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Heme oxygenase-1 (HO-1) is constitutively up-regulated in top alpinists

Gota Miura [a], Kazunori Kato [b], Takahiko Shimizu [c], Daniela Shiga [a], Takuji Shirasawa [a,*]

[a] Department of Aging Control Medicine, Juntendo University Graduate School of Medicine, Bunkyo-ku, Tokyo 113-0033, Japan
[b] Center for Biomedical Engineering Research, Toyo University, Kawagoe, Saitama 350-8585, Japan
[c] Molecular Gerontology, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo 173-0015, Japan

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ABSTRACT

Alpinists who challenge Mt. Everest need adaptation to hypoxia before the attack of Mt. Everest. Although this adaptation is important for the success of climbing Mt. Everest, the molecular mechanism on the adaptation to hypoxia is not well understood. In order to clarify this mechanism, we investigated hypoxia-induced gene expressions specific for top alpinists using microarray analyses. We report here that heme oxygenase-1 (HO-1) is significantly higher in the blood of top alpinist compared with non-alpinists. Although HO-1 expression of non-alpinists is also up-regulated in response to hypoxia, HO-1 level of the top alpinists are constitutively higher than that of non-alpinists. Serial examinations of HO-1 in one top alpinist revealed that the higher expression of HO-1 is maintained in high-level several months after the attack of top mountains. Taken together with the biochemical function of HO-1 that catalyzes heme into CO and bilirubin, HO-1 expression may improve the circulation and compensate with oxidative tissue damages induced by hypoxia. These data also suggest that peripheral blood has the memory on hypoxia independent of antigens by maintaining the high-level of HO-1 expression in top alpinists, which merits the rapid adaptation to hypoxia for 8000 m climbing.

1. Introduction

Those who attack Mount Everest empirically perform the acclimatization before attack in order to prevent acute mountain sickness (AMS) [1,2]. AMS manifests headache, fatigue, nausea, pulmonary edema, and cerebral edema, which sometimes give rise to fatal consequences on the mountain [3]. However, it is not well understood what kind of molecular mechanisms underlies with this condition. In order to elucidate the molecules involved in acclimatization and AMS, we applied microarray analysis in the present study with top alpinists who attack Mount Everest and compared the gene expression with non-alpinists under hypoxic condition.

We use a normobaric hypoxic chamber to acclimatize in Tokyo before the attack of high altitude climbing. One of the advantages of the chamber is the flexibility to control the extent and the duration of hypoxia as we design. Then we used the hypoxic condition of 12.7% O_2, which is equivalent to the altitude of 4000 m. The highest stationary medical assistance is available for alpinists at 4000 m on Nepalese side of Mount Everest.

One of the subject alpinists of this study is Miura Yuichiro who climbed Mount Everest twice at ages of 70 and 75 [4]. The aim of this research is thus to further reduce the risk of AMS for the climbing of elderly people. The elderly people generally show decreased cardiopulmonary function as well as decreased muscle strength, which generate the higher risk to develop AMS. The majority of AMS symptoms come from the vasoconstriction of the pulmonary artery as well as the cerebral artery [5–7]. Thus the understanding of molecular mechanism for vasoconstriction or hypoxia-induced tissue damages in AMS would help to improve AMS symptoms of elderly people. The molecular mechanism of AMS would also help senior alpinist perform safer climbing with the knowledge of the risk for AMS.

We discovered in the present study that HO-1 protein is constitutively expressed in the peripheral lymphocyte of top alpinists but not in non-alpinists. Together with the physiological function of HO-1 as CO generator and antioxidant gene, we suggest that HO-1 is a good biomarker for AMS, which would help senior alpinist to perform safer climbing.

2. Materials and methods

2.1. Subjects for microarray analysis

Four alpinists are the members of 2008 Miura Everest Expedition Team (http://www.qomolangma2008-kddi.com/). Average age of alpinist group was 52.5 ± 16 years old. All alpinists have the experience of climbing peaks over 8000 m. Three male and one female control group are low altitude sojourn who have no previous experience of climbing high altitude more than 3000 m

* Corresponding author. Fax: +81 3 3814 1134.
E-mail address: shirasawa@shirasawa-acl.net (T. Shirasawa).

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for past 10 years. The average age of control group was 48 ± 7. The subjects were exposed to normobaric 12.7% hypoxia, which is equivalent to altitude of 4000 m, for 1 h.

2.2. Subjects for HO-1 analysis

Four alpinists are high altitude climbing guides who had climbed peaks above 5000 m within 6 months. Three out of four alpinists climbed peak above 8000 m within a year. Their average age was 47.4 ± 7.8 years old. Five subjects of control group live in Tokyo (50 m above sea level). They have no previous exposure to high altitude or hypoxia. The average age of control group was 47.75 ± 7.83.

2.3. Subject for serial sampling

One of the elite alpinists, 48-year-old male, was followed for 9 months. During this period, he successfully climbed Mt. Cho-Oyu (8201 m), Mt. Kenya (5109 m), Lobche East (6119 m). The blood was collected three time in-between expeditions. HO-1 expression from peripheral blood was serially examined.

2.4. Hypoxia induction

The subjects were exposed to normobaric 12.7% hypoxia for 1 h in Normo Baric Hypoxic Chamber (NBHC) installed in Miura Dolphins (http://www.snowdolphins.com/).

2.5. Microarray analysis

2.5.1. RNA isolation

Blood (2.5 ml) was collected into PAXgene Blood RNA Kit (QIAGEN, Valencia, CA), kept at room temperature for 2–4 h, and followed by cooling. Total RNA was extracted according to the manufacturer's protocol. RNA quantity and quality were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) and an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA), as recommended.

2.5.2. cRNA amplification and labeling

Total RNA was amplified and labeled with cyanine 5 (Cy5) for test samples and with cyanine 3 (Cy3) for the reference using Agilent's Quick Amp Labeling Kit (Agilent Technologies, Palo Alto, CA) following the manufacturer's instructions. Briefly, 250 ng of total RNA was reversed transcribed to double-strand cDNA using a poly dT-T7 promoter primer. Primer, template RNA and quality-control transcripts of known concentration and quality were first denatured at 65 °C for 10 min and incubated for 2 h at 40 °C with 5× first strand buffer, 0.1 M DTT, 10 mM dNTP, MMLV RT, and RNase-out. The MMLV RT enzyme was inactivated at 65 °C for 15 min. cDNA products were then used as templates for in vitro transcription to generate fluorescent cRNA. cDNA products were mixed with a transcription master mix in the presence of T7 RNA polymerase and Cy5 labeled or Cy3 labeled-CTP and incubated at 40 °C for 2 h. Labeled cRNAs were purified using QIAGEN's RNeasy mini spin columns and eluted in 30 μl of nuclease-free water. After amplification and labeling, cRNA quantity and cyanine incorporation were determined using a Nanodrop ND-1000 spectrophotometer and an Agilent Bioanalyzer.

2.5.3. Sample hybridization and data processing

For each hybridization, 825 ng of Cy3 labeled cRNA and 825 ng of Cy5 labeled cRNA were mixed, fragmented, and hybridized at 65 °C for 17 h to an Agilent 4 × 44 K Whole Human Genome Microarray. After washing, microarrays were scanned using an Agilent DNA microarray scanner. Feature extraction software version 9.5.3.1 (Agilent Technologies, Palo Alto, CA) was used to assess fluorescent hybridization signals and to normalize signals using linear regression and a Lowess curve-fit technique. Reproducibility and reliability of each single microarray was assessed using Quality Control report data (Feature extraction, Agilent Technologies).

The data analyses were performed using GeneSpring GX 7.3 (Agilent Technologies, Palo Alto, CA). Statistical analysis of between groups was performed by Welch's *t*-test. The data were considered to be significant if $P < 0.05$. Further, we applied 1.5-fold difference.

2.6. HO-1 expression

Human HO-1 protein levels in peripheral blood mononuclear cells (PBMCs) were measured using ELISA Kit (R&D Systems, Minneapolis, MN) according to manufacture instruction. Human peripheral blood mononuclear cells were purified from heparin treated blood from volunteers via density gradient centrifugation in Histopaque 1077 (Sigma Chemical Co.). Briefly, isolated 1×10^7 of PBMCs were solubilized on ice for 30 min in 1 ml of buffer containing 1% Triton X-100, 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, and protease inhibitor cocktail (Roche Diagnostics). Nuclear fragments were removed by centrifugation for 20 min at 15,000*g* at 4 °C. The supernatant of each sample was then subjected to ELISA Kit specific for human HO-1. The O.D. of each well at 450 nm was determined using an ELISA microplate reader (Molecular Devices, Menlo Park, CA).

3. Results

3.1. Hypoxia-induced gene expression in top alpinists

In order to investigate the genes which play an important role in the acclimatization of top alpinists who attack Mt. Everest, we searched for the genes induced by hypoxia in 12.7% hypoxic chamber by using whole genome DNA chips and compared their gene expressions with those of non-alpinists. As shown in Volcano plot (Fig. 1), 18 genes are significantly down-regulated and 22 genes are significantly up-regulated with top alpinists after 1 h stimulation with hypoxia in comparison with induction ratio of non-alpinists (folds < 0.67, or folds > 1.50, $P < 0.05$). As shown in Table 1, we detected the lowest ratio of hypoxia induced gene expressions (control/alpinist) in heme oxygenase 1 (HO-1) gene (2.14), BCL2-associated X protein (1.87), AT-hook transcription factor (1.73), and other 15 genes (Table 1, upper panel). We also detected the highest ratio of hypoxia induced gene expressions in V-set and immunoglobulin domain containing 1 (0.58), PTD016 protein (0.60), zinc finger protein 709 (0.61), and other 19 genes (Table 1, lower panel).

Among the induced genes from top alpinists, we focused on HO-1 because the ratio of the induction is lowest (2.14-folds) among whole genes investigated. In addition, it is well known that HO-1 play an important role in vasodilatation in hypoxia, suggesting that HO-1 play an pivotal role in the acclimatization of top alpinists. In order to further clarify whether the suppressed ratio of gene induction result from the reduced induction of HO-1 expression or the increased constitutive expression of HO-1 in top alpinists, we measured the protein expression of HO-1 from top alpinists and compare those from non-alpinists.

3.2. Heme oxygenase-1 (HO-1) is constitutively higher in the peripheral blood of top alpinists

We investigated HO-1 expression of peripheral bloods from another set of top alpinists (age = 47.4 ± 7.8, $n = 4$) and compared

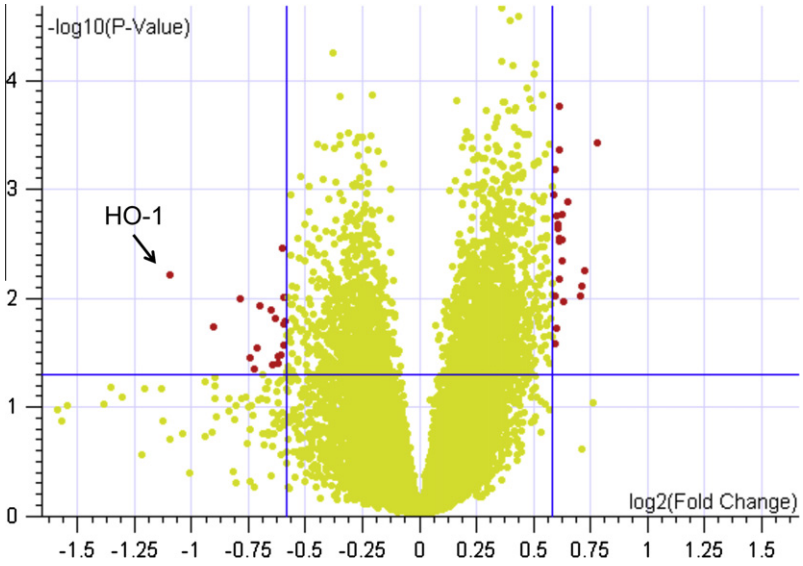

Fig. 1. Volcano plot. Eighteen genes are significantly down-regulated (left panel) and 22 genes are significantly up-regulated (right panel) with top alpinists after 1 h stimulation with hypoxia in comparison with induction ratio of non-alpinists (folds < 0.67, or folds > 1.50, $P < 0.05$). HO-1 is indicated by an arrow.

Table 1
Gene expression profiles.

Gene symbol	Description	Fold change (control/alpinist)	*P*-Value
Down-regulated genes			
HMOX1	Heme oxygenase 1	2.14	0.006
BAX	BCL2-associated X protein, transcript variant epsilone	1.87	0.018
AKNA	AT-hook transcription factor	1.73	0.010
PDLIM2	PDZ and LIM domain 2	1.68	0.036
CSTF2T	Cleavage stimulation factor, 3′ pre-RNA, subunit 2, 64 kDa, tau variant	1.65	0.045
TNKS2	Tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase 2	1.64	0.029
BAX	BCL2-associated X protein, transcript variant sigma	1.63	0.012
APH1A	Anterior pharynx defective 1 homolog A	1.57	0.013
NFATC1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	1.57	0.041
ERBB2	v-erb-b2 Erythroblastic leukemia viral oncogene homolog 2	1.55	0.015
AP2B1	Adaptor-related protein complex 2, beta 1 subunit	1.54	0.035
BRD2	Bromodomain containing 2	1.54	0.040
APAF1	Apoptotic peptidase activating factor 1	1.52	0.033
COPZ2	Coatomer protein complex, subunit zeta 2	1.51	0.003
C1orf83	Chromosome 1 open reading frame 83	1.51	0.027
TBX21	T-box 21	1.51	0.010
AKR1C3	Aldo–keto reductase family 1, member C3	1.51	0.017
VIT	Vitrin	1.50	0.016
Up-regulated genes			
CHD9	Chromodomain helicase DNA binding protein 9	0.67	0.001
MIA3	Melanoma inhibitory activity family, member 3	0.66	0.001
NXT2	Nuclear transport factor 2-like export factor 2	0.66	0.026
STCH	Stress 70 protein chaperone, microsome-associated, 60 kDa	0.66	0.009
ARID4B	AT rich interactive domain 4B	0.66	0.002
CHD7	Chromodomain helicase DNA binding protein 7	0.66	0.019
SMARCA5	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5	0.66	0.002
ATG5	Autophagy related 5	0.66	0.002
TMEM123	Transmembrane protein 123	0.65	0.003
PAQR3	Progestin and adipoQ receptor family member III	0.65	0.003
SGPP1	Sphingosine-1-phosphate phosphatase 1	0.65	0.007
CCAR1	Cell division cycle and apoptosis regulator 1	0.65	0.000
ZZZ3	Zinc finger, ZZ-type containing 3	0.65	0.000
ZNF44	Zinc finger protein 44	0.65	0.002
LACTB2	Lactamase, beta 2	0.65	0.003
PRKD3	Protein kinase D3	0.65	0.005
HBA1	Hemoglobin, alpha 1	0.64	0.011
SMCHD1	mRNA for KIAA0650 protein	0.64	0.001
MANEA	Mannosidase, endo-alpha	0.61	0.010
ZNF709	Zinc finger protein 709	0.61	0.008
LOC51136	PTD016 protein	0.60	0.006
VSIG1	V-set and immunoglobulin domain containing 1	0.58	0.000

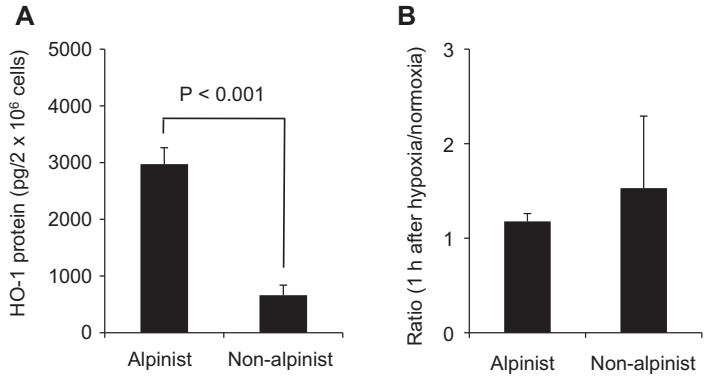

Fig. 2. Constitutive expression and induction of HO-1 in alpinists and non-alpinists. (A) Constitutive expression of HO-1 from top alpinists was significantly higher than that from non-alpinists (2973 ± 178 vs. 662 ± 662, $n = 4$, $P < 0.001$). (B) No difference in the ratio of HO-1 expression in hypoxia/normoxia, 1 h after the hypoxic stimulation between alpinist and non-alpinists (1.178 ± 0.083 vs. 1.530 ± 0.762, $n = 4$).

with those of non-alpinists (age = 47.75 ± 7.8, $n = 4$). As shown in Fig. 2A, constitutive expression of HO-1 from top alpinists was significantly higher than that from non-alpinists (2973 ± 178 vs. 662 ± 662, $n = 4$, $P < 0.001$). However, we failed to detect any difference in the ratio of HO-1 expression in hypoxia/normoxia between alpinist and non-alpinist (1.178 ± 0.083 vs. 1.530 ± 0.762, $n = 4$, Fig. 2B). The data suggest that top alpinists express enough amount of HO-1 before hypoxic stimulation so that they no longer need to express more HO-1 to respond the hypoxia while non-alpinists constitutively express low amount of HO-1 and induce the expression of HO-1 upon hypoxic stimulation to adapt hypoxic condition. The data is compatible with DNA microarray that showed the reduced induction of HO-1 gene expression of top alpinists upon hypoxic stimulation.

3.3. Chronic expression of HO-1 to hypoxia for one top alpinist

In order to investigate how top alpinists keep their high constitutive expression of HO-1 in the peripheral blood, we investigated the longitudinal follow up of one top alpinist by serial sampling of blood. As shown in Fig. 3, HO-1 expression is clearly associated with duration of the time after each descent. We could detect 3699 pg of HO-1/2 $\times$ 10^6 cells, as shown by the 3rd black cross in Fig. 3, eight days after the peak of Lobche East, 6109 m in altitude and 4 days after the descent, in May 2010. The data suggested that HO-1 expression is extremely high right after the descent from high altitude mountain.

The data also showed that HO-1 expression gradually decreased after descent as shown by the first cross in Fig. 3, 47 days after the descent of Mount Cho-Oyu, 8201 m in altitude. Paradoxically, the data still showed a stable of expression of HO-1, 98 days after the descent of Mount Kenya, 5109 m in altitude, as shown by the 2nd cross in Fig. 3.

These data imply that HO-1 expression decreases more rapidly just after the descent but reached to a plateau level several months after the descent. This data is compatible with the fact that top alpinists showed higher constitutive expression of HO-1 than non-alpinists as shown in Fig. 2.

4. Discussion

In order to clarify the mechanism of acclimatization of top alpinists who attack Mount Everest, we investigated the hypoxia-induced gene expressions specific for top alpinists using microarray analyses, revealing that HO-1 expression is significantly higher in the blood of top alpinists compared with non-alpinists.

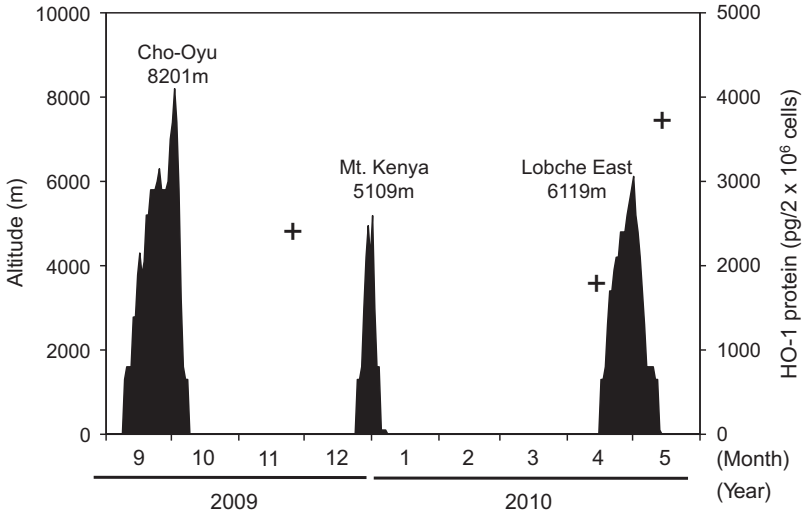

Fig. 3. Longitudinal follow up of one top alpinist by serial sampling of blood. The climbing of the mountain is displayed by the altitude and duration in the graph. HO-1 protein expression in peripheral blood is indicated by black cross.

Serial examinations of HO-1 in one top alpinist demonstrated that the higher expression of HO-1 is maintained in high-level for several months after the descent from top mountains.

4.1. HO-1 may compensate for the hypoxia-induced vasoconstriction

We discovered that HO-1 expression is constitutively high with top alpinists by means of microarray analysis. The initial microarray analysis of the genes specific for top alpinists vs. non-alpinists indicated that HO-1 showed the most suppressed induction of gene expressions upon 12.7% hypoxia. However, the analysis of HO-1 protein expression with top alpinist strongly suggested that HO-1 protein is maintained high with top alpinists while HO-1 protein is constitutively low with non-alpinists, which is induced upon the stimulation of hypoxia.

In general, the blood vessels physiologically constrict in response to hypoxia [8]. It is well known that the endothelial cells and surrounding tissues secrete a lot of chemical mediators to dilate the blood vessels such as NO, prostaglandin I2 and other mediators in order to maintain the tissue circulation [9]. Empirically, top alpinists use NO inducer, Viagra, to treat the pulmonary hypertension to reduce the pulmonary pressure in high altitude. However, in our study, we could not detect any significant alterations in eNOS gene expression in top alpinists by microarray analysis, suggesting that NO play a less significant role in the vasodilatation of pulmonary artery in high altitude. Instead of that, CO may play a pivotal role in the vasodilation of pulmonary artery since HO-1 catalyzes heme into biliverdin, iron, and CO [10]. HO-1 is an inducible isoform in response to stress such as oxidative stress, hypoxia, heavy metals, cytokines, etc. HO-1 cleaves heme to form biliverdin, which is subsequently converted to bilirubin by biliverdin reductase, and CO, a putative neurotransmitter [11]. CO is one of the potent vasodilators bio-synthesized in the body in addition to NO [12].

However, the physiological role of CO is not well understood to date. This is the first report to elucidate the physiological role of CO in the vasodilatation in high altitude. In this context, the drug that induces HO-1 expression or enhances CO production in endothelial cells may help to improve the pulmonary hypertension in high altitude. Furthermore, bilirubin, the other by-product of HO-1, plays an important role in the protection of tissue damages caused by oxidative stress with hypoxia. In general, hypoxia not only induces the ischemic damage but also the oxidative stress in the damaged tissues [13,14]. Thus it is reasonable to speculate that endothelial cells express HO-1 in response to the critical conditions induced by severe hypoxia, which is associated with oxidative tissue damages.

4.2. TOP alpinist may have the memory of hypoxia by constitutively high expression of HO-1 in lymphocytes

The animal has two kinds of memory systems: brain and lymphocytes. The brain maintains the memory by sustaining the neuronal circuits connected by synapsis [15].

The contents of memory is thus stored in the specific neuronal circuits, as either short term or long term memories, both of which is regulated by the electrical activities of synaptic membrane [15,16]. However in lymphocytes, specific clones of lymphocyte correspond to specific antigens are maintained, sometimes for life long in such cases as measles or mumps [16]. The repertoire of the immune system is comprised of a lot of specific lymphocytes, which constitute the total immune memory of the body [16]. In the system of the lymphocytes, the context of memory is based on the collection of antigens and self-recognition molecules expressed on cell surface [16]. In this paper we described a novel type of memory system for the first time other than the neuronal memory or cell memory of lymphocyte. We discovered here that lymphocyte can memorize the hypoxia for several months. Although we cannot exclude the possibility that specific lymphocytes which express high amount of HO-1 maintained in the peripheral blood of top alpinists for several months after the descent, the cell number of specific lymphocytes is too insignificant to be represented in ELISA assay from peripheral blood. In addition, the memory lymphocytes usually correspond to specific antigens, however antigens cannot express the stimulation of hypoxia. Thus, the memory of hypoxia stored in lymphocytes of top alpinists cannot be expressed by the conventional memory functions of lymphocytes. Thus, it is a huge surprise that lymphocyte maintain the memory of hypoxia irrespective of the antigens. We therefore hypothesize here that lymphocytes have another memory function for this kind of life threat to survive in extreme environments such as Mount Everest. We suggest that the sustained alteration of gene expression in some specific genes can be attributable to the sustained adaptation for an extreme environment such as severe hypoxia. Since the lifespan of lymphocytes, except for memory lymphocytes, is too short to maintain the memory of several months, the memory of HO-1 expression in lymphocytes is transferred to their offspring for several months. This novel type of memory system is quite different from those of nervous system or the conventional memory lymphocytes.

As we have known the phenomenon that alpinists who reached certain altitude once are more prone to acclimatize faster in the second attack. This novel type of memory may help to explain it and to prevent AMS. Further studies are needed to elucidate the molecular mechanism underlining this novel type of memory system.

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