminished TNF-α secretion from rat macrophages stimulated with endotoxin in the study of Lahat et al. (11) and decreased mortality in the zymosan-induced shock model of rats with a marked reduction of serum TNF-α levels in the study of Luongo et al. (14). It seems that HBO brings about cytokine induction in the normal state but inhibits it in stress conditions. Luongo et al. suggested that HBO might act as an immune modulator, but the precise mechanism was unknown.

It was reported as early as in 1939 that administration of oxygen is effective for shock (5), and this effect was confirmed by many other researchers. In 1964, Blair et al. (3) first showed that the survival rate after hemorrhagic shock was significantly improved by HBO. In their study, dogs were transferred to a hyperbaric chamber after stabilization of MABP at 30 mmHg for 30 min, and fluid resuscitation was begun after 2 h of HBO. By their protocol, the survival rate was improved 17–74%. In this study, rats were subjected to 40 ml/kg hemorrhage after introduction of HBO to negate the effects of tissue hypoxia. In clinical settings, however, HBO should be applied after hemorrhage. To examine the efficacy of HBO as a treatment for hemorrhagic shock, other protocols, i.e., posttreatment, must be tried with a combination of various forms of fluid resuscitation.

In this study, we detected the changes in cytokine mRNA in total homogenates of the liver, and the cell types responsible for cytokine production in the liver were not examined. Because hypoxia increases TNF-α production by macrophages in vitro, it is quite possible that Kupffer cells in the liver produce cytokines after massive hemorrhage. However, overlying hepatocytes and endothelial cells also seem to produce cytokines in sepsis models of rats (1). The cellular localization of cytokine production in the liver after massive hemorrhage still needs to be elucidated.

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