Hyperbaric stress during saturation diving induces lymphocyte subset changes and heat shock protein expression

H. MATSUO, N. SHINOMIYA, and S. SUZUKI

Department of Microbiology, First Department of Physiology, National Defense Medical College, 3-2 Namiki, Tokorozawa 359-8513; Educational Division, Japan Self-Defense Force Hospital Yokosuka, 2-7-1 Nagase, Yokosuka 239-0826; and Japan Maritime Self-Defense Force Undersea Medical Center, 2-7-1 Nagase, Yokosuka 239-0826, Japan

Matsuo H, Shinomiya N, Suzuki S. Hyperbaric stress during saturation diving induces lymphocyte subset changes and heat stroke protein expression. Undersea Hyper Med 2000, 27(1):37–41.—To clarify the cellular responses and biochemical markers of hyperbaric stress, we investigated heat shock protein (hsp) expression and subset changes of human peripheral blood lymphocytes during saturation diving. Five healthy male subjects underwent a 39-day saturation dive to the maximal storage pressure of 4.1 MPa [400 meters of sea water (msw)]. During the saturation dive, lymphocyte subset changes were detected using a flow cytometer, and increased expressions of hsp 72/73 and hsp 27 were observed by Western blot analysis. Lymphocyte subset changes included a decrease in CD4/CD8 ratio and in the fraction of CD4+ T cells as well as an increase in NK cells, especially during the 400-msw bottom phase. An increased expression of hsp 27 compared to hsp 72/73 was obvious, especially during the hold period at 100 msw. These results suggest that changes in lymphocyte subsets and hsp expression are useful markers for stress responses during saturation diving. These changes may also be useful for testing the barotolerance of divers for saturation diving.

saturation diving, hyperbaric stress, heat shock protein, lymphocyte subset

During saturation diving, divers are exposed to extremely stressful conditions under high pressure. Saturating divers are in danger of various complications, such as high pressure neurologic syndrome (HPNS) (1) or infectious diseases (2–4). However, the stress responses under such a hyperbaric condition have not been extensively investigated, and biochemical markers for hyperbaric stress have not been established. These markers are required for detecting cellular-level stress responses during saturation diving and for testing the barotolerance or fitness of divers for saturation diving. The expression of heat shock protein (hsp), also known as chaperoning protein (5–7), has been shown to increase under oxidative stress (8–10). Although saturation divers are subjected to extremely high pressure (as much as 4 MPa or greater), the oxygen partial pressure (PO2) during a saturation dive is lower (42 or 50 kPa) than that used in hyperbaric oxygen (HBO2) therapy in which the PO2 is as high as 280 kPa. We have previously reported that lymphocyte subset changes are induced by hyperbaric stress rather than by oxidative stress during deep saturation diving (11–13). Moreover, one kind of hsp has been shown to be associated with the growth of lymphocytes (14). To clarify the biochemical markers for hyperbaric stress at the cellular level, hsp expression and subset changes of peripheral blood lymphocytes of five healthy divers were investigated during a 400-meters of sea water (msw) (4.1 MPa) saturation dive.

MATERIALS AND METHODS

Diving profile: A 400-msw heliox saturation diving simulation was done using a deep diving simulator (Fig. 1) at the Japan Maritime Self-Defense Force Undersea Medical Center in Yokosuka in 1997. The dive and decompression followed the profile shown in Fig. 2. To suppress the occurrence of potential symptoms of HPNS, the compression rate was gradually lowered as depth increased, and pressure was held at five stages. The PO2 was maintained at 42 kPa throughout the bottom phase of the dive, and at 50 kPa during the decompression phase. Decompression was performed according to the modified Duke-GKSS procedure (15). Ten excursion dives were performed during the bottom phase at 400 msw and pressure-hold period at 100 msw. No occurrence of severe HPNS or decompression sickness was observed during the dive.

Sampling of peripheral blood: Anticoagulated periph-
chamber was carefully managed as follows: A service lock was decompressed at a rate of 0.2 MPa · min⁻¹ linearly to half of the storage depth with the several additional 4-min pressure hold steps during the remainder of the decompression. Biochemical and hematologic changes (including membrane integrity) were not affected by this recovery procedure (11–13).

**Lymphocyte subset analysis**: Blood samples were stained with fluorescence dye (fluorescein isothiocyanate or phycoerythrin)-conjugated monoclonal antibodies (Becton Dickinson Immunocytometry Systems, San Jose, CA) that bind to specific lymphocyte surface molecules (Leu-4/Leu-12 for T/B cells, Leu-3a/Leu-2a for CD4/CD8 T cells, Leu-7/Leu-2a and Leu-4/Leu-11c + 19 for NK cells, and anti-TCR-γ/δ -1 for γ/δ T cells) at 4°C for 30 min. Samples were treated with FACS lysis solution (Becton Dickinson Immunocytometry Systems) at room temperature for 10 min to lyse erythrocytes and to fix leukocytes. Subsequently, cells were washed twice and analyzed by a FACSCalibur cytometer (Becton Dickinson Immunocytometry Systems) using a 488-nm argon ion laser. Data were acquired using selective gating (forward light scatter and side scatter) and analyzed with CellQuest.

![Image of diving profile](image-url)

**FIG. 2**—Diving profile of 400-msw saturation diving. Divers were exposed to a 400-msw (4.1 MPa) helium–oxygen hyperbaric environment. Rate of compression speed decreased with depth, and the pressure was held for a while at five steps to minimize the symptoms of HPNS. Decompression was performed according to the modified Duke-GKSS procedure. Ten excursion dives were performed at 400 and 100 msw. Arrows indicate days on which blood samples were taken.
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software. The fraction of peripheral blood leukocytes was calculated according to the cell volume and structure. Using this method, we were able to divide the peripheral blood leukocytes into three fractions: polymorphonuclear (PMN) leukocytes, lymphocytes, and monocytes. The percentage of each lymphocyte subset was calculated by dot plot analysis.

Expression of hsp by Western blot analysis: Mononuclear cells were purified from whole peripheral blood by using a Sodium Diatrizoate/Ficoll centrifugation method. To purify mononuclear cells, 4 ml of whole peripheral blood was diluted with the same volume of phosphate buffered saline (PBS). The diluted blood was carefully layered on top of 3 ml of lymphocyte separation medium (LSM, Organon Teknika Co., Durham, NC), and this was centrifuged at 20°C for 30 min at 400 g. The mononuclear cells, lymphocytes and monocytes, were removed from a layer on top of the separation fluid and washed twice with PBS. The extract of mononuclear cells was prepared as follows. Briefly, cells were fixed by 10% trichloroacetic acid for 15 min in an ice-cold bath. After the cells were washed with PBS, 80 μl of lysis buffer (9 M urea, 2% Triton X-100, 5% 2-mercaptoethanol) was added to the cell pellet. Then the samples were processed with a sonicator (Insonator model 200M; Kubota, Japan) at 200 W for 10 min. We obtained clear lysate by adding 20 μl of 10% lithium dodecyl sulfate. After neutralizing the pH by adding 1 μl of 2 M Tris, we stored the lysate at −80°C until use. For Western blot analysis, cell extracts containing 10 μg of protein from each sample were separated on 12% sodium dodecyl sulfate/polyacrylamide electrophoresis gel, and then transferred to polyvinylidene difluoride membrane (Clear Blot Membrane-p; ATTO Co.) by a semidry electroblotter (Horizionblott AE-6645, P/N type; ATTO Co.). We detected the proteins by using the KPL Western blot kit (horseradish peroxidase system; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), following the manufacturer’s instructions. Monoclonal antibodies to hsp 27 and hsp 72/73 were purchased from Calbiochem. After being treated with the Western blotting detection reagent (ECL, Amersham International Plc, Buckinghamshire, England), the membrane was flushed with Fuji medical x-ray film (New RX). The membrane image was digitized, incorporated into a Macintosh computer, and analyzed using NIH image software (version 1.60).

Statistical analysis: Results were expressed as the mean value ± the standard error. Where appropriate, data were analyzed using repeated analysis of variance, followed by Dunnett’s test. \( P < 0.05 \) was accepted as statistically significant.

RESULTS

Lymphocyte subset changes: The number of peripheral blood leukocytes significantly decreased from 5,520 ± 528 cells · ml\(^{-1}\) before diving to 4,120 ± 349 cells · ml\(^{-1}\) on Day 7. This decrease was transient, and the level rose to 5,146 ± 963 cells · ml\(^{-1}\) on Day 25. In contrast, the number of lymphocytes did not change significantly during or after saturation diving. The changes of lymphocyte subsets were serially observed during diving and after surfacing (Fig. 3). Obvious cell damage was not detected by flow cytometric analysis. The fraction of T (Leu-4\(^{+}\); CD3\(^{+}\)) lymphocytes decreased from 59.3 ± 9.5% before diving to 47.3 ± 7.9% on Day 7 and to 46.4 ± 9.8% on Day 39 (Fig. 3 A). The percentage of CD4\(^{+}\) (Leu-3a\(^{+}\); helper) T cells was increasingly suppressed with the increase in ambient pressure (depth) and reached 19.6 ± 5.2% on Day 7. The percentage of CD4\(^{+}\) (Leu-2a\(^{+}\); killer) T cells increased reciprocally (Fig. 3 B). The CD4:CD8 ratio also decreased from 0.86 ± 0.19 before diving to 0.45 ± 0.11 on Day 7 (Fig. 3 B). The fraction of Leu-7\(^{+}\) natural killer (NK) cells, Leu-11c + 19\(^{+}\) NK cells and \( \gamma/\delta \) T cells showed two peaks, on Day 3 or 7 and on Day 39 (Fig. 3 C and D). Only the Leu-11c + 19\(^{+}\) NK cells showed a significant increase on Day 7.

![Graphs](http://rubicon-foundation.org)

**FIG. 3—Effects of 400-msw saturation dive on the lymphocyte subsets.** Data represent the mean % ± SE of each lymphocyte subset of five divers. \(* \text{P} < 0.01\) and \(* \text{P} < 0.05\), compared with the prediving value. A: fraction of T cells or B cells; B: CD4:CD8 ratio and fraction of CD4\(^{+}\) T cells or CD8\(^{+}\) T cells; C: fraction of Leu-7\(^{+}\) NK cells; D: fraction of Leu-11c + 19\(^{+}\) NK cells or \( \gamma/\delta \) T cells.
Changes of hsp expression: We observed the effects of saturation diving on hsp 27 and hsp 72/73 expression during diving and after surfacing (Fig. 4). Percentages of hsp expression were calculated by comparison with the prediving level. The enhancement of hsp 27 expression (maximum enhancement of 219.5 ± 32.4% on Day 25) was greater than that of hsp 72/73 expression (maximum enhancement of 182.5 ± 13.6% on Day 39). These enhancements consisted of two peaks, an early peak right after compression or during the 400-msw bottom phase and a late peak during the pressure hold at 100 msw or right after surfacing. A dip in hsp expression was observed between the two peaks during the decompression phase. Unlike the lymphocyte subset changes, the enhancement of hsp expression was more obvious at the late peak, and the early peak did not increase significantly. Moreover, the enhancement of hsp expression tended to continue even after the divers had surfaced.

DISCUSSION
In this study, we demonstrated that 400-msw saturation diving induced lymphocyte subset changes and enhanced the expression of heat shock proteins. Previously, we reported that the CD4+ T cell subpopulation and the CD4:CD8 ratio significantly decreased during a 440-msw saturation dive (11). Similar changes in lymphocyte subsets are sometimes observed in patients in an immunosuppressed state, such as acquired immunodeficiency syndrome (16). In saturation diving, we often encounter infectious problems such as otitis externa, due mainly to Pseudomonas aeruginosa infection (2–4), that are believed to result from increased susceptibility and decreased resistance to microorganisms. Although Pseudomonas external otitis seen in people who participate in saturation diving may be related to the high humidity rather than the high ambient pressure, it is very important to investigate immunologic changes during saturation diving because they are still poorly understood.

Lymphocyte subset changes can also be induced by heat stress (17) or nutritional effects (18). Heat stroke and heat stress induce a decrease in the CD4+ T cell subpopulation and an increase in the NK cell subpopulation (17), and both effects were also observed in the present study. We showed previously that there was no significant decrease in the CD4:CD8 ratio during a 100-msw (1.1 MPa) saturation dive (12) in which divers were exposed to almost the same conditions as in a 440-msw (4.5 MPa) saturation dive (12), including PO2, humidity, temperature, and nutrition. This result suggests that a decrease in the CD4:CD8 ratio during deep saturation diving might be induced by hyperbaric stress rather than by other kinds of stress such as oxidative stress or heat stress. The total elapsed time for compression might also play an important role in the magnitude of lymphocyte subset changes, similar to the incidence of HPNS (1), because a saturation dive with a shorter compression period tended to produce a significant decrease in the CD4:CD8 ratio in the comparative analysis of two 330-msw saturation dives (13). In this study, we demonstrated two new findings. First, the fraction of T lymphocytes showed two negative peaks (Fig. 3 A), although previous analyses showed only one negative peak during saturation diving deeper than 330 msw (3.4 MPa) (12,13). Second, NK cells significantly increased with compression (Fig. 3 C and D). The γδ T cells also tended to increase with compression (Fig. 3 D). These phenomena may be due to the short compression period and the additional 100 msw (1.1 MPa) pressure-hold stage for 15 days. In this diving profile, the compression period to the storage depth was much shorter than that of previous 440- or 330-msw saturation dives, which might have resulted in significant changes to NK cells in addition to changes to CD4+ T cells. The second peak of lymphocyte subset changes might have resulted from the persistent hyperbaric stress without decompression during the long 100-msw pressure-hold stage and/or repetitive excursion dives at this depth.

In this study, we first demonstrated that expression of hsp 27 and hsp 72/73 increased during a 400-msw saturation dive (Fig. 4). Heat shock proteins are known as molecular chaperoning proteins (5–7), and they are induced by various stimuli such as heat (19,20) or oxida-
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tive stress (8–10). Recently, hsp 70 was shown to be
induced by hyperbaric stress (21), but it may also result
from severe hyperbaric oxidative stress. Although in our
study saturation divers were under very high pressure (as
much as 4 MPa), the ambient Po2 was much lower (43 or
51 kPa) than that used in HBO2 therapy or other in vitro
experimental setups. Under such a condition during
saturation diving, we can investigate the effects of hyper-
baric stress with minimum oxidative stress. Heat shock
protein 27, one of the small hsp’s, has been shown to be
associated with B cell growth arrest (14), which implies
that hsp is also related to the growth or subset changes of
lymphocytes. Our data showed that the enhancement of
hsp 27 expression was more distinct than that of hsp
72/73. Both hsp expression and lymphocyte subset changes
tended to show two peaks during saturation diving,
but the enhancement of hsp expression was more
obvious at the second peak during the 100-msw pres-
sure-hold stage and even after surfacing. These observa-
tions suggest we can use lymphocyte subset changes
during the early phase of saturation diving and increased
expression of hsp, especially hsp 27, during the late phase
of saturation diving as reliable markers for hyperbaric
animal stress. These markers may be useful not only for
detecting cellular stress responses to saturation diving but
also for testing the barotolerance of divers for saturation
diving.

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REFERENCES
1. Edmunds C, Lowry C, Pennefather J. High pressure neurologi-
sical syndrome. In: Diving and subaqueous medicine. Oxford:
2. Alcock SR. Acute otitis externa in divers working in the North
Sea, a microbiological survey of seven saturation divers. J Hyg
3. Dibb WL. In vitro efficacy of Otic Domeboro against Pseudo-
4. Shinomiya N, Suzuki S, Oiwa H. Relationship between the
change in microflora and the onset of otitis externa in 400 msw
5. Ellis RJ, Vies SM. Molecular chaperones. Annu Rev Biochem
proteins are molecular chaperones. J Biol Chem 1993;
268:1517–1520.
7. Rao PV, Horwitz J, Ziegler J. Chaperone-like activity of
8. Sciandra JJ, Subjeck JR, Hughes CS. Induction of glucose-
regulated proteins during anaerobic exposure and of heat-shock
proteins after reoxygenation. Proc Natl Acad Sci USA 1984;
81:4843–4847.
9. Drummond IAS, Steinhardt RA. The role of oxidative stress in
the induction of drosophila heat-shock proteins. Exp Cell Res
induces ischemic tolerance in gerbil hippocampus. Brain Res
saturation diving on the lymphocyte subsets of healthy divers.
12. Shinomiya N, Suzuki S, Ikeda T, Oiwa H. Immunologic capaci-
ties during deep saturation diving: changes of lymphocyte
subsets under high pressure. In: Çimientos, ed. XXth annual
meeting of the European Underwater Baromedical Society on
diving and hyperbaric medicine. Istanbul, Turkey: Hyperbaric
speed on the lymphocyte subset change during deep saturation
diving. In: Sipinen SA, Meinö M, eds. XXlst annual meeting of
EUBS on diving and hyperbaric medicine. Helsinki, Finland:
B lymphocytes is accompanied by induction of the low molecular
weight mammalian heat shock protein (hsp 28). J Immunol
15. Vann RD. Decompression from saturation dives. In: Proceedings
of the third annual Canadian ocean technology congress.
subsets and adherence molecules expression in heatstroke and
and immune effects of dietary arginine, glutamine and omega-3
fatty acids supplementation in immunocompromised patients. J
Induction of heat shock protein synthesis at febrile temperature
is correlated with enhanced resistance to hyperthermic stress but
not to heavy metal toxicity or dexamethasone-induced immuno-
Protection of translation and DNA replication against some
influences mRNA expression of transforming growth factor-β1
and heat shock protein 70 in chondrocyte-like cell line. J Orthop