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Mechanisms in the Pathophysiology of Diving

Bubble Formation and Cardiovascular Effects of Simulated Diving, Exercise-Induced Muscle Injury and Hyperbaric Oxygen Preconditioning

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ISBN 978-82-326-0086-1 (printed version)
ISBN 978-82-326-0087-8 (electronic version)
ISSN 1503-8181

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Printed by Skipnes Kommunikasjon as

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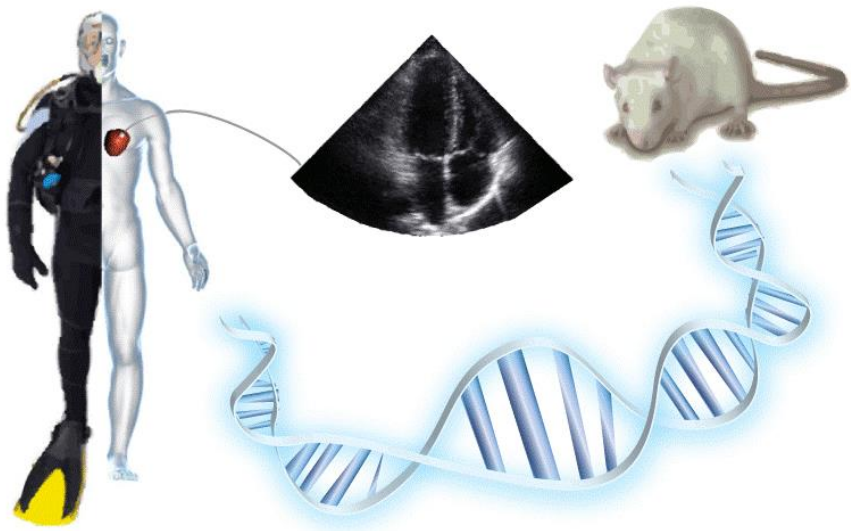

A Cotutelle Agreement providing joint supervision of a doctoral level degree between the

Norwegian University of Science and Technology and the University of Texas Medical Branch

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Mekanismer i Utvikling av Trykkfallsyke etter Dykking

Millioner av mennesker verden over driver med dykking. Mennesket er ikke tilpasset et liv under vann, og i løpet av et dykk usettes kroppen for flere ulike stressfaktorer som kan påvirke helsen i negativ retning. Under et dykk vil økt omgivelsestrykk komprimere pustegassen, slik at økte mengder gass løser seg i kroppens blod og vev. I de fleste dykk blir luft, som i hovedsak består av nitrogen og oksygen, brukt som pustegass. Nitrogenet hoper seg gradvis opp i kroppen ved økende dybde og varighet av et dykk, og under oppstigning fra dykket reduseres omgivelsestrykket og den økte mengden oppløst nitrogen kan ved for rask oppstigning danne bobler i kroppen. Det er allment akseptert at disse boblene er årsak til trykkfallsyke, en svært alvorlig tilstand som kan føre til permanent skade og død. Til tross for at dykkere følger dagens gjeldende retningslinjer for hva som anses som trygg dykking, så kan de likevel risikere å utvikle trykkfallsyke av ukjente årsaker. Nyere forskning tyder på at utviklingen av trykkfallsyke er en svært kompleks prosess, og for å kunne forhindre utvikling av trykkfallsyke så må man forstå denne prosessen. Man tror at stresset fra høye oksygennivå under dykking kan sette i gang skadelige prosesser som kan bli ytterligere forverret ved dannelse av bobler etter dykking. I tillegg til oksygen og gassbobler, så spiller fysisk aktivitet en viktig medvirkende faktor i utvikling av trykkfallsyke, og observasjoner kan tyde på at muskelskade på grunn av fysisk aktivitet kan øke risikoen. Mekanismene for utvikling av trykkfallsyke og hvilke faktorer som spiller en viktig medvirkende rolle er fortsatt uklare.

Hensikten med denne studien var å undersøke hvordan fysisk aktivitet før dykking kan påvirke bobledannelse, og hvordan høye nivåer av oksygen i pustegassen og bobledannelse kan påvirke risikoen for trykkfallsyke.

For å simulere dykking, ble rotter plassert i et trykkammer der omgivelsesluften ble komprimert. Trykket ble så gradvis redusert, og mengden sirkulerende gassbobler ble målt i hovedpulsåren og lungeåren ved hjelp av ultralydteknikk. Prøver fra blod og vev ble analysert.

Vi fant at muskelskade fra fysisk aktivitet før dykking ikke ser ut til å øke bobledannelse eller redusere overlevelse etter dykking (paper I). Vi fant videre økt aktivitet av gener og høye nivå av proteiner i blodåreveggen og blodet som tyder på at oksygenstress under dykking kan føre til en skadelig tilstand med økt betennelse i blodårene, økt aktivitet av immunforsvaret og økt risiko for dannelse av blodpropp (paper II). Vi fant også en klar sammenheng mellom økte mengder sirkulerende bobler etter dykking og økte nivå av stressmarkører for vevsskade i hjertet, men at dyr som hadde pustet høye oksygennivåer en stund før dykket hadde lavere uttrykk av stressmarkører til tross for samme mengde med bobler (paper III).

Funnene i denne studien viser at stress fra et høyt oksygennivå under dykking setter i gang potensielt skadelige prosesser som kan forverres ved bobledannelse etter dykking, men at forbehandling med oksygen før et dykk kan potensielt forhindre utvikling av trykkfallsyke og vevsskade. Ved å forstå hvordan fysisk aktivitet, høye nivå av oksygen og bobledannelse kan påvirke utviklingen av trykkfallsyke, kan man utvikle bedre prosedyrer for å redusere både akutte skader og langtidsskader forårsaket av dykking.

Kandidat: Arve Jørgensen
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Hovedveiledere: Professor Alf O. Brubakk og Assisterende Professor Philip P. Foster
Biveiledere: Professor Ulrik Wisløff og Forsker Ingrid Eftedal

Ovennevnte avhandling er funnet verdig til å forsvares offentlig
for graden Doctor Philosophiae
Disputas finner sted i Auditoriet MTA, MTFS
TIRS.dag dato 25.02, *kl.* 12.15

Mechanisms in the Pathophysiology of Diving

Bubble Formation and Cardiovascular Effects of Simulated Diving, Exercise-Induced Muscle Injury and Hyperbaric Oxygen Preconditioning

by

Arve Jørgensen

Medicinae Doctor, Trondheim, Norwegian University of Science and Technology, 2007

Submitted to the Department of Circulation and Medical Imaging at the Norwegian University of Science and Technology, and to the Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine at the University of Texas Medical Branch, in partial fulfillment of the requirements for the degree of Philosophiae Doctor in Medical Technology

July 2013

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July 15, 2013

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Executive Summary

Currently, there are millions of recreational divers and underwater workers worldwide. Although humans cannot live naturally underwater, certain physiological mechanisms can adapt the human body so that it may explore the underwater environment. During diving, increased pressure surrounding the body leads to high levels of different breathing gasses, for example nitrogen (N_2) and oxygen (O_2). During a dive, N_2 accumulates in the tissues. On ascent to the surface, the ambient pressure decreases (decompression) and bubbles may form due to excess N_2. The general opinion is that these gas bubbles are the cause of the clinical manifestations termed decompression sickness (DCS), which is a major health risk to divers. However, even when dives are performed in compliance with today's procedures, which are commonly accepted as safe, bubble formation and DCS still occur. Despite many years of study, there is still limited knowledge of the mechanisms by which bubbles form and how they are involved in DCS development. Previous studies have shown that physical exercise and exposure to increased levels of O_2 in the breathing gas may have a significant impact, in both reducing and increasing bubble formation and DCS risk. It is believed that exercise-induced muscle injury may enhance bubble formation and that high levels of O_2 during diving may enhance DCS risk. In contrast, exposure to high O_2 levels prior to diving may reduce DCS risk. However, little is known about the mechanisms behind these observations.

In the present study, we investigated whether physical exercise may lead to enhanced vascular bubble formation (paper I), how diving-induced bubbles may initiate DCS (paper II and III), and how exposures to high levels of O_2 in the breathing gas may increase or reduce injury from diving (paper II and III). To simulate diving, rats were exposed to a high ambient pressure in a hyperbaric chamber, then a rapid pressure reduction to induce bubble formation. Immediately after the dive, the rats were anesthetized and the amount of vascular bubbles was evaluated by ultrasonic imaging. Blood and tissues were collected and analyzed. The first findings were that eccentric exercise-induced skeletal muscle injury made prior to diving did not seem to affect vascular bubble formation (paper I). Thus, our results do not endorse a link between exercise-induced muscle injury and vascular bubble formation. Analysis of the effects of diving on the early genetic responses in vascular tissue after diving, using full genome gene expression profiling (paper II), found that high levels of O_2 in the breathing gas are likely to be an important contributor in DCS etiology. In addition, it was noted that enhanced coagulation and inflammation may be involved in DCS development. Finally, we found that high bubble loads after diving led to elevated cardiac stress-marker levels (paper III). Further, it seemed that that preconditioning with hyperbaric O_2 prior to diving prevented cardiac injury induced by vascular gas bubbles, which was indicated by an attenuated rise in stress-marker levels. Thus, the factors that were identified as being important in DCS development included exposure to high levels of O_2 prior to and during the dive, as well as high bubble loads occurring post-dive.

The main implications of the present study are that eccentric exercise causing muscle injury prior to diving does not seem to increase vascular bubble formation after diving and that exposure to high levels of O_2 during diving seem to be involved in the adverse effects of diving. However, the adverse effects may be prevented by prophylactic exposure to high O_2 levels prior to the dive. Understanding how physical exercise and exposure to high levels of O_2 affect the risk of bubble formation and DCS is important, as it will facilitate preventive measures to help increase the safety of divers. Furthermore, it is our hope that this study may yield new and broader insight into the mechanisms of bubble formation and the pathogenesis of DCS after diving.

Acknowledgments

The present PhD study was carried out during the years 2009 – 2013 at the Department of Circulation and Medical Imaging, Norwegian University of Science and Technology (NTNU), Norway, and at the Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Texas Medical Branch (UTMB), Texas, USA.

First of all I would like to thank my supervisor **Alf O. Brubakk**. In 2002, as a medical student, Alf introduced me to research and the fascinating world of diving physiology. He has enthusiastically shared his great knowledge about diving medicine and life in general. He has been open minded for new and radical ideas, which has resulted in many interesting, exciting and educational experiments. He has been a good supervisor; fair, supportive and a good friend.

I would also like to thank my Cotutelle supervisor **Philip P. Foster** who introduced me to aerospace medicine as a Fulbright Fellow at UTMB, in the years 2009-2010. He has contributed significantly to this work with his great knowledge about diving and aerospace medicine throughout this project. His unique insights in the mechanisms of bubble formation and DCS development and all the fruitful discussions have made me understand that the mechanisms in the pathogenesis of diving are very complex. I really appreciate his warm welcome and for taking really good care of me during my exchange year in USA.

I am greatly indebted to my co-supervisors **Ulrik Wisløff** and **Ingrid Eftedal**. Ulrik for his critical and to-the-point advice as well as his exceptionally good sense of what is important to consider in planning experiments, writing articles and the thesis. His unique ability to see the overall importance of the projects and to put things into perspective is highly appreciated. Ingrid for the invaluable help with all parts of this work, which include applications, protocols, articles, this thesis, as well as planning and conducting the experiments, collecting and analyzing the blood and tissues, as well as frequent meetings and discussions that finally led to the completion of this thesis. Without her help, this work would have been considerably more difficult. I hope for continued good cooperation in the future.

Thank you to the Medical Faculty and NTNU that gave me the opportunity and support to start doing research during medical school. Special thanks go to **Vegard J. Berge**, my good friend and research fellow during medical school. I will never forget all the fun times we had together.

Also highly appreciated were all the hours in the laboratory with **Marianne B. Havnes**, the advice given by and discussion with **Andreas Møllerløkken, Svein Erik Gaustad, Christian Gutvik** and **Erney Mattsson**. Thanks to **Ragnhild Røsbjørgen** for immunohistochemistry and ELISA, **Toril Rolfseng** for gene expression analyses, **Arnar Flatberg** for statistics and figures, **Gøran Paulsen** for his expert input about eccentric exercise and heat shock proteins, **Astrid Hjelde** for statistics and laboratory techniques, **Arnfinn Sira** for technical assistance with the equipment, **Anne Berit Johnsen** for confocal microscopy, **Håvard Knappskog** for the software enabling automatic control of the diving chamber, **Rune Haaverstad** for helping me with applications, the staff at the NTNU Animal Department for their professional handling of the animals, the Department of Clinical Chemistry at St. Olav's University Hospital for analysing blood samples, the Proteomics and Metabolomics Core Facility (PROMEC) at NTNU for help with gene expression profiling and protein analyses, the FUGE Writer's Club, and to **Edmund Søvik** who gave me the opportunity and time to finally write this thesis. Thanks to all the

fantastic colleagues and friends here at the Department of Circulation and Medical Imaging, NTNU, and at St. Olav's University Hospital.

Thanks to the US-Norway Fulbright Foundation for the Fulbright Flagship Grant and the possibility to do one year of research in Texas, USA. This fulfilled my dream of studying and researching aerospace medicine. Thanks also to the Norwegian Space Centre for their support and help in the application process. I had a fantastic year in Texas and want to thank **Richard Jennings** and **James Vanderploeg** as well as **Philip P. Foster** for the opportunity to attend grand rounds, journal clubs, courses, seminars and lectures in Aerospace Medicine at UTMB, Baylor College of Medicine, Universities Space Research Association, National Buoyancy Laboratory and Wyle Laboratories. Thanks to **Johnny Conkin** and **Joseph Dervay** for inspiring discussions at NASA, **Michael Gernhardt** for sharing his knowledge about bubble growth, **Leslie Elgin** and **Gene Felter** for administrative help, my good friends **Chris** and **Wendy Cleboski** for all the fun times together when not doing research, and Philip's family, **Dominique** and **Roman**.

I wish to thank my parents, **John** and **Anita** and my brother, **Frank,** for your support and for stimulating my curiosity and interest in science, my best friends, **Kristian**, **Svein Erik** and **Kai Arne** who always support and stand up for me during good and bad days and Åsne's parents **Geirmund** and **Anne** for your help and support.

Finally, to the most important person in my life, I would like to thank my beloved fiancé **Åsne**, for always being there for me, for your support, inspiration and patience, and for staying with me in Texas. You and our two lovely children **Ida** and **Jon** have taught me what is really important in life.

Arve Jørgensen, Trondheim, September 2013.

The study was supported by grants from:

FUGE Midt-Norge

Kontaktutvalget, St. Olavs University Hospital/NTNU

Samarbeidsorganet HMN-NTNU

Statoil, ExxonMobil and Gassco under the Competence Program Diving

The Norwegian Air Ambulance Foundation

US-Norway Fulbright Foundation for Educational Exchange (Fulbright Flagship Grant)

Contents

Abbreviations

AGE:	Arterial gas emboli
ANP/BNP:	Atrial/brain natriuretic peptide
ATA:	Atmosphere absolute
CNS:	Central nervous system
CO_2:	Carbon dioxide gas
cTnT:	Cardiac troponin T
DCI:	Decompression illness
DCS:	Decompression sickness
DNA:	Deoxyribonucleic acid
ECLIA:	Electrochemiluminescence immunoassay
ELISA:	Enzyme-linked immune-sorbent assay
HIF-1α:	Hypoxia inducible factor-1alpha
HBO:	Hyperbaric oxygen
HBO-PC:	Hyperbaric oxygen preconditioning
HSP:	Heat shock protein
IHC:	Immunohistochemistry
NF-κB:	Nuclear factor-kappa B
MIAME:	Minimum information about a microarray experiment
MPs:	Microparticles
mRNA:	messenger ribonucleic acid
N_2:	Nitrogen gas
NO:	Nitric oxide
***Nppa/Nppb*:**	Natriuretic peptide precursor a/b
O_2:	Oxygen gas
PAI-1:	Plasminogen activator inhibitor-1
pN_2/pO_2:	partial pressure of nitrogen/oxygen gas
qRT-PCR:	real-time reverse transcription polymerase chain reaction
ROS:	Reactive oxygen species
TNF-α:	Tumor necrosis factor-alpha
VGE:	Venous gas emboli

Definitions

Decompression: reduction of the ambient pressure.

Decompression Illness: systemic or local clinical manifestations that may occur after decompression. It includes decompression sickness, arterial gas embolism and barotrauma.

Decompression Sickness: a disease caused by gas bubbles formed by excess inert gas coming out of solution in the tissues and vessels.

Eccentric Exercise: lengthening contractions of muscles, where muscles elongate while under tension due to an opposing force greater than that generated by the muscles.

Gene Expression: production of RNA from DNA in the biochemical process of transcription. Proteins are made by further translation of mRNA into polypeptides. The level of mRNA at a given time and place reflects the cellular responses to different stimuli.

Gene Expression Profiling: simultaneous measurement of the expression of all genes in a particular genome, usually made by microarray analysis. Details about microarray analysis can be found on the homepage of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Hyperbaric: increased ambient pressure.

Hyperoxia: increased inspiratory partial pressure of oxygen gas (pO_2).

kPa: kilopascal. Pascal is the SI derived unit of pressure where 1 kPa = 1000 pascal. Pressures in diving are often given in Bar, ATA or psi where 100 kPa = 1 Bar, 1 ATA=101,325 kPa = 1,01325 Bar = 14,696 psi.

Oxidative Stress: imbalance between the production of reactive oxygen species (free radicals) and the body's antioxidant defenses. Oxidative stress can damage tissues and disrupt normal cellular signaling.

Saturation Dive: in which the tissues absorb the maximum amount of gas at a given depth and the amount of gas dissolved in the tissues is at equilibrium with the breathing gas. Increased depth will increase tissue gas uptake, additional time at that depth will not.

Simulated Diving: by increasing the ambient pressure in a pressure chamber, the individual placed in the chamber can be exposed to the same partial pressures of gases corresponding to actual diving.

Preface

This study was carried out during the years 2009 - 2013 at the Department of Circulation and Medical Imaging, Norwegian University of Science and Technology (NTNU), Trondheim, Norway, and the Department of Internal Medicine, Division of Pulmonary and Critical Care, University of Texas Medical Branch (UTMB), Texas, USA. It was formally based on a Cotutelle Agreement providing joint supervision of a doctoral level degree between NTNU and UTMB. This agreement intends to increase the universities' level of internationalization and to create collaboration between NTNU and UTMB. The goal of such an agreement is to educate the candidate at two institutions to raise the quality of both the training and the research in the academic field in question, and should provide both institutions with added value.

The candidate was enrolled in both institutions, including one year as a Fulbright Fellow joining UTMB's program in the field of Aerospace Medicine, Decompression Physiology and Biology, following a decision made on 2nd of November 2009.

It is a requirement that the entire dissertation and its attachments are made available in the public domain.

The papers listed below will be referred to by their roman numerals in this study.

List of papers

I. Exercise-induced myofibrillar disruption with sarcolemmal integrity prior to simulated diving has no effect on vascular bubble formation in rats.

Arve Jørgensen, Philip P. Foster, Ingrid Eftedal, Ulrik Wisløff, Gøran Paulsen, Marianne B. Havnes and Alf O. Brubakk.

Eur J Appl Physiol. 2013 May;113(5):1189-98. doi: 10.1007/s00421-012-2537-z. Epub 2012 Nov 6.

II. Early genetic responses in rat vascular tissue after simulated diving.

Ingrid Eftedal, Arve Jørgensen, Ragnhild Røsbjørgen, Arnar Flatberg, and Alf O. Brubakk.

Physiol Genomics. 2012 Dec 18;44(24):1201-7. doi: 10.1152/physiolgenomics.00073.2012. Epub 2012 Nov 6.

III. Effects of hyperbaric oxygen preconditioning on cardiac stress-markers after simulated diving.

Arve Jørgensen, Philip P. Foster, Alf O. Brubakk and Ingrid Eftedal.

Submitted to Physiological Reports.

Contributions

All authors contributed to drafting the manuscripts or revising them critically, and have approved final versions of the manuscripts.

Paper I: A.J., P.P.F., G.P., I.E. and A.O.B conception and design of research; A.J., U.W., G.P., M.B.H. and I.E. collection, analysis and interpretation of data.

Paper II: I.E., A.J., A.F., and A.O.B. conception and design of research; I.E., A.J., and R.R. performed experiments; I.E., A.J., R.R., and A.F. analyzed data; I.E. and A.J. interpreted results; I.E. prepared figures.

Paper III: A.J., P.P.F., A.O.B. and I.E. conception and design of research; A.J. and I.E. collection, analysis and interpretation of data.

1 Introduction

The purpose of this baromedical study is to understand better the mechanisms that initiate and prevent decompression sickness (DCS), which poses a major risk of injury during diving, caisson work, aviation, spaceflight and emergency evacuation from pressurized vessels. The study investigates the role of exercise-induced muscle injury as a possible mechanism of gas bubble formation during diving (paper I), how diving-induced oxidative stress and bubbles affect gene expression and markers of vascular function (paper II), and cardiac stress/injury (paper III). An understanding of DCS etiology is important for DCS prevention and treatment to avoid acute and long-term injury.

1.1 History of Baromedicine

"A fundamental human urge has always been to understand the mysteries of strange new worlds, to boldly go where no one has gone before"(228).

Human exposure to the challenges of the underwater world extends back at least 2000 years, where Japanese art and literature tells the story of the Ama people who practiced breath-hold diving (155). The first historical mention of diving without breath-holding is attributed to Alexander the Great, who descended in a diving bell at the siege of Tyre (68). With the invention of self-contained breathing apparatus (scuba) in 1864 by the Frenchmen Rouquayrol and Denayrouze and later, in 1943, the development of an open circuit aqualung by Jacques Cousteau and Emile Gagnan, new possibilities emerged for the exploration of the fascinating and beautiful underwater world (75, 221).

Humans are not readily adapted to live in and explore the underwater or space environs. The development of caisson work in the 18th and 19th century provided some of the first observations of the effects of raised ambient pressure on humans. In 1782, the divers of the UK Royal Engineers reported musculoskeletal pain when trying to salvage the *Royal George* which had sunk at Portsmouth (68). In 1841 Triger, a French mining engineer, reported pain during articulation of the limbs and muscle cramps

in coal miners after leaving the mine shaft, while four years later, the two French physicians, Pol and Watelle, recognized that recompression ameliorated the symptoms (233). In 1878, Bert provided the first clues as to pathophysiology of hyperbaric induced disease, demonstrating that decompression of animals produced bubbles in the blood and that administration of oxygen would resolve them (179). Sir Robert Boyle (1627-1691) was the first to observe bubble formation *in vivo* in a viper's eye during decompression to low ambient pressures in a hypobaric chamber. The invention of passenger carrying balloons and aircraft travelling at high altitude brought the clinical syndrome of pain, as a result of decompression, into the realm of aerospace medicine (233). Windmills, kites, parachutes and the rocket were early inventions brought about for the pursuit of human flight. The first flight of a heavier-than-air powered aircraft, piloted by the Wright Brothers on December 17th 1903, launched the base for an explosive growth in aviation. On April 12th, 1961, the first flight orbit of the Earth by Yuri Gagarin opened the era of human space flight. Man's fascination with travelling into and exploring space was cemented by the NASA Apollo moon landing on July 20th 1969, when Neil Armstrong and Edwin Aldrin Jr. became the first two humans to walk on a heavenly body other than the Earth (54).

1.2 Baromedical Challenges

Why should we invest time and effort to understand how extreme environmental conditions experienced in diving affects the organism?

Diving is a relatively novel challenge to human physiology. It is increasingly popular and there are currently millions of recreational divers and underwater workers worldwide. In addition, commercial diving is necessary for the offshore production of oil and gas, our society's most important energy sources. Even when performed in compliance with accepted safety procedures, diving is not completely without risk.

The major risk of injury associated with diving is the formation of intra- and extra-vascular gas bubbles on reduction of the ambient pressure (decompression) during ascent to the surface after completion of a dive (208, 252). These gas bubbles initiate adverse pathophysiological, systemic responses by mechanical distortion of tissue, vascular obstruction and biochemical effects, which give rise to the clinical manifestations termed DCS. The terminology describing the clinical manifestations that may occur after decompression can be confusing. However, it seems to be agreed that decompression illness (DCI) encompasses DCS, arterial gas embolism (AGE) and other gas-related forms of decompression induced barotraumas (105). DCS is believed to be caused by the formation of bubbles evolved from gases dissolved in tissues of the body following decompression (233). AGE can occur when expanding gas stretches and ruptures alveolar capillaries (252), following right-to-left cardiac shunts (180, 248) and when large amounts of VGE overcome the pulmonary capillary filter (27, 254). The proportion of cases of DCI attributable to AGE is rare compared to the incidence of DCS, which is thought to constitute most of the cases. This study investigates the mechanisms in the pathophysiology of DCS, and throughout this thesis the term DCS (and not DCI) will be used about the clinical manifestations that may occur after diving.

The epidemiology, diagnosis and treatment of DCI and DCS have been extensively described elsewhere by Vann et al. (252). Manifestations of DCS comprise a collection of signs and symptoms ranging from musculo-skeletal discomfort to debilitating neurological and cardiovascular damage. A large number of circulating bubbles can cause cardiopulmonary DCS and chokes with cough, dyspnea, pulmonary edema, shock and death (186). Hypobaric exposure (e.g., aviation and spaceflight) can also cause DCS, and prevention of DCS is important. Within the past few years, NASA has invested considerable resources to prevent and manage DCS, as there is a growing interest, development and activity in exploring space both through national space agencies and private companies. In the near future, the space tourism industry will probably emerge and there may be an increase in humans traveling in space. There are also plans for returning to the Moon and to explore Mars. The purpose of this study is to

investigate the effects of diving and vascular bubble formation to better understand the mechanisms that initiate DCS. It is important that recreational and work activities can be carried out with a minimal risk of DCS and that physicians have enough expertise in this field to be well prepared to handle any health problems that may occur.

1.3 Mechanisms of Bubble Formation

William Henry (1775-1836) found that the amount of a gas in a solution varies directly with the partial pressure of that gas over the solution (233). This relationship follows Henry's Law and is of great importance during hyper- and hypobaric exposures. Increased ambient pressure will lead to the accumulation of dissolved inert breathing gas (nitrogen and occasionally helium) in the body's tissues and the amount of dissolved gas will increase as the gas pressure increases until tissue saturation is reached (252). When the ambient pressure changes once again, the gas in the tissues will always try to find equilibrium. Therefore, during and after decompression from hyperbaric to normobaric pressure in diving, or when moving from normobaric to hypobaric pressure in aviation or extravehicular activity in space ("spacewalk"), excess dissolved gas (supersaturation) will diffuse out from peripheral tissues and into the blood circulation, travel to the lungs and leave the body in the expired air. If the excess supersaturated gas is "large enough", the dissolved gas will evolve from solution and form bubbles. The probability of bubble formation will increase as supersaturation augments with reduced ambient pressure (233).

Bubble formation is considered to be the initiating cause of DCS (191, 247), and so preventing the formation of bubbles can avoid DCS. To minimize the risk of bubble formation and DCS after diving, decompression procedures and tables have been developed. The procedures are based on conservative calculations from numerous mathematical models and measurements of circulating bubbles and symptoms of DCS after diving (247). However, diving within the limits of today's diving tables is no guarantee of protection from DCS (252). Calculations in the commonly used decompression models are usually based on the depth and duration of the dive. However, there are other important factors to consider that may

affect the DCS outcome, for example, the effect of altered surrounding temperature, immersion, the degree of physical activity at different stages of a dive, the number of preformed bubble nuclei in the body, *de novo* bubble formation and biochemical mechanisms (61, 76, 79, 208, 247, 252). The complexity of the processes involved in bubble formation and initiation of the pathways that ultimately lead to DCS are not completely understood and are simplified substantially in the following sections of the study.

1.3.1 Micronuclei Hypothesis

Numerous experiments and models indicate that bubbles grow from preformed gaseous micronuclei (5, 70, 76, 247, 253, 280, 281) and/or they may form directly (*de novo*) from cavitation processes in the body (32, 110, 112, 116, 173-175). These bubbles may form and grow in areas such as crevices and cavities that are hydrophobic and/or in areas that are hydrodynamic or exposed to great mechanical pressure (110, 247).

In 1670, Robert Boyle observed that bubbles formed at certain points on surfaces in liquids after decompression. In the early 1900s, Hill and Twort (114) further discovered that bubbles will not form in supersaturated solutions unless "points" were given for the bubbles to form on. Harvey and colleagues (110) observed that if a supersaturated liquid comes into contact with dry surfaces, bubbles will arise from points were small gas masses or gas nuclei remain sticking to the surface. However, if the surface had been first boiled in water to remove its air films and then exposed to the supersaturated liquid, not a single bubble would appear. They suggested that the sticking of gas nuclei to a surface seems to be a matter of contact angles between the gas nuclei and the liquid surroundings. In fact, inside hydrophobic surface cracks or acute angled cavities, gas nuclei could probably form in liquids without any supersaturation and may remain stable in the cracks for indefinite periods of time (109, 110, 115, 150).

Brubakk et al. (25) suggested that micronuclei may be stabilized and form in lipid-rich areas on the inside of the blood vessels. In fact, models of nucleation of bubbles demonstrate that hydrophobic

crevices in the vasculature may act as possible sites for bubble formation (35, 37). Bubbles would be expected to grow under decompression until they emerge from the crevice and are swept away by the blood stream. Subsequently, bubbles may be generated at the same site and also seed into the blood (35-38). Based on the idea that liquid-surface interfaces could contain populations of stable nuclei, Evans and Walder (70) published a study in Nature indicating that such micronuclei were present in living organisms at all times and that the number present at any one time was dependent on a balance between the rate of spontaneous formation and elimination. By removing a substantial number of these nuclei in shrimps using high hydrostatic pressures prior to diving, they reduced decompression-induced bubble formation dramatically. Vann et al. (253) performed experiments to demonstrate that it was likely that preformed nuclei also exist in higher species such as rats.

Experiments indicate that for spontaneous or *de novo* formation of bubbles in homogeneous liquids at rest to occur without preformed micronuclei, the liquid has to be decompressed from pressures higher than 100-1000 ATA (110). However, under certain conditions, further experiments implied that bubbles could form directly from cavitation processes in organisms following relatively moderate decreases in barometric pressures, similar to those experienced during decompression in diving and altitude exposures (106, 110, 116, 265, 268). Several mechanisms could explain how micronuclei form spontaneously from cavitations, including high energy particles, stimulation with ultrasound, turbulent flow, random nucleation and mechanical stress (32, 41, 110, 112, 116, 269). Even though Harvey et al. (110) could not directly demonstrate *de novo* bubble formation, they suggested that bubbles could theoretically form *de novo* in animal tissues by a huge decrease in local hydrostatic pressure. The tearing of a liquid requires negative pressures as high as 150 ATA before the liquid breaks, leaving a cavity. However, if the pressure develops in a very short time interval in a very small area in biological systems, local pressures high enough to tear the liquid could appear. In a number of animal experiments, where preformed nuclei were first removed prior to decompression, bubbles could be created *de novo*, possibly due to enormous negative mechanical pressures in small areas in tissues that were pulled apart, cut and

crushed. Harvey et al. (110) and Whitaker et al. (265) demonstrated that trauma-induced muscle injury before decompression resulted in profuse bubble formation and it is speculated that this could be due to the formation of micronuclei. However, the knowledge of the mechanisms involved is limited. Therefore, one of the aims of the present study was to investigate the potential bubble producing effects of muscle injury prior to diving (paper I).

1.3.2 Physical Activity

Robust experimental evidence demonstrates the role of muscle exercise and movement in bubble formation and DCS occurrence, and it has long been recognized that muscular activity prior to altitude exposure increases the risk of both bubble formation and DCS (48, 76, 110, 119). However, recent diving experiments indicate that exercise prior to diving may decrease decompression-induced bubble formation and DCS risk in animals (22, 219, 272, 274) and man (11, 13, 56, 123).

Normal day-to-day ambulation and exercise is thought to produce populations of micronuclei that may grow into bubbles during decompression (247). Physical activity and movement seems to increase the rate of micronuclei formation, probably through nucleation processes induced by mechanical pressure when one tissue surface rubs against another, i.e., tribonucleation (110, 112, 116). Experiments by Hayward (112) and Ikels (116) demonstrated that bubbles could form *de novo* on surfaces in liquids free of micronuclei by tribonucleation, under conditions that required moderate decreases in barometric pressures that potentially could occur in numerous sites in biological systems. Ikels suggested that these sites could be articulating surfaces of joints, inside circulatory vessels, or at muscle tendon insertions to bone joints. McDonough et al. (173-175) suggested that physical movement or activity was necessary for bubbles to form. In a series of hyperbaric studies using fish and various crustaceans, such as crabs and shrimps, they demonstrated that on total immobilization of animals or animal limbs, then decompression from pressures higher than 100 ATA were necessary for bubbles to form. In contrast, if animals were able to move their limbs or joints, bubbles could form following decompression from only 2-10 ATA. Ample

evidence demonstrates that physical exercise prior to (51, 109, 265), after altitude decompression (48, 99, 109, 113, 213) and after decompression from diving (106, 215, 250), increases the risk of bubble formation and DCS. Furthermore, Wilbur et al. (268) provided direct evidence recently that physical exercise could lead to the formation of micronuclei even without any changes in ambient pressure. However, whether micronuclei are formed *de novo* or from preformed nuclei due to physical activity is not clear, but both scenarios are probably possible.

It is speculated that exercise-induced skeletal muscle injury prior to diving may enhance bubble formation during decompression (76). Certain forms of exercise involving eccentric contractions and movement (e.g., downhill running), resulting in muscle disruption and soft tissue damage (207, 218), may expose hydrophobic surfaces and/or facilitate viscous adhesion or cavitation, generating gas micronuclei (76). Trauma-induced muscle injury and vigorous exercise that could theoretically injure muscle tissues before altitude exposure, have each been shown to result in profuse bubble formation (110, 265). Previous diving experiments demonstrating exercise-induced protection against bubble formation (11, 56, 272, 274), have all applied concentric exercise modalities (e.g., uphill running) which provoke very little muscle injury (90, 189). Thus, eccentric exercise regimens may enhance decompression-induced bubble formation. Whether pre-dive eccentric exercise that induces skeletal muscle injury will lead to increased amounts of decompression induced vascular bubbles after diving is not known, and is one of the questions posed in this study (paper I).

1.4 Vascular Gas Bubbles in DCS pathology

Bubbles initiate DCS, but little is known about the mechanisms that cause the initiation (179). Growing evidence suggests that DCS is a result of complex interplays of gas bubbles, biochemical and immunological reactions, which eventually lead to the symptoms of DCS (7, 25, 76, 79, 233, 243). Venous bubbles can be observed following nearly all dives. However, in most dives, no acute clinical symptoms occur (191) and recent studies have also detected arterial bubbles present in subjects without

any acute DCS symptoms (55, 158). Even if acute clinical symptoms are lacking, bubbles may trigger biochemical and immunological effects that could form the basis for possible long-term injuries (7, 25, 191). Therefore, genetic and biochemical reactions in response to diving and bubble formation that may be involved in DCS development were also investigated (paper II).

1.4.1 Cardiopulmonary and CNS Manifestations

The presence of a large number of VGE in the body can cause serious cardiopulmonary DCS (186, 252). Growing bubbles may detach from the endothelial surface, circulate downstream into the venous circulation, through the right chambers of the heart and then become effectively trapped in the small vessels of the pulmonary circulation (27, 254).

In animal experiments, circulating bubbles trapped in the lungs are shown to increase pulmonary artery pressure and decrease oxygen content in blood, which may lead to cardiac overload and heart failure and tissue ischemia (17, 27, 128, 254). Furthermore, neurological manifestations are a serious and relatively common decompression-related problem (79). They can occur when on occasion, venous gas bubbles cross over and enter the left (arterial) chambers of the heart and then the systemic circulation (86, 270, 271). It is generally assumed that venous bubbles trapped in the pulmonary circulation will have no further effects on the systemic circulation (24). However, they may become arterialized when the filtering capability of the pulmonary vascular bed is overwhelmed by massive venous bubbling (27, 254). Circulating bubbles may also pass through right to left shunts in the heart (180, 248, 271), and very rarely through the lungs during exercise after diving (67, 199, 234). AGE are known to often affect the brain but can occasionally affect the heart and other organs (252).

Arterialization of bubbles is considered to dramatically increase the risk of serious DCS, especially neurological DCS (78, 79). However, it is puzzling that the majority of the DCS symptoms in sport divers requiring treatment are from the central nervous system (CNS), even though arterial bubbles

are rarely detected (78, 252). This indicates that bubbles either have to be present in the systemic circulation but are not detected, and/or that venous bubbles may incite some kind of systemic biochemical reactions that eventually results in CNS symptoms. Brubakk et al. (24) have demonstrated that diving, with a minimum of circulating venous bubbles, resulted in vascular dysfunction in the systemic circulation even though no bubbles were detected by ultrasound imaging in arteries. Martin et al. (169) demonstrated that immunological processes seem to be involved in the development of DCS symptoms, while recently Thom et al. (243) suggested that circulating microparticles, generated on decompression, initiate immunological processes that may further lead to vascular damage in several sites in the organisms, including the brain and skeletal muscles. Several studies have shown that vascular bubbles trigger a cascade of local and systemic biochemical reactions involved in the pathogenesis of DCS (7, 25, 79, 233).

1.4.2 Enhanced Coagulation and Thrombi Formation

The blood circulates in the vascular bed, a closed system, which communicates with all tissue cells and exchanges oxygen, nutrients, wastes and provides the necessary components for host defense (220). Any opening or disruption of this fragile closed system due to tissue injury may lead to serious infection and blood loss. To minimize bleeding, components of the vascular system are activated in a process called hemostasis. The components include platelets, endothelial cells and plasma coagulation factors; they may be activated on exposure to foreign surfaces (e.g., gas bubbles) and endogenously released compounds to prevent bleeding.

One of the many mechanisms of DCS is probably bubble-induced mechanical vessel obstruction resulting in hemodynamic and biochemical changes. However, Pontier et al. (217) suggested that the pathological events in DCS cannot be explained by bubble occlusion alone, but are a result of bubble-induced platelet aggregation and adhesion. It has been shown that the surface of decompression-induced gas bubbles seems to activate the process of coagulation (79, 201). Philp et al. (210, 211) and Warren et al. (260) demonstrated that vascular bubbles acquired a coating of fibrinogen and lipids leading to

adhesion of platelets directly to the bubble surface, further progressing to platelet aggregation. Platelet aggregation can result in clogging and obstruction of the blood vessels by microthrombi. Philp et al. (212) demonstrated formation of microthrombi in rat lungs after diving and showed that there was a positive correlation between the extent of microthrombi and severity of DCS. Olsznaki et al. (202) demonstrated increased platelet aggregation and increased number of aggregates in humans after diving. Tanoue et al. (238) found a decreased platelet count from a possible over-destruction of platelets and the formation of platelet thrombi in the lungs of rabbits. They therefore suggested that bubbles from decompression interact with platelets and participate in the formation of thrombi in DCS development. Further to this, Pontier et al. (216) showed that platelet activation and thrombin generation were increased in a rat model of DCS, and that administration of anti-thrombotic drugs increased survival and decreased DCS symptoms (217). These findings support the hypothesis that bubble-induced platelet aggregation with thrombus formation may be a significant cause of DCS. However, the genetic reactions behind these findings are unknown. In paper II, the acute genetic reactions to diving and bubble formation in relation to increased coagulation and thrombi formation are investigated.

1.4.3 Innate Immune System Activation and Endothelial Dysfunction

It is suggested that vascular bubbles would trigger many of the same effects as microbial intruders coming into contact with the blood (211). Bubbles in blood may represent a foreign surface that can activate leukocytes and the complement system (10, 211, 258, 260). The complement system represents a chief component of innate immunity, participates in inflammation and enhances the adaptive immune response (34). Circulating leukocytes, especially neutrophils, are selectively recruited to injured or inflamed tissues in a tightly-regulated cascade-like fashion, which is named the leukocyte adhesion cascade (142). During inflammation, neutrophils attach and roll along the endothelial wall and integrate inflammatory signals (282). Neutrophils crawl and transmigrate into the underlying inflamed tissue (sequestration) and start the

production of reactive oxygen species (ROS), enhancing the inflammatory processes, which may cause endothelial injury and dysfunction (226, 259).

It has been demonstrated that vascular bubbles can cause endothelial injury and dysfunction through mechanical and biochemical mechanisms (24, 195-198). Furthermore, endothelial dysfunction has been associated with the incidence of DCS (243) and as the number of gas bubbles increases, the likelihood of endothelial injury increases (198, 211, 260). Endothelial damage as a result of circulating bubbles has been seen microscopically in the venous circulation (198), while endothelial dysfunction has been measured by decreased effects of vasoactive compounds (197) and reduced flow-mediated dilatation (24). The mechanisms in which diving leads to endothelial or vascular dysfunction is unclear; as to how these processes are orchestrated at the genetic level has not been investigated. The acute vascular genetic reactions that may be involved in innate immune reactions and vascular dysfunction after diving are investigated in the present study (paper II).

1.5 Hyperoxia and Oxidative Stress in DCS Pathology

Strict regulation and utilization of O_2 is necessary to maintain a balance between the generation of energy and production of potentially toxic oxidants (229). Oxygen over-utilization or over-delivery generates ROS that may lead to oxidation of lipids, nucleic acids and proteins that can result in cellular dysfunction and death. However, ROS generated by mitochondria is essential in cell signaling and O_2 sensing, leading to activation of adaptive processes that will enhance survival (101).

Both low (hypoxia) and high (hyperoxia) O_2 levels may result in the generation of excess ROS. ROS contribute to the regulation of hypoxia-inducible factor-1 (HIF-1), a transcription factor that plays an essential role in development, physiology and disease (101, 229). This transcription factor consists of two subunits, HIF-1α or HIF-2α; they partner with the constitutively expressed subunit HIF-1β that together forms a HIF DNA-binding complex (69). The half-lives of the HIF-α-subunits are short, but on exposure

to hypoxia the subunits can accumulate and translocate to the nucleus, where they may bind to HIF-1β and stabilize in the promoter regions of target genes. The main purpose of HIF gene regulation is to optimize cell energy use and homeostasis, for survival and function in oxygen-poor environments (193). HIF-1 is known to regulate the expression of more than hundred genes that function in various responses triggered by hypoxia. These responses involve metabolism, angiogenesis, vascular tone, cell differentiation and apoptosis (124, 130). Hyperoxia also causes activation of pathways mediated via HIF-1 (100, 209, 227), thus it may trigger many of the same mechanisms as hypoxia, through the generation of excess ROS and HIF-1 stabilization. In addition, stabilization of HIF-1α seems to induce an anti-inflammatory and tissue-protective response (224).

Divers are exposed to an elevated partial pressure of O_2 (pO_2), as they breathe compressed gas while diving. Hyperoxia denotes that the pO_2 of the breathing gas is higher than 21 kPa (normoxia), which is the pO_2 of air at sea level. A diver carries a tank with a fixed volume of breathing gas, usually comprising of oxygen, nitrogen and occasionally helium or other inert gases; due to the increased ambient pressure during diving, the partial pressures of all of the gases will increase with increased diving depth. The Boyle-Mariotte law describes the inversely proportional relationship between the absolute pressure and volume of a gas, if the temperature is kept constant within a closed system. This means that during a dive, the pO_2 in the breathing gas from the tank increases by a factor of two at 10 meters depth, of three at 20 meters depth and so on. It is suggested that exposure to hyperoxia with a subsequent oxidative stress during diving may initiate DCS and that decompression-induced bubbles are not the causative agent in the progression of DCS, but rather an exacerbating factor (163). An alternative hypothesis may be that oxidative stress enhances DCS susceptibility. However, oxidative stress alone does not lead to DCS, since DCS does not occur in the absence of bubbles (63). The role of hyperoxia and oxidative stress during diving in DCS etiology is not clear and the involvement of HIF-1 is unknown; therefore it is investigated in the present study (paper II).

1.6 Hyperbaric Oxygen Preconditioning

The use of hyperbaric oxygen (HBO) therapy, where O_2 is breathed at a raised partial pressure in a hyperbaric chamber at raised ambient pressure, induces a number of beneficial biochemical, cellular, and physiologic effects (246). HBO therapy is widely used to treat conditions such as carbon monoxide poisoning, radiation-induced injury, infections, ischemic injuries and wounds, as well as DCS. Patients who have symptoms of DCS are treated with HBO, breathing oxygen (100% O_2 at 2.5-3.0 ATA) for several hours and sometimes with repeated treatments as necessary until they are symptom-free or there is no further clinical improvement (181).

Recent studies have shown beneficial and protective effects of HBO if administered prior to injury, which is commonly referred to as HBO pre-conditioning (HBO-PC) (209). HBO-PC prior to ischemia has been shown to provide wide-scale cardio- and neuro-protective effects (30, 143, 144, 278, 279) and recent diving studies have shown that HBO-PC may provide promising results in reducing the incidence, severity and complications of DCS after simulated diving (28, 71, 169).

One of the protective mechanisms of HBO-PC seems to be related to the activation of heat shock proteins (HSPs), which is caused by oxidative stress (192, 239). It is well documented that HSPs play a key role in protecting against myocardial ischemic damage (14, 136). The small, oxidative stress activated heat shock protein, αB-crystallin (170), protects the heart against ischemic injury by binding to cytoskeletal structures, where it preserves the integrity of microtubules (14). Ischemia leads to the phosphorylation of αB-crystallin on serine 59, enhancing its ability to protect myocardial cells from damage (267). Whether HBO-PC may protect the heart against injury after diving is unknown. In the present study, the cardioprotective role of HBO-PC in relation to diving and bubble formation is investigated (paper III).

2 Aims and Hypotheses

The overall aim of this study was to investigate the effects of diving and vascular bubble formation on the cardiovascular system and to further investigate whether the stressful pre-dive interventions, eccentric exercise and hyperbaric oxygen exposure, could modify the outcome. ***The overall hypothesis*** was that injury from diving is initiated by oxidative stress during diving and further exacerbated by decompression-induced bubble formation, and that pre-conditioning with a pre-dive oxidative stress may protect against the adverse effects induced by diving.

Specific Aims:

1. To investigate the effects of eccentric exercise prior to diving on:
    a. the degree of muscle injury at the time of decompression.
    b. the degree of vascular bubble formation and survival.
2. To determine the effects of diving and vascular bubbles on the cardiovascular system through:
    a. identifying which vascular genes and pathways that are differentially expressed (up or down regulated) immediately after diving.
    b. identifying markers of cardiac stress and injury.
3. To determine the effect of hyperbaric oxygen preconditioning prior to diving on the heart.

Specific Hypotheses:

1. It was hypothesized that exercise-induced skeletal muscle injury prior to decompression from diving would increase vascular bubble formation and lower survival rates after decompression.
2. It was hypothesized that diving would lead to immediate early vascular gene expression changes that are involved in DCS etiology.
3. It was hypothesized that diving-induced bubble formation would lead to elevated levels of cardiac stress-markers.
4. It was hypothesized that hyperbaric oxygen preconditioning would induce a cardioprotective effect, detectable as a reduction of cardiac stress-marker expression in response to diving-induced bubble formation.

3 Methodological Considerations

3.1 Ethical Considerations

The purpose of this study was to obtain a novel and improved knowledge of the mechanisms involved in DCS etiology. The use of a physiologically relevant and standardized animal model was necessary to obtain this information. We always strive to *Replace* animals with *non-sentient* alternatives if possible, to *Reduce* to a minimum the number of animals used and to *Refine* our experiments to cause the minimum of pain and distress. Appropriate design of experiments to minimize variation, to provide standardized optimum conditions of animals care and to minimize unnecessary stress or pain will improve the quality of research (73).

All experimental protocols were reviewed and approved by the Norwegian Committee for Animal Experiments, and conform to the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes.

3.2 Experimental Animals

In this study rats were exposed to high ambient pressures in order to simulate diving, and then further exposed to severe decompression stress. A standardized, established and validated experimental animal model, previously used in diving studies in our laboratory (9, 80, 272, 274, 275), was used. The use of this animal model provides better control of genetic, biometric and nutritional factors than human studies, and permits study of physiology, biochemistry, genomics/epigenomics and the proteomics of specific tissues and organ systems.

Female outbred Sprague-Dawley rats (Taconic, Denmark), of ~16-18 weeks old and of weight 250-300 grams were used. Animal weight seems to be single most important factor influencing the risk of bubble formation and mortality in our experimental diving model (151, 152, 275). Rats weighing less than 250 grams are unlikely to produce any bubbles, while animals heavier than 300 grams will most likely

produce massive amounts of bubbles resulting in severe DCS with cardiopulmonary collapse and death. Rats were chosen for each experiment to achieve an optimal balance of weights between all groups. Female rats were chosen due to their much lower weight than male rats and because the weight gain of female Sprague-Dawley slows down when reaching ~260 grams.

The rats arrived at the animal facility 6-8 weeks prior to the experiments in order to allow them to reach the weight appropriate to induce severe bubble formation. Randomization and group allocation of the rats is described in more detail in the papers I, II and III.

3.3 Exercise Protocol

To determine the role of exercise-induced skeletal muscle injury on bubble formation after diving, we adapted an eccentric exercise protocol that previously had been shown to generate immediate skeletal muscle injury in Sprague-Dawley rats (6, 237). A downhill running protocol was chosen, as this form of eccentric exercise in individuals unaccustomed to such exercise, seems to induce the greatest and most widespread degree of muscle damage and systemic responses (207). In addition, a treadmill running protocol was preferable, as the laboratory is set up for this purpose and we have comprehensive experience with this type of protocol (9, 272-274).

During eccentric exercise, the contracting muscle is forcibly lengthened (218). When rats are running downhill, the contracting quadriceps and triceps muscles control the rate of limb flexion against the force of gravity and with each step, the muscles undergo eccentric contractions resulting in accumulating damage to muscle fibers, if the rats are unaccustomed to such movements. Different techniques were used to verify that the exercise protocol resulted in muscle injury. This is described in detail in the method section of paper I.

3.3.1 Downhill Intermittent Treadmill Running

Pilot rats were used to test, modify and adapt the exercise protocol to ensure that all rats would run to total exhaustion, and also to ensure that a high degree of muscle injury was achieved. By careful handling, unnecessary stress to the rats before and during the exercise bout was avoided, as this could cause them to refuse to run. To avoid bias due to stress from handling, the control rats were handled in exactly the same way and placed on the treadmill but not made to run. The exercise protocol is described in detail in paper I.

Treadmill angle, speed and duration of the exercise bout was adapted from an experiment by Armstrong et al. (6) that demonstrated extensive muscle injury compared to similar horizontal or uphill running. However, a protocol with shorter durations and lower angles has also demonstrated severe injury to skeletal muscles (149). Thus, we believe that the protocol used in our study was strenuous enough to produce a high degree of muscle injuries.

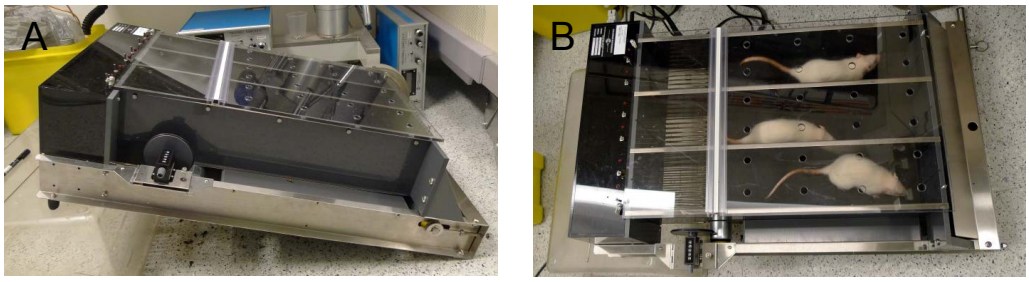

Figure 1. A. *Showing the treadmill inclination (16° decline).* **B**. *Rats running downhill on the treadmill.*

3.3.2 Rest Interval between Exercise and Diving

Physical activity may lead to the generation of micronuclei that could theoretically grow and form bubbles on subsequent decompression (110, 268). Generated microbubbles seem to be short-lived, ranging from a few minutes (110, 268) up to a maximum of two hours (51). To be able to study the sole effect of muscle injury on bubble formation and to exclude the effects of potential micronuclei generation during the

exercise bout, the rats were rested for 140 min prior to decompression (including 90 min of rest in their cages followed by 50 minutes of diving). In addition, rats had free access to water after the exercise bout to enable rehydration, mainly to counter any possible effects of dehydration on bubble formation (12, 82).

3.4 Chamber Exposures

3.4.1 Simulated Diving

To simulate diving, rats were exposed to high ambient pressures in a dry hyperbaric chamber. At the end of the dive, the pressure was gradually reduced to induce decompression stress and gas bubble formation. The diving protocol used produces severe decompression stress with massive vascular bubble formation in female Sprague-Dawley rats. The diving protocol is described in detail in paper I-III.

The bottom time in all dives in the present study (Paper I-III) was 50 min. Studies using similar simulated diving protocols (152) show that DCS risk increases with increased bottom times up to ~45 min. Extending bottom time beyond this period does not significantly increase DCS risk or death, indicating that it takes around 45 min to saturate the tissues responsible for bubble growth. However, tissue saturation time may vary, since gas uptake is dependent on tissue blood perfusion and how blood is distributed to tissues (247). Thus, several factors may affect gas uptake and elimination during diving e.g., physical activity, stress (59, 262) or alterations in surrounding temperature (87). To avoid bias from environmental factors, all animals were handled equally and the animal exposures differed only in in terms of ambient pressure and breathing gas compositions. We strived to keep the stress level at a minimum and constant room and chamber temperatures were kept throughout the experiments.

A slightly puzzling observation is that this simulated diving protocol can produce a huge range of different bubble grades across the subject group, even though rats are dived together, are handled equally, have the same weight and are of the same sex and age. Individual bubble loads may vary from no detectable bubbles (scan grade 0), to massive bubble loads (scan grade 5). The reason for this is unknown,

but it is speculated that different endogenous factors affect bubble formation, such as genetic composition and fat distribution, or biochemical responses to handling and diving (79). The variation in bubble loads is potentially problematic, since it may lead to a huge variation in genetic reactions and injury after diving. However, it may also be advantageous, since it enables the possibility to investigate the link between the degree of bubble loads and the parameters measured in this study, for example, the association between bubble loads and the genetic reactions after diving (paper II), or the level of cardiac stress-markers (paper III).

3.4.2 HBO-PC

To determine the effects of HBO-PC prior to diving on the cardiovascular system, a protocol previously shown to reduce DCS risk in rats was adapted (28, 169). The HBO-PC protocol is described in detail in paper III.

HBO exposure prior to decompression may reduce bubble formation during decompression due elimination of N_2 (denitrogenation) from tissues (76). The purpose of the last paper in the present study (paper III) was to investigate any protective effects of oxygen other than denitrogenation before decompression. Thus, we had to avoid the effects of N_2 elimination prior to diving. At surface, the body tissues are equilibrated and saturated with N_2, but during exposure to HBO, the fraction of inspired oxygen gas is increased relative to the fraction of N_2, leading to a gradual elimination of N_2 dissolved in tissues (76). For this reason, exposure to increased O_2 pressures prior to decompression may lead to a reduction in decompression-induced bubbles (262). Thus, to avoid the protective effects of nitrogen elimination prior to the dive, enough time for N_2 uptake between the HBO-PC exposure and diving was needed to ensure that the partial pressure of N_2 (pN_2) in tissues prior to the dive was the same in all groups. The easiest way to do this was to add a short period of hyperbaric air breathing at the end of the HBO-PC protocol exposure to ensure a rapid uptake of N_2. The tissue pN_2 was calculated by the use of the classic

exponential model as described by Foster et al. (77). A tissue half time of 10 min was assumed based on studies by Lillo et al. (152). Fig. 3 illustrates the HBO-PC and diving protocol.

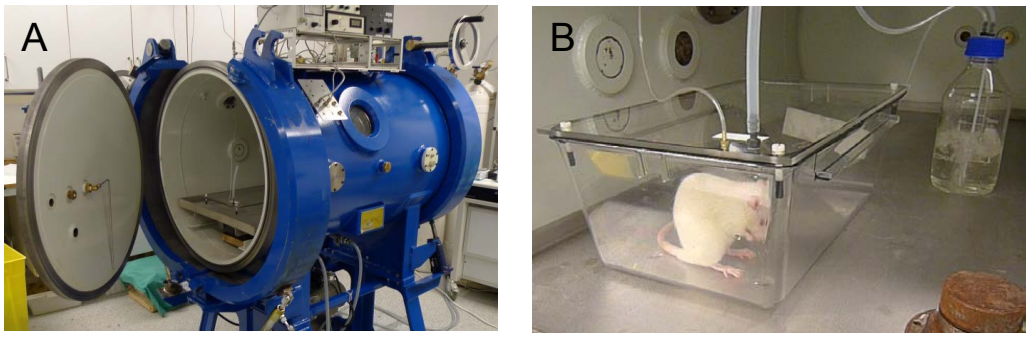

Figure 2. A. *The rats were placed inside a hyperbaric chamber and the chamber air was compressed to simulate diving.* **B**. *Within the chamber, the rats were placed inside an airtight box flushed with 100% oxygen, and the air surrounding the box were compressed to enable exposure to hyperbaric oxygen.*

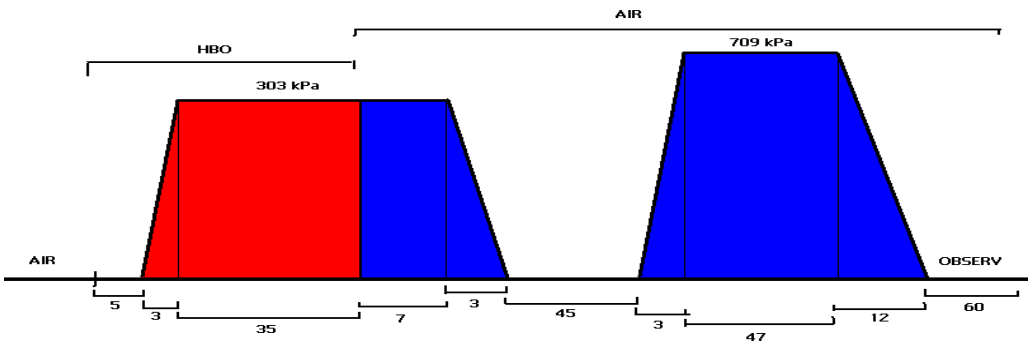

Figure. 3. *Illustration of the hyperbaric oxygen preconditioning (HBO-PC) and diving protocol used in this study (papers I-III). The HBO-PC rats first breathed 100% normobaric oxygen for 5 min, then were compressed to 303 kPa, breathing 100% oxygen for 38 min (red). To renitrogenate tissues, the rats breathed hyperbaric air for 7 min at the same pressure and were decompressed back to the "surface". The rats then rested in their respective cages for either 45 or 180 min prior to the dive (50 min at 709 kPa, blue).*

For practical purposes, a one compartment model (whole rat) was assumed to be an adequate model to calculate gas uptake and elimination in this study, however more advanced calculations could have been used. The concepts of decompression theory are described in detail in Bennett and Elliott's Book: Physiology and Medicine of Diving (247).

3.5 General Anesthesia

In this study, the animals were observed under general anesthesia for up to four hours after the dives. At the end of this observation period, various surgical interventions were performed. To avoid unnecessary discomfort, pain and stress for the animals, proper surgical anesthesia had to be ensured. Details about the anesthetics, surgical procedures and euthanasia are described in papers I-III.

A mixture of midazolam, fentanyl and haloperidol were used. Midazolam is a short-acting benzodiazepine that induces sedation and amnesia and is a commonly used benzodiazepine for sedation. Fentanyl is a very potent synthetic analgesic opioid and is often used in combination with midazolam as an anesthetic. Haloperidol is an antipsychotic drug. A similar combination has previously been shown to produce excellent surgical anesthesia in rats (74). General anesthesia unavoidably generates hemodynamic changes, however the anesthetics used in this experiment are shown to preserve cardiac output and tissue perfusion (231). During anesthesia there is also a risk of a significant heat loss. To preserve the body core temperature, the animals were covered with an electrical heating blanket during the observation period.

Tail and pedal withdrawal reflexes were used to monitor the adequacy of anesthesia. Experience from previous experiments with Sprague-Dawley rats that had received the same type of anesthetics, indicated that the tail and pedal withdrawal reflexes are a valid and reliable method for monitoring anesthetic depth (120). Using reflexes to monitor the depth of anesthesia is also advantageous as it is a simple clinical method. However, the response to stimuli is very variable between different animal species and within the same species, which can be due to variations in pharmaco-kinetics and -dynamics resulting

in a wide range of drug concentrations at the receptor site. The reflex response is also dependent on the type of anesthetics and route of administration (232).

3.6 Detection of Vascular Gas Bubbles

Circulating bubbles are easily detected by ultrasonography and the amount of bubbles detectable in the venous system draining the tissues is widely used as an objective and reliable indicator of decompression stress (62, 64, 191). No studies have actually observed where decompression induced bubbles form, but it is believed that they may grow on the endothelial surface in small peripheral blood vessels or capillaries, or in tissues in areas supersaturated with gas. The bubbles may detach from the endothelial surface and circulate through the right (venous) side of the heart to the lungs where they are trapped and eliminated. Thus, most of the circulating bubbles can be detected by ultrasound when they are moving through the pulmonary artery, and if there are systemic/arterial bubbles present, they can be detected in the ascending aorta (191).

Although circulating bubbles can be used as an index of decompression stress, they are not equivalent to DCS. DCS refers to symptoms initiated by bubbles, not just their presence, and the association between circulating bubbles detected by Doppler or ultrasonic imaging and DCS is weak. However, it is known that the more circulating bubbles detected, the greater the risk of DCS, especially severe DCS. The correlation improves when the number of venous bubbles detected are scored according to a grading system (45, 46, 191). Large quantities of venous gas bubbles and small amounts of arterial bubbles can be present in divers with no DCS symptoms (156, 158) and the absence of bubbles is highly associated with the absence of DCS symptoms (46, 64, 191). In this study, we chose to detect vascular bubbles instead of observing DCS symptoms. The advantage of vascular bubble detection is that it enables relatively objective measurement of decompression stress, while offering the possibility to observe correlations between vascular bubble loads with survival (paper I), genetic responses (paper II) and tissue injury (paper III). Another advantage is that bubble detection is most easily performed in anesthetized

animals and therefore causes a minimum of pain and distress (151, 152). The disadvantage of vascular bubble detection is the weak correlation between low bubble loads and DCS symptoms.

3.6.1 Ultrasonic Imaging and Doppler

Doppler ultrasound is widely used to monitor circulating bubbles (191). A distinct sound is heard when bubbles pass the Doppler probe and they can be seen as bright spots in the Doppler signal. The drawback with Doppler is that small bubbles may not be detected, as the signals may be quieter than the sound from background blood flow. The Kisman-Masurel or Spencer codes are typically used to grade the amount of vascular bubbles when using Doppler ultrasound (191). However, for visual 2D-ultrasound imaging, we use a scoring system developed by Eftedal and Brubakk (62). The scoring system is composed of the following grades: 0, no bubbles; 1, occasional bubbles; 2, at least one bubble per fourth heart cycle; 3, at least one bubble per heart cycle; 4, at least ten bubbles per heart cycle; and 5, "signal whiteout" where individual bubbles cannot be seen. The advantage of 2D-ultrasound in comparison to Doppler is that the number of bubbles can be counted, as individual bubbles can be seen in the image. The disadvantage, in our opinion, is that 2D-ultrasound is not as sensitive as Doppler in terms of detecting occasional bubbles, which would typically be graded on a scan as grade 1. Therefore, it is preferential to use a combination of Doppler and ultrasonic imaging techniques.

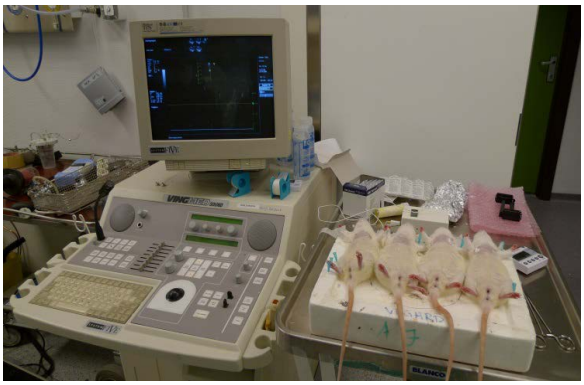

Figure 4. *After the simulated dive, the rats were anesthetized and vascular gas bubbles were detected by ultrasonography.*

3.7 Tissue Collection and Analyses

Blood and tissues were sampled and analyzed in order to answer the overall and specific aims of this study. Animals that died during the post-dive observation period were excluded from the analyses.

3.7.1 Paper I

In paper I, the role of pre-dive exercise induced skeletal muscle injury on vascular bubble formation and survival after decompression was investigated. To induce muscle injury, the rats performed an eccentric exercise protocol on a treadmill. The degree of muscle injury was evaluated by the presence of signs of disruption to myofibrils and muscle cell membrane (sarcolemma) using microscopy. Three markers of muscle injury, αB-crystallin, NF-κB, and TNF-α, were evaluated by the use of immunohistochemistry (IHC) and real-time reverse transcription polymerase chain reaction (qRT-PCR). The methods are described in more detail in paper I.

Tissue Collection

Muscles from both the front (*Musculus triceps brachii caput mediale*) and hind limbs (*Musculus vastus intermedius*), which had demonstrated the greatest damage in previous eccentric exercise treadmill protocols, were selected for evaluation (3, 6, 31, 237). As it is possible that the hind limbs may touch the electrical grid positioned behind the rat at the beginning of the exercise bout, forelimbs, which were at less risk of electrical stimulation, were also chosen for analysis (6). Mild electrical stimulation is commonly used to spur the animals in treadmill running experiments and it is shown that electrical muscle stimulation may lead to muscle injury (49). However, mild electrical stimulation is not believed to influence bubble formation (265). Non-exercising rats were not exposed to electrical stimulation to the same degree as exercising rats, thus we cannot exclude the possibility that exposure to some extra electric stimulation during running could have influenced the outcome. To avoid muscle injury and tissue

degradation during sampling, muscles for IHC analysis were dissected out carefully and quickly frozen in isopentane, cooled by liquid nitrogen, and stored at -80°C. To avoid RNA degradation, muscles for gene expression analysis were immediately immersed in 'RNAlater' to ensure RNA integrity, kept at room temperature over night to ensure complete permeation of the RNAlater and then stored at -20°C. Blood and tissue collection are described in detail in paper I.

Tissue Analysis

Several studies show that protein accumulation and mRNA upregulation of αB-crystallin are valid and reliable markers to verify muscle injury as a response to eccentric exercise (72, 129, 205, 206). Eccentric exercise induces an immediate translocation and accumulation of the small heat shock protein αB-crystallin to cellular structures prone to disruption. αB-crystallin seems to function as a stabilizer of disrupted myofibrillar structures, and translocation of this heat shock protein is associated with phosphorylation. Protein phosphorylation is a way of regulating protein function by the addition of a covalently bound phosphate group (1). The protein then switches from an unphosphorylated to a phosphorylated form; one of these two is an active form, while the other is inactive. Increased levels and granular appearance of αB-crystallin in skeletal muscle tissue is associated with acute skeletal muscle injury. In contrast, no such translocation, phosphorylation, accumulation and granulation of αB-crystallin are observed after concentric or isometric exercise (129, 184, 256). Previous studies have demonstrated that an immediate increase in muscle tissue levels of αB-crystallin correlate well with other commonly used markers and signs of muscle injury such as: increased muscle pain, reduced muscle force, increased creatine kinase serum activity and myoglobinaemia, loss of specific force-bearing cytoskeletal proteins and myofibrillar disorganization as seen in micrographs. Thus, this marker of injury was chosen for study due to its immediate response to muscle injury.

Eccentric exercise causes direct mechanical damage to muscles followed by increased proteolytic activity and inflammatory processes (207, 218). We chose to investigate the two major pro-inflammatory cytokines TNF-α and NF-κB, since they are shown to be crucial in the pathogenesis of the inflammatory responses in injured skeletal muscles. TNF-α emerges as a key cytokine that promotes peripheral muscle proteolysis (96, 159) and activates NF-κB (146, 147), a central regulator of innate immune responses, cell growth and cell death (125, 193). NF-κB activity and TNF-α mRNA expression in skeletal muscle tissues is found to be markedly increased immediately after an acute bout of eccentric exercise due to muscle injury (149, 207).

3.7.2 Paper II

In paper II, the effects of diving and vascular bubbles on the vasculature were investigated by identifying the immediate early gene expression changes in the abdominal aorta and subsequent protein expression in the blood. This was done by combining global gene expression profiling and protein detection with monitoring of physiological parameters. The genetic reactions to diving were analyzed by full genome microarray analysis. Increased transcription of selected genes was verified by qRT-PCR. Stabilization of HIF-1 in the aorta was examined by the use of microscopy and IHC. Increased protein expression of plasminogen activator inhibitor-1 (PAI-1) in the blood was evaluated by enzyme-linked immune-sorbent assay (ELISA) technique. The methods are described more in detail in paper II.

Tissue Collection

In anesthetized animals, the abdomen was carefully opened and blood was collected from the abdominal aorta for protein and blood gas analysis; the aorta was quickly and carefully excised to avoid damage to the vessel. To minimize endothelial activation, the blood flow in the aorta was maintained during

harvesting. The aorta was immediately stored in RNAlater at room temperature for RNA preservation before storage at −80°C.

There were several reasons for choosing the abdominal aorta for analysis; the aorta is often used as a model in studies of vascular function in vitro, it is easily excised with a minimum of damage, can be rapidly harvested in order to avoid non-random mRNA degradation prior to stabilization and enables enough tissue for a global gene expression analysis and IHC. In addition, the majority of vascular bubbles formed after diving are on the venous side of the circulation where they may cause direct mechanical damage to the endothelium (198). Thus, to avoid this damage, an arterial vessel was chosen instead of a venous vessel for analysis. However, a possible disadvantage of choosing the abdominal aorta is that it may not reflect all underlying mechanisms in DCS development, so some may be missed. It is suggested that the direct contact between gas bubbles, the blood and the endothelium are involved in the vascular reactions behind DCS development (139, 166, 197, 257), inducing activation of the immune system and enhanced coagulation (24, 195, 196, 210, 216, 217, 238, 243). Furthermore, gas bubbles are believed to be formed in peripheral vessels in the venous circulation, thus, a peripheral vein may have been more physiologically representative. However, endothelial dysfunction after diving is observed in both venous and arterial vasculature (24, 197). Therefore, we believe that the aorta is a good representative blood vessel when studying most of the genetic reactions behind DCS development.

Tissue Analysis

HIF-1 is suggested to regulate the transcription of anti-inflammatory and/or tissue-protective signaling pathways in the vasculature (69, 224). During a dive, rats are exposed to hyperoxia, and after the dive, they are potentially exposed to hypoxia and inflammation. Since it has been shown that hyperoxia, hypoxia and inflammation can activate and/or stabilize HIF-1 in vascular tissues (69, 209, 224), we aimed to examine the stabilization of HIF-1 in the wall of the aorta after diving.

PAI-1 promotes clot formation by the inhibition of fibrinolysis in blood and is essential for maintaining vascular tissue integrity (52). Since bubble formation and DCS is associated with increased coagulation and thrombi formation, we aimed to measure the level of PAI-1 in blood after diving.

3.7.3 Paper III

In paper III, the impact of simulated diving and vascular bubble formation on cardiac stress/injury was investigated and the cardioprotective role of HBO-PC was evaluated. This was done by examination of vascular bubble formation after simulated diving and the association with the levels of three different cardiac stress-markers in serum and cardiac tissue; serum cardiac troponin T (cTnT), cardiac gene expression of the natriuretic peptide precursor B (*Nppb*) and cardiac gene and protein expression of αB-crystallin. The effect on cardiac gene expression of the natriuretic peptide precursor A (*Nppa*) was also investigated, but is not included in paper III. Subsequently, the effect of HBO-PC on the levels of these stress-markers was analyzed. The level of cTnT in serum was measured by electrochemiluminescence immunoassay (ECLIA) technique. *Nppa/Nppb* and *Cryab* (αB-crystallin) mRNA expressions in cardiac tissue were measured by the use of qRT-PCR. Protein expression of αB-crystallin in cardiac tissue was measured by the use of Western blot. The methods are described more in detail in paper III.

Tissue Collection

Blood was sampled by puncture of the abdominal aorta, and to avoid cardiac ischemia, the heart was rapidly excised while still beating. Blood and tissue collection are described in detail in paper III.

Tissue Analysis

Cardiac troponins are the preferred biomarkers for the diagnosis of cardiomyocyte necrosis/injury, and have nearly absolute cardiac tissue specificity and high clinical sensitivity (88, 245). We chose to use a high sensitive cTnT assay that permits measurements of concentrations that are lower by a factor of 10 than those measurable with conventional cTnT assays (203). The measuring range of the assay is 10-25000 $ng \cdot L^{-1}$. This assay is routinely used at our hospital (St. Olavs Hospital, Trondheim, Norway) to measure human cTnT, however, the same assay is also shown to be a valid and reliable assay to study myocardial ischemia in rats (122).

The natriuretic peptide precursors A and B (*Nppa/Nppb*) encode for atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) and are well established biomarkers of acute heart failure (19). These natriuretic peptides are synthesized and released by cardiomyocytes in response to hemodynamic stress when the ventricles are subject to increased wall tension. Decompression-induced VGE may cause increased pulmonary artery pressure, cardiac overload and acute heart failure (17, 186, 254) and it is shown that the expression of *Nppa* and *Nppb* is rapidly increased in both the atria and the ventricles in response to cardiac overload (187). Thus, we aimed to measure the expression level of *Nppa* and *Nppb* in cardiac tissue after diving.

In cardiac tissues, αB-crystallin seems to stabilize myofibrillar proteins during stress conditions and prevent them from loss of function (93-95). αB-crystallin is the most abundant constitutively expressed myocardial stress protein in the heart, and it is likely to play a key role in protecting the heart from injury (14, 40, 134, 136). In the heart, both ischemic and oxidative stresses induce an immediate increase in αB-crystallin levels as well as activation by phosphorylation (267). Thus, we aimed to measure the gene and protein expression levels of both total and phosphorylated αB-crystallin in cardiac tissue after diving (phosphorylated αB-crystallin results are not included in paper III).

3.8 Blood and Tissue Analyzing Techniques

Microscopy

A range of microscopy techniques provide an essential tool in studying tissues and cells (1). In this study, microscopy was used to visualize injury to muscle fibers (paper I) and specific immunostained proteins in muscles and aorta (paper I and II). To visualize a tissue, it is usually immobilized and preserved by fixation, cut into very thin slices then laid flat on the surface of a glass microscope slide. The slices can then be visualized by staining them with dyes (e.g., hematoxylin). Specific substances of interest can be detected and located in tissues by coupling fluorescent dyes with antibodies. Confocal laser scanning microscopy (CLSM) can be used to obtain high-resolution images with depth selectivity (paper I). In paper I, CLSM was used in addition to conventional light microscopy to study the structure of muscle fibers. The quality of CLSM images is greatly enhanced over conventional microscopy because image information (e.g., muscle fibers) is not superimposed. The use of microscopy in the present study is described in detail in papers I and II.

3.8.1 RNA Analysis

In this study (papers I-III) different techniques were used to study the expression of different genes. mRNA was extracted from muscle tissues (paper I), the aorta (paper II) and cardiac tissue (paper III). Then, the level of mRNA expression from specific genes were determined by qRT-PCR. In paper II, microarray analysis and data processing were performed to enable global gene expression profiling.

Total RNA Preparation

Prior to microarray analysis or PCR, total RNA had to be prepared. Immediately after sampling, the tissues were rinsed and mechanically mashed (homogenized) and total RNA was extracted. To ensure good RNA quality, the RNA purity and concentration were determined by the use of a spectrophotometer

measuring the absorbance of different wavelengths in the extracted sample. Low purity of a sample can be due to contaminants from the extraction procedure, such as alcohol or phenol, or to remaining proteins or DNA.

Microarray Gene Expression Analysis

In paper II, the aim was to screen any changes in vascular gene expression as a response to a simulated dive by microarray gene expression profiling. Microarrays are well suited for simultaneous monitoring of multiple gene expressions to study the effects of certain treatments, diseases, and developmental stages. They can be used to find genes that change in response to pathogens or other organisms by comparing infected groups against uninfected groups. In the present study, a comparison of the genes that differed in expression after diving and no diving was made. Disadvantages of the microarray technology are that it is expensive and time-consuming and it can be difficult to achieve satisfactory quality and quantity in the samples.

Microarray Data Processing

Gene expression profiling was used to identify candidate hypotheses for future studies. However, due to the enormous amount and complexity of the microarray data, a variety of interpretations are possible. In order to identify alternative hypothesis, similar genes were grouped together in heat maps (Fig. 3, paper II) and all of the gene expressions that changed the most between the diving and non-diving groups were listed. The data was processed using advanced statistics and bioinformatics analysis packages. Important considerations during analysis of this data are good experimental design and adequate biological replication. Use of this technology allowed us to find the genes that were differentially expressed, however, of more biological relevance is the knowledge of the precise proteins made by the cells. The rat

genome, which consists of thousands of genes, can probably generate millions of protein variants, but the technology of screening and analyze millions of proteins at once is not yet available. By placing the expression profiling results in a microarray database, it is possible to identify expression patterns. Our results were submitted to the ArrayExpress database, which is a peer reviewed, public accessible repository that adheres to academic or industry standards. The collected data in this database conforms to the MIAME standards.

qRT-PCR

In this study, qRT-PCR was used to verify microarray results and quantify the level of expression of specific genes. The principles of the different PCR techniques are described in detail in the books of Campbell (33) and Lewin et al. (141). qRT-PCR is a widely used tool in gene expression profiling to quantify the level gene expression, and is used in the diagnosis of genetic diseases. An advantage of qRT-PCR is that it provides a highly sensitive technique to detect very low numbers of RNA. A challenge with qRT-PCR is the exponential amplification of cDNA during PCR, which may lead to an inaccurate quantification of RNA content in the sample. Due to the very high sensitivity of this method, small levels of contaminations may lead to undesirable results. qRT-PCR assays are considered the gold standard to quantify specific gene expressions in a sample. However, poor standardization of the method may lead to bad quality of the data, and the study will then be almost impossible to replicate for others. It this study, simultaneous expression of the gene of interest along with a so-called “housekeeping-gene” was carried out to minimize the effects of inter-sample variation in RNA quantity and quality.

3.8.2 Protein Analysis

In this study (paper I-III) a range of different techniques were used to study proteins. Proteins are the main components of the physiological metabolic pathways of cells and are the functional expression of genes

(140). Four different approaches to obtain protein expression in different tissues were used; IHC, Western immunoblotting, ELISA and ECLIA. The methods are described in detail in paper I-III.

Immunohistochemistry

IHC was used to detect specific proteins in different tissue samples, HIF-1α in the aortic wall (paper II) and αB-crystallin and dystrophin in muscle tissues (paper I). IHC is a semi-quantitative method where antibodies bind specifically to antigens (e.g., proteins) in cells of a tissue section; the antibody-antigen complex can then produce a color reaction that can be visualized under a microscope (1). The advantages of IHC are that it is an excellent detection technique, showing exactly where the protein of interest is located in the tissue sample. For this reason it is widely used in the diagnosis of abnormal cells such those present in cancerous tumours. It is also widely used in basic research to look at the distribution and localization of biomarkers in different parts of a biological tissue. Disadvantages of ICH may include: difficulties in separating variants of the same protein, strong background staining, weak target antigen staining and autofluorescence. These aspects of the IHC method must be systematically addressed to identify and overcome staining problems.

Western Immunoblotting

Western immunoblotting was used to measure the protein level of αB-crystallin in cardiac tissue in paper III. It is a semi-quantitative method to detect specific proteins in sampled tissues of interest (141). Western immunoblotting is a reliable and widely accepted technique. However, the disadvantages of this method may include the fact that it is time consuming, while optimization of each step is necessary to obtain valid and reliable results. Antibodies can also be expensive.

ELISA

ELISA was used to measure the level of the protein, PAI-1, in the blood after diving (paper II). This technique is a commonly used method to detect the presence of a substance, usually an antigen, in a liquid sample (1). An advantage of ELISA is that it is a quantitative as well as a qualitative test. ELISA has high sensitivity, which enables detection of very small amounts of antigens in a sample. For this reason, ELISA is a widely used tool for determining antigen or antibody concentrations or the presence of an antibody/antigen in serum (e.g., in HIV). It is also used in the food industry to detect food allergens, and in toxicology when screening for drugs. A disadvantage with the test is that due to its high sensitivity it can detect other, substances similar to that targeted, giving a false positive result. It is often necessary to decide a "cut-off" value between what is considered a positive and a negative result. It may also be impossible to separate different variants of the same protein of interest. An ELISA kit is often expensive and cannot usually be reused.

ECLIA

ECLIA was used to determine the level of cTnT in rat serum after diving (paper III). It is a high sensitivity in vitro method for quantitative determination of the presence of a substance. Advantages to the method is that it is fast, has high sensitivity, is qualitative and quantitative and the analysis can be done by an automatic analyzer. A disadvantage is that hemolysis may result in false low results. A study by Gencbay et al. (83) suggests that vascular bubble formation may lead to hemolysis. Thus, this is an important consideration when investigating the association between vascular bubble formation and cTnT in serum.

4 Results and Discussion

The primary findings of this study were that exposure to high levels of oxygen during simulated diving, together with bubble formation after simulated diving, seem to be involved in DCS pathology in rats. The alterations in gene expression in the arterial vasculature were characteristic of cellular responses to oxidative stress, activation of immune responses and coagulation. Furthermore, diving with subsequent venous gas bubble formation resulted in increased levels of cardiac stress-marker expression. Pre-dive eccentric exercise with subsequent muscle injury did not modify bubble formation or survival as hypothesized; however, pre-dive exposure to hyperbaric oxygen appeared to provide remarkable cardioprotection.

4.1 Eccentric Exercise Prior to Diving

The specific aim (paper I) of this study was to investigate the effect of exercise-induced muscle injury on post-dive vascular bubble formation and survival. It was hypothesized that eccentric exercise prior to a simulated dive would induce skeletal muscle injury that would lead to an increase of vascular bubbles and lower survival rates after decompression. The primary findings were that pre-dive eccentric exercise that caused myofibrillar injury with preservation of sarcolemmal integrity and inflammatory activation, did not increase vascular bubble formation or reduce survival after simulated diving in rats. Thus, our results do not endorse a link between exercise-induced muscle injury and increased vascular bubble formation after diving.

4.1.1 Eccentric Exercise-Induced Muscle Injury

Protein and gene expression analysis and additional microscopic examination of muscle tissues verified that the eccentric exercise protocol generated skeletal muscle injury that persisted through the decompression phase of the simulated dives. Compared to non-exercised skeletal muscles, the staining of

exercised muscles was more granular in appearance, more immunostained for total and phosphorylated αB-crystallin (fig. 2, paper I) and there were irregular sarcomere structures (fig. 3 I and II, paper I). In the micrographs, there was increased sarcomere distance (fig. 3A, paper I) and increased gene expression of αB-crystallin, NF-κB and TNF-α (fig. 5), indicating a greater degree of muscle injury in exercised animals.

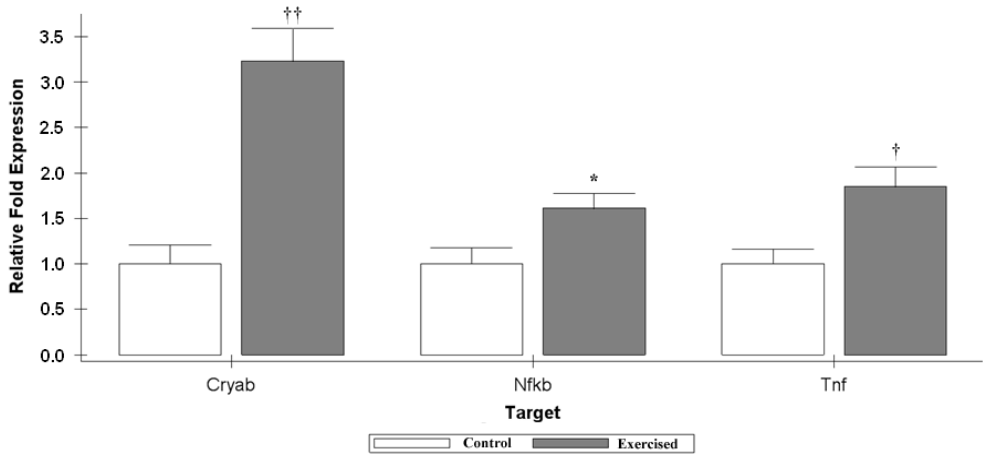

Figure. 5. *Gene expression of markers associated with muscle injury; αB-crystallin, NF-κB and TNF-α were increased in muscle tissue of eccentric exercised (gray bars) compared to non-exercised animals (white bars). *P < 0.05, †P < 0.005, ††P < 0.001 significantly different from non-exercised control (see paper I for details).*

Increased αB-crystallin Gene and Protein Expression

The small HSP αB-crystallin is thought to be involved in the protection of the cytoskeleton and the contractile machinery of muscles during exercise stress (72, 183). Koh and Escobedo (129) exposed mice to eccentric contractions and sampled the exposed muscles immediately after the contractions. They demonstrated that there is a correlation between αB-crystallin translocation and reduced force-generating

capacity which is known to reflect muscle damage (261). In contrast, muscles exposed to isometric contraction showed no evidence of translocation. Similar findings were found in humans 30, 60 and 240 min after eccentric contractions (205, 206), and in addition, there was an immediate strong immunostaining of αB-crystallin in exercised, but not sedentary muscles. Furthermore, Feasson et al. (72) showed that a muscle-damaging treadmill protocol in humans resulted in increased αB-crystallin levels, but Morton et al. (184) showed that a non-damaging protocol did not. In contrast to αB-crystallin, other HSPs (e.g., HSP70, HSP60) are increased in response to both damaging and non-damaging exercise regimens (183, 184). To avoid erroneous conclusions, care has to be taken when evaluating our results compared to others, where the results presented were found in different species, thus may not be directly comparable. However, previous findings demonstrate that increased αB-crystallin levels seem to be specific to eccentric contractions. Therefore, the finding in the present study (paper I), that increased αB-crystallin expression immediately after eccentric exercise reflects muscle injury, is in accordance with previous findings in mice and humans.

Increased Expression of NF-κB and TNF-α

To provide support for the αB-crystallin measurements, additional markers of muscle injury were evaluated. Liao et al. (149) demonstrated that a similar downhill treadmill protocol as used in the present study (paper I), resulted in an immediate increase in TNF-α gene and protein expression and activation of NF-κB activity in injured skeletal muscles of rats. Our results are in accordance with the results of Liao et al. and in addition, are comparable when it comes to sex, age, weight, strain and species, which are factors that may be important in influencing the exercise outcome. For example, studies have demonstrated that there are sex differences in the degree of muscle damage in response to eccentric exercise (42). A possible limitation of the study by Liao et al. was that the rats were accustomed to treadmill running every day for two weeks prior to the experiment. It is well known that exposure to concentric as well as eccentric exercise may lead to muscle adaptations, called “the repeated bout effect”, which could attenuate muscle

injury when a second more strenuous bout of eccentric exercise is performed up to several weeks later (43, 72, 91, 194, 205). Thus, we chose not to accustom the rats to treadmill running. A limitation of a protocol where the rats are unaccustomed to running is that it may lead to large variations in individual performance, with the potential of causing variation in the degree of muscle damage, and may, for that reason, hide exercise-induced changes due to a low number of tested animals. However, all of our rats completed the exercise protocol and the number of animals used in the present study (paper I) did suffice to demonstrate significant differences in markers of muscle injury between the groups.

Irregular Sarcomere Structures and Increased Sarcomere Distance

Irregular sarcomere structures and increased sarcomere distance were seen in some areas in sections of exercised muscles, but not in non-exercised muscles. In previous studies, light micrographs rarely show morphological changes immediately after eccentric exercise (72). Thus, light microscopy is probably not a good method to visualize disrupted muscle fibers. Moreover, mechanically induced muscle tissue trauma during tissue sampling can probably lead to similar results as seen after eccentric exercise, and must be considered. However, areas with increased sarcomere distance in light micrographs clearly show increased immunostaining of αB-crystallin (Fig. 3A, paper I), indicating that damage to muscle fibers was induced prior to tissue sampling. Increased sarcomere distance as seen in our study has also been observed previously in light micrographs immediately after eccentric exercise (6). Although signs of muscle injury were demonstrated in light micrographs, electron microscopic observations are probably a better method to visualize damaged muscle fibers. Numerous studies using electron microscopy clearly demonstrate that eccentric exercise leads to immediate disorganization of the myofibrillar ultrastructure with sarcomere disruption (72, 205, 237). For better visualization and direct verification of muscle injury, electron microscopy rather than light microscopy, should probably have been used.

Dystrophin Staining of Muscle Cells

To visualize the sarcolemma, muscle tissue was immunostained with dystrophin. In the early phase of exercise-induced myofibrillar damage, there is typically sarcolemmal disruption, disorganization of contractile components and cytoskeletal damage (137). One of the cytoskeletal proteins, dystrophin, has been shown to be vulnerable in the early phase after eccentric exercise (131). Dystrophin is attached to the sarcolemma and is important for force transmission during contraction (276). Disruption and loss of dystrophin have been seen after eccentric overload in anesthetized rat and mouse models using electric stimulation to induce muscle injury (129, 131, 160). In contrast, no significant loss of dystrophin immunostaining in any of the groups was observed (fig. 3C and D). However, it might be speculated that this could be due to differences in eccentric exercise protocols (electric stimulations *vs.* downhill running). Supka et al. (235) did not find any loss of dystrophin in a human eccentric exercise experiment, but Lehti et al. (137) did see a non-significant tendency of loss in rats after downhill running. Whether the exercise protocol in our study (paper I) resulted in an immediate or delayed damage to the sarcolemmal structures is not completely known; however, strong continuous dystrophin-staining indicates no gross injury.

Time Course of Muscle Injury

Skeletal muscles were examined 140 min after the exercise bout. However, muscle injury is a dynamic process, in that the degree and type of injury may change during the hours and days after unaccustomed eccentric exercise (207). The immediate damage caused due to mechanical stress leads to disruption of the sarcolemma and sub-cellular and segmental damage of the fibers in the affected skeletal muscle (6, 182). Sarcolemmal damage leads to an influx and elevation in intracellular calcium, which activates a number of proteolytic and phospholipolytic enzymes that cause cell necrosis. Damaged connective tissue and necrotic muscle fibers will secondarily attract neutrophils that release cytotoxic factors and generate ROS, which can cause further damage over the next hours and days through inflammatory processes (172, 176).

Peak injury is shown to be most prominent two days after the exercise bout in rats (6, 172). Thus, to investigate the impact of peak muscle injury and inflammatory damage on the risk of bubble formation and survival, we suggest a follow up study were the rats are exposed to a simulated dive two days after unaccustomed eccentric exercise.

Summary of the Protocol for Eccentric Exercise-Induced Muscle Injury

By using an intermittent downhill running protocol, gene and protein expression analyses and microscopic examination, it was demonstrated that we were able to induce injuries to skeletal muscles in rats that persisted through the decompression phase of the simulated dives.

4.1.2 Effects of Exercise on Vascular Bubble Formation and DCS Development

In the present study, there were no differences observed in bubble amounts or survival rates after diving in the exercised animals in comparison to the non-exercised, despite evident muscle injury at the time of decompression (fig. 1, paper I). However, maximum bubble grades in exercised animals were associated with decreased survival time and survival rate, but no such association was seen in non-exercising rats.

Dual Effects of Exercise

No previous experiments have investigated the effects of exercise-induced muscle injury on bubble formation and survival. However, there have been numerous studies investigating the effects of exercise prior to, during and after decompression from diving and to altitude, producing a range of findings. It has been found that physical exercise during and after decompression from diving may result in increased bubble formation and DCS symptoms (106, 173-175, 215, 250). However, other diving experiments have demonstrated protective effects of exercise prior to (11, 13, 22, 56, 123, 219, 272, 274), during

decompression from (59, 117, 118), and after diving (57). Studies examining altitude decompression demonstrate that physical exercise immediately prior to, during and after may result in increased bubble formation and DCS symptoms (48, 51, 99, 109, 113, 213, 265), and exercise in combination with oxygen-breathing prior to altitude decompression has been shown to reduce bubble formation and/or DCS risk (76, 262, 264).

This seemingly dual effect of exercise on bubble formation, harmful or protective, is a striking feature of decompression and knowledge of the mechanisms responsible for this duality is limited. From previous studies, it seems apparent that the timing of exercise and the choice of exercise modality become critical factors in whether or not the effects are harmful of beneficial. In addition, whether exercise is performed in relation to diving or altitude exposure seems crucial. Moreover, previous studies suggest that pre-dive eccentric exercise may increase DCS risk. However, there is a growing body of evidence that there might be other mechanisms occurring, simultaneously preventing DCS and protecting the body, for example exercise-induced production of nitric oxide (NO), discussed in detail in paper I.

The present study was designed to test the adverse effects of exercise in relation to diving, which is in contrast to previous experiments in our laboratory investigating the protective effects of exercise (272, 274). Even though we did not find any adverse effects of pre-dive eccentric exercise, we cannot exclude the possibility that it may enhance bubble formation under other circumstances. For example, choosing different diving protocols, exercise modalities, timing of the exercise bout, and animal species.

Altitude vs. Diving Decompression

When discussing the effects of exercise on bubble formation and DCS risk, it is important to differentiate between previous findings from diving and altitude experiments, because hyperbaric and hypobaric exposures are very different. There is a tendency to indiscriminately transfer information and inferences from one field of research to another, which may lead potentially to erroneous conclusions (233). The

differences between diving and altitude exposure are described in detail by Foster et al. (77) and Pilmanis et al. (214). However, despite such differences, the mechanisms investigated in the present study may be attributed both to diving and altitude.

The Choice of a Proper Diving Protocol

The diving protocol used in the present study resulted in high bubble loads and a 22% mortality rate during the first hour after diving (paper I: 21%, paper II: 19%, paper III: 25%). Since the rats were exposed to such high loads of bubbles, it is not unlikely that this may have "overshadowed" possible effects of the pre-dive interventions; exercise (paper I) and HBO-PC (paper III). For example, in paper III, the cardioprotective effect of HBO-PC was only evident in the rats with bubble grades less than 4, but no such protection was found in rats with higher bubble loads.

Rats have been used widely as an experimental animal to study simulated diving. Lillo et al. (151, 152) have studied the effects of different depths, gas mixtures, and bottom times on DCS outcome extensively. By comparing previous studies and using simple decompression theory, we can assume that similar vascular bubbles loads might have been provoked by using a shallower dive with a faster decompression rate, or by using a deeper dive with a slower decompression. In addition, an increase in bottom time (> 50 min), would likely have increased saturation in the slow tissues. However, even if alternative protocols induced similar bubble loads, the distribution of bubbles in the tissues might have been different (76, 247), so could change the site of any injury and have the potential to affect DCS outcome and survival. For example a shallow, long dive with a slow decompression is more likely to lead to bubble formation in slow tissues (e.g., bones and joints). However, a deep, short dive with a rapid decompression is more likely to affect the fast tissues (e.g., the brain, heart and muscles). Therefore, it may be speculated that a deeper and shorter dive with a more rapid decompression might have served the study better when observing the effects of exercise-induced muscle injury.

Type of Exercise

Results from altitude experiments have shown that the generation of micronuclei and tissue damage may be involved in the enhanced risk of bubble formation in relation to physical exercise (265). Findings from experiments by Harvey et al. (109) indicated that micronuclei formation through negative mechanical pressure was likely to be the most important mechanism. Physical activity or movements seem to generate short-lived micronuclei that might be trapped and remain stable in hydrophobic regions in tissues and on their surfaces; on injury, a momentary exposure of hydrophobic regions might occur (109, 110, 116, 150, 268). Reports from experiments involving trauma-induced muscle injury (110) , violent exercise (265) and humans performing deep knee squats (51) immediately before altitude decompression, seem to favor bubble formation. Deep knee squats have an important eccentric component, which is potentially disruptive to muscle tissues. Thus, eccentric exercise-induced disruptions or damage to muscle tissues could lead to exposure of hydrophobic surfaces that stabilize gas micronuclei, creating enhanced bubble growth when exposed to decompression (76). However, in diving experiments, rats have been exposed to both non-damaging concentric (9, 272, 274) and damaging eccentric exercise (paper I), while humans have performed level running involving similar amounts of concentric and eccentric contractions (11, 13, 56) immediately prior to diving without modifying bubble formation in either case. Thus, it seems likely that exercise-induced muscle injury does not play a significant role in the production of vascular bubbles in relation to diving. However, whether exercise-induced muscle injury will enhance local bubble formation in joints and muscles or enhance bubble formation in relation to altitude exposures is still unknown.

Altitude experiments have demonstrated that a high local concentration of metabolic CO_2 resulting from physical exercise is an important mechanism in enhancing bubble growth during and after decompression (107, 113). However, in diving conditions, metabolic gases like CO_2 and O_2 seem to play only a minor role in initiating bubble growth, due to the large fraction of dissolved N_2 (77). ***This reduced***

impact of CO_2 in hyperbaria might explain why exercise in diving experiments fails to induce bubble growth. The protective effects of physical exercise during (59, 117, 118) and after (57) decompression in diving experiments, may result from increased blood perfusion, which will improve the rate of denitrogenation and prevent bubble formation (213). However, in altitude exposures, tissues are already denitrogenated due to O_2 prebreathing (76) and the beneficial effect of increased tissue blood perfusion through exercise is attenuated. Instead, the negative effect of CO_2 production will probably tend to increase bubble formation.

Additional experiments indicate that the exercise modality is of importance in modifying bubble production in relation to diving. Rattner et al. (219) found that treadmill running prior to diving reduced bubble formation in mice, but swimming did not. Moreover, Jurd et al. (123) and Gennser et al. (85) showed that jogging on the spot combined with star jumps prior to diving reduced bubble formation, but ergometer cycling did not. Which type of exercise is most effective in reducing bubble formation after diving, or whether certain types of exercise modalities will increase bubble formation is still unclear. However, it seems that not only the type of exercise may be of importance, but also the timing of exercise.

Timing of Pre-Dive Exercise

We chose to end the exercise bout 140 min prior to decompression in the present study. Eccentric exercise closer to the dive could have resulted in increased bubble formation due to an increased number of micronuclei, as explained in the methodological considerations section. In addition, bubble formation could also have been increased when exercising closer to the dive, due to a possible increase in blood perfusion at depth increasing tissue nitrogen uptake and consequently DCS risk (59). However, Jankowski et al. (118) did not find any adverse effects of exercise immediately prior to decompression from diving. Moreover, since the diving protocol used in the present study is considered a saturation dive for the rat model, it is possible that a slight increase in nitrogen uptake due to exercise might have minor effects on bubble formation. To test whether a summation of these factors (increased nitrogen uptake, increased

numbers of micronuclei and induction of muscle injury) would increase bubble formation, the least possible interval between eccentric exercise and diving has to be performed. The timing of exercise seem to be a critical factor in whether or not exercise modifies bubble formation and DCS outcome, and is discussed further in paper I.

Anthropometric Considerations in Rats vs. Humans

It is not unlikely that characteristics related to species, such as fat tissue distribution, leg size and decompression dose play a significant role in susceptibility of bubble formation and DCS (47, 263). The fact that heavier rats are more susceptible to bubble formation may be due to increased body fat (275). Because the solubility of all gases, including N_2, is greater in pure fats and oils than in water and blood, great amounts of N_2 stored in adipose tissues can be washed out during decompression (135, 251). The pN_2 in our current diving protocol (pN_2 = 560 kPa) is very high compared to human diving protocols, so our protocol is likely to produce a massive N_2 tissue wash-out evolving to bubble formation and growth. Most of the N_2 wash-out may occur in adipose tissues mainly located in the abdomen rather than in the legs of the rats. Thus, the possible bubble producing effects of muscle injury may be smaller and not evident compared to the bubble production from adipose tissues. In addition, micronuclei may be generated during exercise in skeletal muscles (112, 116), but because humans have much longer limbs than rats, producing greater torque during exercise and potentially greater micronuclei production, increased effects of physical exercise may be found in human experiments compared to those on the rat. Therefore, careful interpretation has to be taken to translate these findings to humans, due to these possibly important anthropometric differences. Whether unaccustomed eccentric exercise in humans gives similar results as in rats is not known.

Summary of the Effects of Eccentric Exercise on Bubble Formation and Survival

No differences in vascular bubble amounts or survival rates were observed between the eccentric exercised rats in comparison to the non-exercised after diving, despite evident muscle injury at the time of decompression. It is apparent that the effects of exercise on bubble formation are dependent of proper timing and exercise modality. Yet there are no studies that have demonstrated any harmful effects of pre-dive physical exercise. Further studies are still needed to make any recommendations regarding the effects of exercise prior to diving.

4.2 Cardiovascular Effects of Diving and Vascular Bubbles

The specific aims of this part of the study were to determine the effects of diving and vascular bubbles on the cardiovascular system through identification of the vascular genes and pathways that are differentially expressed (up- or down-regulated) immediately after diving, and identification of markers of stress and injury in the blood, vascular wall and cardiac tissue. It was hypothesized that diving would lead to immediate early vascular gene expression changes that may be involved in DCS etiology. Further, it was postulated that diving-induced gas bubble formation would lead to elevated levels of cardiac stress-markers. The results indicate that the gene expression changes found after diving were characteristic of cellular responses to oxidative stress, with functions of up-regulated genes, including activation and fine-tuning of stress-responsive transcription, cytokine/cytokine receptor signaling, molecular chaperoning, and coagulation. In addition, elevated cardiac stress-marker levels were found after diving, and a strong positive correlation was found between the amount of venous gas bubble loads and cardiac stress-marker levels in the blood and cardiac tissue. Thus, it seems likely that both exposure to high O_2 levels and gas bubbles in relation to diving is involved in DCS etiology.

4.2.1 Diving-Induced Vascular Genetic Reactions

More than 22,500 transcripts were screened by use of a full genome microarray analysis of the aorta, one hour after a simulated dive. A total of 23 genes were significantly up-regulated, and 19 of these seem to be targets of transcription factors involved in hyperoxia and/or oxidative stress responses (fig. 6). The highest up-regulated gene, *Nr4a3*, is involved in pathways enhancing inflammation by induction of adhesion and activation of leukocytes and platelets as well as endothelial activation. This may indicate a genetic link between diving and inflammatory development in the vasculature. The second highest up-regulated gene, *Serpine1*, and its gene product, PAI-1, enhance coagulation and thrombus formation, indicating a genetic link between diving and procoagulant development. In addition, HIF-1α accumulated in the aortic vessel wall after diving, indicating that HIF-1 is involved in the observed genetic responses to diving.

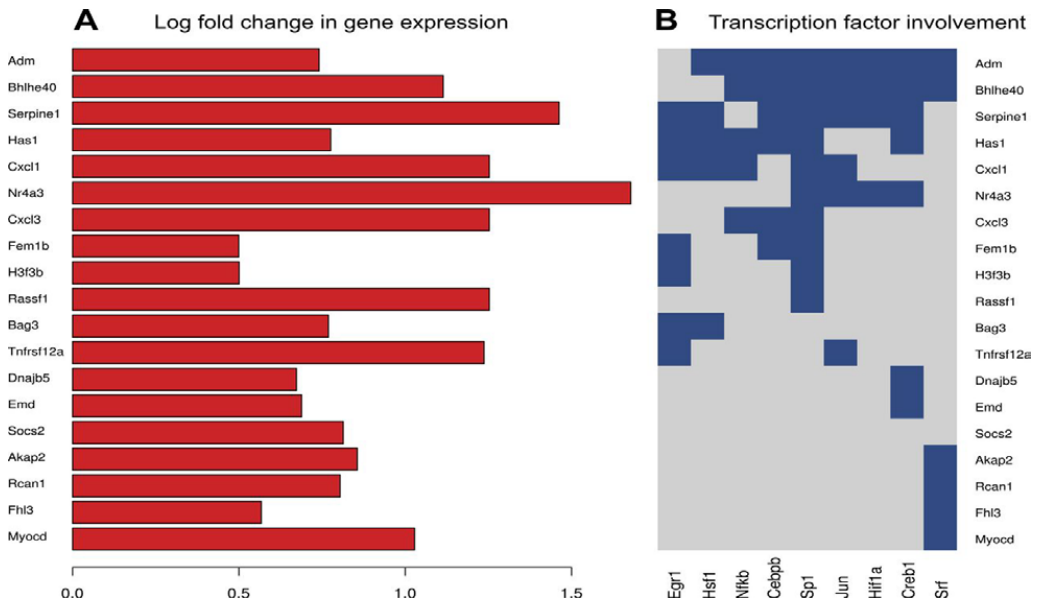

Figure 6. *Showing the 19 significantly upregulated genes in the aorta 1 hour after diving* (**A**) *that are targets of the top 9 transcription factors involved in differential gene expression* (**B**). *The potential involvement of the transcription factors shown in B in the activation of genes in A is indicated by blue squares. All of these transcription factors are involved in hyperoxia and/or oxidative stress responses (see paper II for details).*

Nr4a3 gene expression

When endothelial cells are activated, *Nr4a3* and its gene product, NOR1, cause transcriptional activation of chemokines and adhesion molecules that mediate activation, recruitment and adhesion of leukocytes and platelets to the endothelium (171, 283, 284), which may lead to the killing of endothelial cells through the release of ROS (138). This may in turn lead to endothelial dysfunction, which is an established sign of acute inflammation (65, 185). As mentioned previously, studies have shown that endothelial activation and/or dysfunction, as well as activation and adhesion of leukocytes to the endothelium, is associated with DCS development (24, 169, 197, 243). Thus, the increased expression of *Nr4a3* supports the idea that endothelial and leukocyte activation is involved in DCS development.

Serpine 1 gene and PAI-1 protein expression

PAI-1 is shown to promote thrombosis *in vivo* (23) and increased coagulation and thrombi formation is a hallmark in DCS etiology (210, 216, 217, 238). In addition, persistent elevation of PAI-1 has been observed in injured divers diagnosed with avascular bone necrosis, indicating that PAI-1 may be involved in the pathogenesis of DCS (177). The increased gene and protein expression of *Serpine 1* and PAI-1 in the present study support these previous findings. *Serpine 1* expression is stimulated by several inflammatory signaling pathways, as well as by hypoxia, hyperoxia and ROS (15, 132, 148, 266). Thus, hyperoxia during the dive, or tissue hypoxia after the dive could have contributed to the increased expression.

A possible link between PAI-1 and DCS development may be the formation of microparticles (MPs), which are cell fragments shed from the plasma membrane of different cell types. Recent studies have shown that the number of circulating MPs are increased after diving, and increased levels of MPs may lead to increased DCS risk through neutrophil activation and sequestration, vascular damage and tissue injury (162, 241-244, 277). Furthermore, Brodsky et al. (21) have shown that activated endothelium

will produce MPs that are able to initiate endothelial dysfunction at remote sites. MPs typically range in size of 0.1 to 2 μm, and so those formed on the venous side of the circulation due to venous gas bubble damage, are small enough to pass through the lung filter, entering the systemic circulation with the potential to affect the arterial vasculature. In addition, it is found that PAI-1 seems to promote the formation of MPs in a dose-dependent manner (20). Thus, the findings in the present study could potentially link PAI-1 to the recent findings of increased levels of circulating MPs after diving and DCS development.

HIF-1α

Increased levels of HIF-1α were observed in the aortic vessel wall one hour after diving in comparison to non-diving controls (Fig. 4, paper II). It has been shown that both hypoxia and hyperoxia may cause oxidative stress, leading to a stabilization of HIF-1α in vascular cells as an immediate reaction (229). HIF-1α is shown to play a central role in regulating the innate immune system and optimizing cell metabolism and homeostasis in the hypoxic environments (193). For example, HIF-1α regulates the expression of *Nr4a3* and *Serpine1*; thus, there may be a connection between HIF-1α accumulation in the vascular wall, increased gene expression of *Nr4a3* and *Serpine1* and DCS development.

Decompression-induced bubbles can lead to systemic hypoxia, which can further induce vascular inflammation (69, 102, 108). However, vascular bubbles may also trigger vascular inflammation, inducing a subsequent tissue hypoxia (69). At the site of inflammation, circulating leukocytes adhere to activated endothelium and transmigrate into the underlying tissues, where high immune activity leads to a rapid depletion of both nutrients and oxygen leading to hypoxia (130, 142). In addition, exposure to a seven-fold increase in pO_2 during diving, as in the present study, may induce significant amounts of oxidative stress (225, 229) and endothelial dysfunction (167, 200). Therefore it is suggested that there may be three possible ways that HIF-1α can be activated and accumulate in the vessel wall after diving: 1). hyperoxia

during diving leading to excessive oxidative stress; 2). insufficient oxygen supply from low O_2 content in the circulating blood induced by vascular bubbles after diving; 3). local tissue hypoxia from increased metabolism and inflammation in the vasculature triggered by hyperoxia and/or gas bubbles. However, this study does not fully explain or identify which of the potential mechanisms of HIF-1α accumulation are the most likely involved, and further investigations are needed.

The Role of Hyperoxia and Bubbles in the Observed Genetic Changes after Diving

Eight of the 19 up-regulated genes involved in hyperoxia and/or oxidative stress responses, seem to be involved in endothelial activation/dysfunction. It has previously been hypothesized that hyperoxia is the primary instigator of dysfunction of the vascular endothelium after diving (163), and a recent human study concluded that vascular function after diving was affected by hyperoxia and not by venous bubbles (167). Nossum et al. (195-197) demonstrated that diving and venous gas bubbles could lead to endothelial dysfunction on the venous side of the circulatory system, while a diving study by Brubakk et al. (24) demonstrated further endothelial dysfunction in the systemic circulation despite the fact that no arterial bubbles were present. These findings indicate that diving could trigger mechanisms that lead to systemic endothelial dysfunction, even though bubbles are only present on the venous side. In the study by Brubakk et al. (24), a comparison was made between a diving group and non-diving group breathing gases with a similar pO_2. It was found that both groups had the same degree of arterial vasodilatation. However, the dived group had less flow mediated vasodilatation, which indicated that factors other than hyperoxia explained the observed arterial endothelial dysfunction. There was also a non-significant tendency that subjects with many bubbles had less dilatation than subjects with few bubbles. In a follow up study, Obad et al. (200) demonstrated that a single dive with few venous gas bubbles, no arterial gas bubbles and no symptoms of DCS resulted in arterial endothelial dysfunction that lasted up to 48 hours after the dive, and that the endothelial dysfunction could be attenuated by pre-dive administration of antioxidants. Thus, oxidative mechanisms seem to play a major role in endothelial dysfunction after diving.

In contrast, a study by Marabotti et al. (166) showed that divers with detectable venous bubbles had significantly higher levels of circulating granulocytes in comparison to individuals without bubbles after diving. In addition, it has also been shown that hyperoxic exposures without diving are accompanied by an elevation in leukocytes (154, 223, 236). In conditions where hyperoxia is present in combination with bubbles, it seems that leukocyte levels are increased even more than in the presence of hyperoxia alone (166). Therefore, both hyperoxia and vascular bubbles may play important roles in DCS development. However, the results from the present study indicate that the acute genetic reactions in the arterial vasculature of rats exposed to simulated diving are triggered by hyperoxia and/or oxidative stress. Although the current study did not identify bubble-dependent genetic reactions, a larger group of animals would be required to conclude whether vascular bubbles affect the expression of genes not identified in this study or cause further disturbance of genetic pathways already activated by hyperoxia.

Summary of the Genetic Reactions after Diving

The acute genetic reactions observed in the arterial vasculature after diving seem to be triggered by hyperoxia and or/oxidative stress and may involve activation of inflammation and coagulation. These findings support previous studies that hyperoxia, oxidative stress, enhanced inflammation and coagulation plays important roles in DCS development.

4.2.2 Diving-induced Cardiac Stress and Injury

Strenuous simulated diving with subsequent VGE formation resulted in increased levels of the cardiac stress-markers: serum cardiac troponin T (cTnT) (fig. 2, paper III) the natriuretic peptide precursor B (*Nppb*) (fig 3, paper III) and the small heat shock protein αB-crystallin (fig 4, paper III). In addition, a strong positive correlation was found between the amount of VGE and stress-marker levels in serum and cardiac tissue.

Effects of Vascular Bubbles on Cardiac Stress-Markers

The diving protocol resulted in varying degrees of bubble formation, from no detectable bubbles through to massive bubble loads, with a positive correlation between VGE loads and the cardiac stress-markers, cTnT, *Nppb* and αB-crystallin. These results indicate that VGE formation may cause cardiac injury. In addition, the diving animals had significantly higher heart and respiration rates one hour after diving compared to non-diving animals, again implying that there were cardiopulmonary manifestations.

There are, to our knowledge, no previous studies showing an association between diving-induced vascular bubbles and the cardiac stress-markers measured in this study. However, the findings that chamber diving and vascular bubbles may lead to cardiac stress, are in line with previous studies (26, 27, 29, 186, 254). Circulating bubbles trapped in the lungs may cause a mechanical and a reactive increase in pulmonary vascular resistance, due to vascular obstruction and vasoconstriction mediated by vasoactive substances (17). Butler et al. (26, 27) demonstrated that increasing loads of injected VGE led to increased pulmonary artery pressure and pulmonary resistance in dogs. In the presence of high VGE loads, there was a "spillover effect" in the pulmonary circulation and gas bubbles became arterialized, resulting in a decrease in systemic blood pressure. In addition, they also showed ECG changes that indicated cardiac ischemia in animals both with and without arterial bubbles. Similar findings were observed in pigs in a study by Vik et al. (254), but they also found an association between VGE, decreased pO_2 and increased pCO_2, demonstrating that there was an increasing systemic hypoxia with increasing VGE loads. In another study by Butler et al. (29), where rats were exposed to a similar diving protocol compared to the present study, they found a decreased cardiac output and stroke volume as well as increased systemic vascular resistance. However, they did not investigate the association between gas bubble loads and the observed cardiopulmonary changes.

Based on these previous findings and findings in the present study, it seems the most likely mechanism of the rise in cardiac stress-marker levels and death, is as a result of bubbles trapped in pulmonary vessels causing impaired gas exchange, then leading to systemic, hypoxia-induced cardiac ischemia; this is in addition to pulmonary hypertension leading to right ventricular overload and acute heart failure (186). At even higher bubble loads, this may eventually create a reduced pulmonary venous return with reduced left ventricular pre-load and cardiac output and, finally, cardiovascular collapse and death. Furthermore, numerous non-diving studies have demonstrated that pulmonary embolism may lead to similar findings as in the present study (89, 133). For example, it is shown that a thrombus trapped in the pulmonary vessels can lead to an acute rise in the pulmonary artery pressure with right ventricular failure and subsequent elevation in *Nppb* expression and serum cTnT. Although cTnT is considered a marker of cardiac ischemia, and *Nppb* expression a marker of acute heart failure, both cardiac ischemia and acute heart failure may result in increased levels of each of these markers (19, 92, 103, 245). However, our findings, where high loads of bubbles were detected in the pulmonary artery and no bubbles were found in the aorta of surviving animals, suggest that the rise in the cardiac stress-marker levels is a result of gas bubbles in the pulmonary circulation and not in the coronary or systemic circulation.

To what extent diving-induced gas bubbles result in increased levels of cardiac stress-markers in humans is not known. Human chamber experiments did not show any increase in pulmonary artery pressure, despite relatively high loads of VGE (53, 249). However, reports from diving accidents indicate that highly provocative dives may progress into acute heart failure, cardiopulmonary collapse and death (78), while a diving-induced increase in serum troponins have been described in a case report (39).

Effects of Hyperoxia on Cardiac Stress-Markers

It is well known that increased O_2 levels, such as those found during exposure to normobaric hyperoxia and HBO, lead to hemodynamic changes and ROS production that may induce significant cardiac stress

and injury (8, 188, 230). In addition, several diving studies, including the results from the present study (paper II), suggest that hyperoxia is an important factor in DCS development (163, 167, 200), and that the combination of hyperoxia and gas bubbles is even worse than gas bubbles alone (166).

The present study was not designed to differentiate between the possible adverse effects of exposure to hyperoxia and gas bubbles on the heart. However, to our knowledge no studies have shown any association between exposure to high levels of O_2 and increased serum cTnT. Hyperoxia and HBO is a widely used treatment in pre- and in-hospital settings (18, 230, 246), and troponin is a widely used marker of cardiac injury (245); thus, an association between exposure to hyperoxia and cTnT release would most likely have been discovered if there is any.

We found increased *Nppb* expression after diving. Two hyperbaric experiments by Grassi et al. (97, 98), demonstrated that hyperoxia or HBO did not seem to contribute to increased BNP levels as seen after diving. Thus, hyperoxia does not seem to be involved in the increased *Nppb* expression levels found in the present study.

A few studies have investigated the effect of hyperoxia and HBO on ANP release, with conflicting results. A study by Rico et al. (222) showed that breathing air at 1, 2 and 3 ATA led to increased ANP release into the blood, but breathing of 100% oxygen at the same pressures blunted the ANP release. A study by Lund et al. (161) showed no effect on ANP release by HBO, and finally, Claybaugh (44) showed a decrease in ANP release during chamber diving. Furthermore, in line with our results, *Nppa* expression seems to remain unchanged one hour after cardiac overload and/or cardiac infarction (104, 164, 165, 187). However, there was a large variability in *Nppa* expression levels within each group in the present study, which could have hidden possible differences in *Nppa* expression between the groups (fig. 7). Thus, a larger group of animals would be required to conclude whether the simulated dive could affect the expression of *Nppa* not identified in this study.

To provide support for the cardiac stress-markers cTnT and *Nppa/Nppb* used in the present study, αB-crystallin expression was measured as a general indicator of cardiac stress. Protein levels of αB-

crystallin were increased in the heart after diving and were associated with increased VGE loads. Increased levels of αB-crystallin seem to be induced by a wide array of stresses, including cardiac ischemia and heart failure (40, 134, 136, 170). In addition, oxidative stress seems to play a key role in inducing αB-crystallin expression and activation (267). Thus, whether the increase in αB-crystallin was induced by gas bubbles and/or hyperoxia remains unclear, and requires further work.

In future work investigating the effects of the high pO_2 experienced during diving, groups exposed to HBO without subsequent diving and a group exposed to the same pO_2 as the diving animals will be included, with the latter achieved by breathing 100% oxygen at pO_2 = 149 kPa .

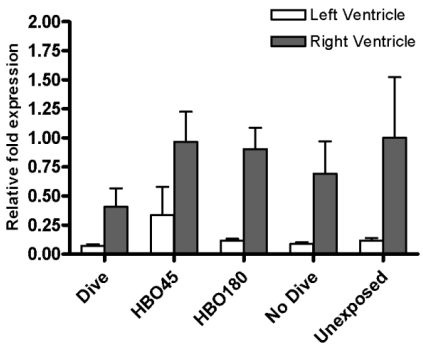

Figure 7. *mRNA expression of the atrial natriuretic peptide precursor (Nppa) in cardiac tissue was unchanged one hour after simulated diving. Differences were shown as the relative fold expression compared to the control gene Hprt. Values were expressed as the mean ± SEM, n = 6-8 in all groups.*

Immersion vs. Chamber diving

In the present study, we chose to study the effects of diving in a dry pressure chamber. Thus, the effects of immersion on the cardiopulmonary system were eliminated (see methodological considerations). The

acute effects imposed by water immersion during diving result in a number of significant changes to the cardiovascular system (16, 208). Immersion induces hemodynamic changes where blood is translocated from the limbs to the central parts of the body and the heart (80, 208, 252). Subsequently, pulmonary artery pressure is shown to be increased (58, 158), and distension of the atria and ventricles results in the release of ANP and BNP (16, 50, 81, 157, 168). Marinovic et al. (168) demonstrated that even low risk dives, with few VGE and no DCS symptoms, were associated with significant cardiac stress and increased levels of BNP. In addition, Grassi *et al.* (98) found that increased plasma BNP levels were observed after a wet-dive, but not after a hyperbaric chamber dive. Although it may be advantageous to eliminate the hemodynamic effects of immersion, allowing differentiation of the effects of immersion and gas bubbles on the cardiovascular system, it might also be a major limitation of this study, as cardiopulmonary stress would be ruled out in the dry chamber dives.

Summary of the Effects of Diving on Cardiac Stress-Marker Levels

Strenuous simulated diving led to increased levels of the cardiac stress-markers, cTnT, *Nppb* and αB-crystallin in the blood and cardiac tissue, indicating that such dives may lead to cardiac injury. ***In addition, a strong positive correlation between stress-marker levels and post-dive VGE loads was found, indicating that the injury was induced by gas bubbles.*** Oxidative stress induced by high pO_2 during the dive may have played an additional role in provoking cardiac stress and injury. However, more work is needed to determine the role of decompression-induced gas bubbles *vs.* hyperoxia behind the observed increase of the cardiac stress-markers in the present study.

4.3 Protective Effects of HBO-PC

The specific aim of this part of the study was to determine the cardioprotective effects of HBO-PC prior to diving. It was hypothesized that HBO-PC would protect the heart against the adverse effects of diving, as

indicated by lower levels of cardiac stress-markers found after diving in preconditioned animals in comparison to non-preconditioned. ***It was found that HBO-PC prior to diving appeared to provide cardioprotection, as indicated by the lower expression levels of the cardiac stress-markers, cTnT and αB-crystallin in blood and cardiac tissue*** (fig. 8). Further, it was observed that the HBO-PC effect was more pronounced when there was a longer (180 min compared to 45 min) interval between HBO-PC and diving.

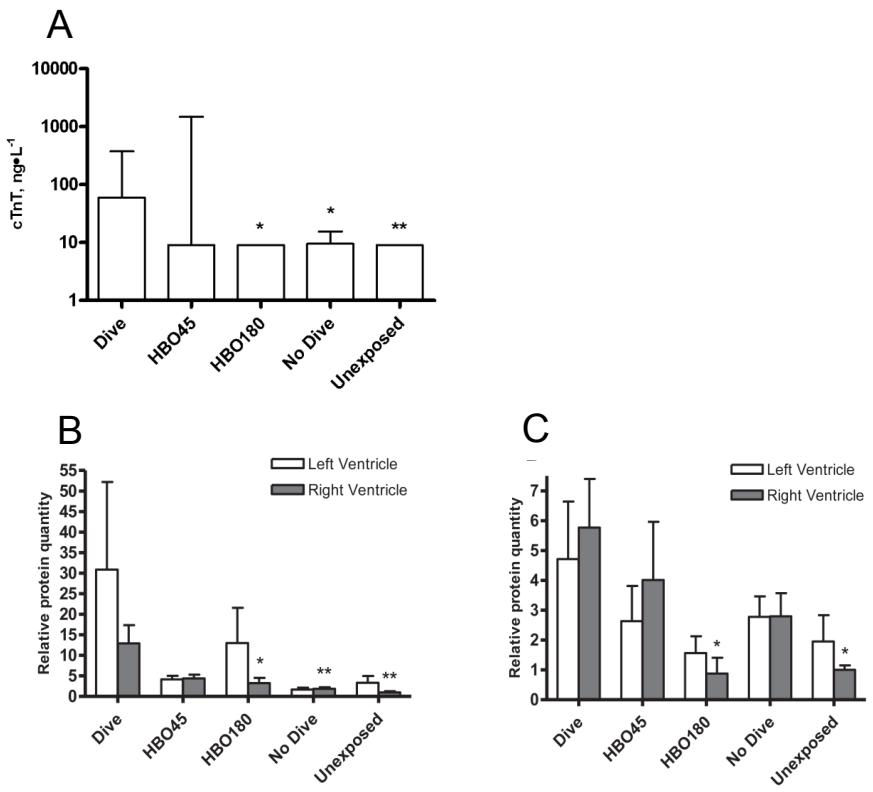

Figure 8. *Cardiac troponin T levels in the blood* (**A**), *total* (**B**) *and phosphorylated* (**C**) *αB-crystallin protein expression in the cardiac tissue were higher in the diving group in comparison to the HBO180, non-diving and unexposed animals. *P < 0.05, **P < 0.01 significantly different from the diving group (see paper III for details).*

Protective Mechanisms of HBO-PC in Diving

The findings in the present study were consistent with those previously observed in rats, demonstrating that HBO-PC may protect against DCS (28, 71, 169, 190). Martin and Thom (169) showed that simulated saturation diving resulted in CNS dysfunction in a cerebral model, most likely due to leukocyte sequestration in the brain, and that HBO-PC (45 min at ~280 kPa) inhibited leukocyte sequestration and protected against CNS DCS. This study does not mention whether there was an interval between HBO-PC and diving. However, the simulated dive was a saturation dive (120 min at 612 kPa) and therefore the HBO-PC did probably not affect the tissue pN_2 prior to decompression, and there were indications that the HBO-PC protocol did not modify gas bubble formation. Butler *et al.* (28) showed that HBO-PC (45 min at ~280 kPa) at both 1 and 18 hours prior to a simulated saturation dive (60 min at 683 kPa) resulted in fewer overall signs of DCS in comparison to control animals, and demonstrated lower levels of inflammatory markers in the blood, lungs and urine. Gross observation at autopsy showed that the animal group with the shortest interval between HBO-PC and diving had significantly less vascular bubbles than controls. Thus, in this group it is unknown whether the DCS protection and reduced inflammation was due to lowered bubble formation and/or other mechanisms. However, the 18 hour group had fewer overall signs of DCS, despite no reduction in bubble formation. Fan et al. (71) used the same 18 hour HBO-PC protocol and found indications that the protective effect was mediated via enhanced NO production. They showed that HBO-PC resulted in less signs of CNS DCS in comparison to controls, and found higher levels of NO in CNS immediately after HBO-PC. They further showed that inhibition of NO production prior to HBO-PC nullified the DCS protection. Their observations were consistent with previous findings, demonstrating a protective effect of NO (60, 178, 274, 275). However, Fan et al. did not measure the amount of bubbles after diving, thus, whether the protection of HBO-PC was due to reduced bubble formation or other mechanisms is unknown. A recent study by Ni et al. (190) showed that the same HBO-PC protocol did not seem to affect bubble formation.

A number of studies have investigated HBO exposure immediately prior to or during decompression and demonstrated protection against DCS in rats (4, 126, 127). These studies suggest that the protection was due to removal of micronuclei and hence less bubble formation during and after decompression. However, the dives used in these studies were probably not saturation dives (33 min bottom times), the amount of gas bubbles was not measured and the decompression profiles varied between the groups. Thus, it is unclear whether the observed protection in these studies was a result of micronuclei removal or other mechanisms.

Cardioprotective Mechanisms of Preconditioning

The concept of cardiac preconditioning has been extensively studied in non-diving experiments (111), and HBO-PC is one strategy used to induce cardiac protection. Exposure to HBO-PC is thought to induce a cardiac stress response of a reduced intensity, thereby lowering the damage caused by subsequent, more severe stress (30, 111). Findings from non-diving studies demonstrate that HBO-PC can protect the heart against injury (145, 278), and one possible protective mechanism may be HBO-PC-induced NO production that reduces tissue neutrophil sequestration, adhesion and the associated injury (121, 255, 279). There are also indications that the involvement of HSPs plays an important role in the cardioprotective effects of HBO-PC (40, 192, 239). From CNS studies, it is suggested that HBO-PC-induced ROS and NO production may induce a long lasting increase in HSP production (66, 153, 239, 240). Two recent diving studies demonstrate an association between HBO-PC-induced increase in NO and HSP70 levels and reduced DCS risk in rats, suggesting that HSPs may be involved in the protective effects of HBO-PC against CNS DCS (71, 190). However, whether HSPs are involved in the cardioprotective effects of HBO-PC in relation to diving, or whether HBO-PC protects humans against the adverse effects of diving is not known.

Characterization of the Cardioprotective Effects by αB-crystallin

One of the most interesting HSPs associated with cardioprotection is αB-crystallin (40, 134, 136, 170). In the present study, increased αB-crystallin protein levels were found in diving animals, with lower levels in animals exposed to HBO-PC and diving, but with no affection of αB-crystallin gene expression. ***The unaltered gene expression may reflect that the acute responses to diving-induced stress were initiated via HSP stabilization and phosphorylation rather than by de novo transcription.*** In a non-diving study by Whittaker et al. (267), it was suggested that both cardiac ischemia and oxidative stress seem to induce the cardioprotective properties of αB-crystallin. In addition, αB-crystallin exhibits anti-apoptotic and immunomodulatory properties, and administration of αB-crystallin is likely to diminish the extent and severity of ischemic lesions, including cardiac infarction, stroke and arterial occlusion (2, 204). Thus, the high αB-crystallin levels in the heart after diving in the present study may reflect that the heart has been exposed to a high level of diving-induced stress, while the significantly lower levels noted after HBO-PC perhaps indicate that HBO-PC induced a mild stress to the heart activating the cardioprotective properties of αB-crystallin. ***The activation of αB-crystallin prior to the dive probably prevented a subsequent higher increase in αB-crystallin levels after diving, because the heart was already preconditioned against the stress from diving.*** Based on these findings, we may speculate that αB-crystallin is involved in protecting the heart from injury after diving, however further studies are necessary to investigate the specific protective roles.

Summary of the Effects of HBO-PC on Diving-Induced Cardiac Injury

The present study indicates that HBO-PC may prevent cardiac injury induced by gas bubble formation after diving. Although these findings are from a diving study, vascular gas bubbles can also result from a reduction in ambient pressure in caisson work, aviation, extravehicular activity during spaceflight, or escape from pressurized vessels (84, 252), as well as from gas entry into the vasculature during in-hospital

procedures, e.g., cardiac surgery with extracorporeal bypass and through central venous and hemodialysis catheters (186, 246). HBO exposure is the main treatment for the adverse effects of gas bubbles, and it is generally accepted that the beneficial effects of HBO are rapid resolution of gas bubbles and increased tissue oxygenation (252). Thus, the implication of the present study is that HBO may not only be used in the treatment of DCS after diving but also in a prophylactic manner to prevent DCS and/or injury due to gas bubble formation for inpatients' procedures. However, the functional basis for the protective effects of HBO treatment is only partially understood, and whether the findings from the present study on rats can be translated to humans requires further investigation.

4.4 Study Limitations

Rats were used as experimental animals to investigate the mechanisms involved in bubble formation and DCS development. To further test the effects of eccentric exercise in rats, testing at time points other than 140 min prior to decompression is necessary. In addition, careful interpretation has to be made to apply these findings to humans, as there may important differences in the mechanisms of DCS development between rats and humans. We also chose to detect vascular bubbles instead of observing DCS manifestations; however, the correlation between low bubble loads and DCS manifestations has been shown to be weak (191). Thus, to better understand the mechanisms that are involved in DCS development, observations of DCS manifestations may have been preferred.

There were indications that pre-dive eccentric exercise and exposure to high levels of oxygen gas during diving may increase DCS risk, and that HBO-PC may reduce DCS risk, although their actual effect on DCS symptoms is unknown. Furthermore, the animals in this study were exposed to a simulated dive in a dry hyperbaric chamber, but divers are usually immersed in water, which leads to significant hemodynamic changes (208). Therefore the effects of in-water diving on the present findings are also unknown.

Recent studies indicate that HBO-PC protects CNS against DCS development (71, 169, 190), and the present study indicates that HBO-PC protects the heart. However, recreational diving accident reports (252) suggest that CNS DCS manifestations are more common than the cardiopulmonary manifestations investigated in the present study (paper III). Thus, one could argue that studying the heart has a limited impact on the search for mechanisms that protect against DCS, and that a study of the CNS would have been more relevant.

4.5 Novelties and Implications

To our knowledge, the present study is the ***first where the effect of exercise-induced muscle injury on bubble formation has been investigated*** (paper I). Our results do not endorse a link between exercise-induced myofibrillar disruption with preserved sarcolemmal integrity and vascular bubble formation after diving. This study may hopefully ***increase our understanding in how physical exercise affects the risk of bubble formation;*** this knowledge is important to understand how physical exercise may facilitate preventive measures to increase the safety of persons in risk of DCS development.

This is also the ***first full genome transcriptional study that describes the responses in the vasculature after diving*** (paper II). We found that genetic pathways sensitive to oxidative stress are induced after simulated diving. The observed responses are characteristic of endothelial activation and enhanced coagulation, and are consistent with previous studies. Reports of severe injury and casualties after diving persist, despite general adherence to procedures generally accepted as safe. Previous and present results show that DCS is a multifactorial disease initiated by events prior to (e.g., physical activity), during (e.g., high oxygen levels) and after diving (e.g., vascular bubbles). Our results indicate that DCS is a staged event that progresses when oxidative stress is followed by exposure to vascular gas bubbles during decompression. These findings may ***alter our understanding of the pathology of diving, underlining the potential risk triggered by breathing high pO_2.***

Furthermore, this study is the first to show ***that HBO-PC protects the heart against injury from vascular gas bubbles,*** and that ***it appears to provide protection against serious cardiopulmonary DCS*** (paper III). It is also the ***first controlled study that demonstrates an association between decompression-induced vascular gas bubbles and increased levels of the cardiac stress-markers; cTnT, Nppb and αB-crystallin.*** The implication of these findings is that ***HBO-PC may be used in a prophylactic manner to prevent cardiac injury due to vascular gas bubbles***.

4.6 Further Perspectives

The mechanisms behind the beneficial and harmful effects of physical exercise in relation to diving are still unknown. Investigation of the harmful effects of pre-dive eccentric exercise would necessitate testing in higher animal species as well as humans, while we should also try to gain a better understanding of the optimal intensity, timing and exercise modality needed to provide a certain degree of protection.

Global gene expression profiling allowed us to discover that the rat aorta responds to simulated diving by activation of target genes for redox and oxygen-sensitive transcription factors, which may be involved in DCS development. We suggest a similar follow-up study in humans. However, there are obvious limitations to the types of biological material that are available. Peripheral blood is accessible and suited for microarray-based transcriptome analysis and can be collected prior to and at several time points after diving. However, larger groups of animals and humans would probably be needed in follow-up studies, to investigate the effects of vascular bubbles on the observed changes in gene expression patterns not identified in this study.

We found that HBO-PC seemed to protect against the adverse effects of diving on the heart, and previous studies have also found that it offers some protection to the CNS. The exact protective mechanisms are unknown, but the involvement of HSPs seem to be central and should be studied further (e.g., HSP70 and αB-crystallin). Studies have also shown protective effects of oxygen pre-breathing (84), pre-dive physical exercise (272) and pre-dive administration of nitric oxide (274). Follow-up studies that combine the different protective regimes may lead to a better understanding of what provides optimal protection against DCS. HBO-PC may also be used to limit serious DCS and injuries in divers that have to do a rapid emergency evacuation from saturation diving (e.g., sunken submarine or underwater installations). The effect of HBO-PC in this regard can be investigated in anesthetized pigs prior to an emergency evacuation in a hyperbaric chamber.

5 Conclusions

I. The first aim of this study was to investigate the effects of eccentric exercise prior to diving on the degree of muscle injury at the time of decompression and the risk of vascular bubble formation and survival. It was hypothesized that exercise-induced skeletal muscle injury prior to decompression from a simulated dive would cause an increase in vascular bubbles and lower survival rates after decompression in rats. ***Despite evident muscle injury with inflammatory activation at the time of decompression, unaccustomed eccentric exercise with exercise-induced myofibrillar disruption and preserved sarcolemmal integrity, did not increase the amount of vascular bubbles after diving.*** However, the presence of bubbles in exercised animals was associated with a decrease in survival time and survival rate.

II. The second aim was to investigate the effects of diving and vascular bubbles on the cardiovascular system by identifying which vascular genes and pathways were differentially expressed (up- or down-regulated), and to measure the levels of cardiac stress-markers, immediately after diving. It was hypothesized that simulated diving would cause immediate early vascular gene expression changes that are involved in DCS etiology, and elevate levels of the cardiac stress-markers, cTnT, *Nppa*, *Nppb* and αB-crystallin. It was found that ***diving induced acute genetic reactions that involved activation of inflammation and coagulation*** processes in the arterial vasculature. In addition, it was observed that ***diving led to increased cardiac stress-marker levels with a strong positive correlation between their levels and the amount of detected post-dive VGE. The vascular genetic reactions seemed to be triggered by diving-induced oxidative stress, possibly due to hyperoxia and/or vascular inflammation.*** However, the elevation of cardiac stress-marker levels seemed to be induced by decompression-induced gas bubbles. Thus, the observed effects on the cardiovascular system after diving seem to involve a complex interplay between hyperoxia, oxidative stress, vascular inflammation and gas bubble formation.

III. The third aim of this study was to investigate the cardioprotective effects of HBO-PC in relation to diving. It was hypothesized that HBO-PC would have a cardioprotective effect, as indicated by lower levels of diving-induced cardiac stress-marker expression. ***When HBO-PC was performed 180 min before diving, it resulted in lower levels of the cardiac-stress markers cTnT and αB-crystallin in comparison to non-preconditioned diving animals, despite no differences in vascular gas bubble loads.*** This study indicates that HBO-PC prevents cardiac injury induced by vascular gas bubbles. Thus, HBO may be used in a prophylactic manner to prevent cardiopulmonary DCS.

The three papers presented and explained in this thesis describe important mechanisms that may be involved in the pathophysiology of diving. The work was performed using a simulated diving rat model, providing a translational approach to investigate possible processes in which the human body may respond to factors prior to, during and after diving.

Traditionally, the development of DCS is attributed to the formation of bubbles after diving. However, the intention of this study was to investigate the role of additional factors of importance in DCS development. Previous diving and altitude studies have revealed two prominent factors that may affect bubble formation and DCS outcome. They are physical exercise and exposure to high levels of oxygen, and both factors appear to have a dual effect, positive or negative, on bubble formation and DCS development. It is apparent that the beneficial or harmful effects of exercise are dependent on the type, intensity and timing of exercise. The protective or harmful mechanisms of exercise are still unclear. The present study advances our understanding of the effects of pre-dive exercise, but a more comprehensive knowledge of the mechanisms is necessary before pre-dive physical exercise could be recommended as a possible way to reduce DCS risk.

Having investigated one aspect of the effect of exercise on the pathophysiology of diving, the role of oxygen stress during a dive was observed. The present study indicates that exposure to high oxygen levels during diving is a likely contributor in DCS development. It further indicates that blood and tissue responses to diving are likely to be extremely multifactorial, including inflammatory and immune responses, as well as activation of coagulation and the endothelium. In addition, a strong positive association between the amount of vascular bubbles and markers of cardiac injury was found. Overall, these findings indicate that injury from diving may be initiated by oxidative stress during diving and then be further exacerbated by vascular bubbles after diving. Consequently, if the potentially harmful downstream events initiated by oxygen stress during diving could be prevented, it is possible that less injury from subsequent bubble formation would occur. Previous non-diving HBO-PC studies have shown that cardiovascular tissues could be protected from injury induced by various stresses, like oxidative stress and ischemia, and in line with these studies it was found in the present study that HBO-PC protected the heart against the adverse effects of diving. The exact cardioprotective mechanisms of HBO-PC are unclear. However, protection against the harmful effects of oxidative stress and vascular bubbles, or both, is possible. It is hoped that the basic and novel findings presented in this study may yield a new and broader insight into the mechanisms in the pathophysiology of diving. Whether these novel findings can be translated to humans is still unknown.

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7 Papers

Paper I

Exercise-induced myofibrillar disruption with sarcolemmal integrity prior to simulated diving has no effect on vascular bubble formation in rats.

Arve Jørgensen, Philip P. Foster, Ingrid Eftedal, Ulrik Wisløff, Gøran Paulsen, Marianne B. Havnes and Alf O. Brubakk.

Eur J Appl Physiol. 2013 May;113(5):1189-98. doi: 10.1007/s00421-012-2537-z. Epub 2012 Nov 6.

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Paper II

Early genetic responses in rat vascular tissue after simulated diving.

Ingrid Eftedal, Arve Jørgensen, Ragnhild Røsbjørgen, Arnar Flatberg, and Alf O. Brubakk.

Physiol Genomics. 2012 Dec 18;44(24):1201-7. doi: 10.1152/physiolgenomics.00073.2012. Epub 2012 Nov 6.

Physiol Genomics 44: 1201–1207, 2012.
First published November 6, 2012; doi:10.1152/physiolgenomics.00073.2012.

Early genetic responses in rat vascular tissue after simulated diving

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Submitted 1 June 2012; accepted in final form 5 November 2012

Eftedal I, Jørgensen A, Røsbjørgen R, Flatberg A, Brubakk AO. Early genetic responses in rat vascular tissue after simulated diving. *Physiol Genomics* 44: 1201–1207, 2012. First published November 6, 2012; doi:10.1152/physiolgenomics.00073.2012.—Diving causes a transient reduction of vascular function, but the mechanisms behind this are largely unknown. The aim of this study was therefore to analyze genetic reactions that may be involved in acute changes of vascular function in divers. Rats were exposed to 709 kPa of hyperbaric air (149 kPa P_{O_2}) for 50 min followed by postdive monitoring of vascular bubble formation and full genome microarray analysis of the aorta from diving rats ($n = 8$) and unexposed controls ($n = 9$). Upregulation of 23 genes was observed 1 h after simulated diving. The differential gene expression was characteristic of cellular responses to oxidative stress, with functions of upregulated genes including activation and fine-tuning of stress-responsive transcription, cytokine/cytokine receptor signaling, molecular chaperoning, and coagulation. By qRT-PCR, we verified increased transcription of neuron-derived orphan receptor-1 (*Nr4a3*), plasminogen activator inhibitor 1 (*Serpine1*), cytokine TWEAK receptor Fn14 (*Tnfrsf12a*), transcription factor class E basic helix-loop-helix protein 40 (*Bhlhe40*), and adrenomedullin (*Adm*). Hypoxia-inducible transcription factor HIF1 subunit HIF1-α was stabilized in the aorta 1 h after diving, and after 4 h there was a fivefold increase in total protein levels of the procoagulant plasminogen activator inhibitor 1 (PAI1) in blood plasma from diving rats. The study did not have sufficient power for individual assessment of effects of hyperoxia and decompression-induced bubbles on postdive gene expression. However, differential gene expression in rats without venous bubbles was similar to that of all the diving rats, indicating that elevated P_{O_2} instigated the observed genetic reactions.

hyperbaric; gene expression; oxidative stress; coagulation

DIVING IS A RELATIVELY NOVEL physiological challenge, it is increasingly popular, and today there are millions of recreational divers and underwater workers worldwide. However, even when performed in compliance with accepted procedures, diving is not without risk of adverse health effects. Divers may develop decompression sickness (DCS), a condition that usually appears shortly after surfacing, often within the first hour and almost always within 24 h (15). The symptomatology, epidemiology, diagnosis, and treatment strategies for DCS have been extensively described (41), yet there is limited knowledge of mechanisms distinguishing pathological development from normal physiological responses. During a dive, vascular function is challenged by altered breathing gas composition and by the demand for rapid gas exchange in body tissues in response to variation in ambient pressure. Healthy vasculature maintains its physiological homeostasis through the concerted action of proteins that regulate vessel structure and tone and controls the interactions with circulating blood cells and plasma components. When exposed to stressful stimuli, the vascular endothelium switches to an activated state to protect tissue structure and promote cell survival (11). Harmful external stimuli may lead to disruption of vascular homeostasis, resulting in a state of reduced endothelial function and enhanced coagulation as it is observed after diving (7, 35). The primary contributors to this effect are likely to be the high partial pressure of oxygen (P_{O_2}) in the diver's breathing gas and inert gas bubbles formed during decompression.

Gas bubbles formed during the decompression phase of a dive trigger innate immune reactions (5) that may have evolved as defense mechanisms against injury from gas forming infections and penetrating trauma (2). The expression of inflammatory markers rises progressively with increasingly stressful decompression from diving, which in turn correlates with higher vascular bubble loads and reduced endothelial function (7, 40, 43). Bubble formation appears to be intrinsic to diving pathology since DCS does not occur in its absence (14), but there is no simple correlation between bubble load and adverse effects of diving (10). Also, bubbles are less common in arteries, yet reduced endothelial function after diving is observed in both venous and arterial vasculature (7, 29). Onset of inflammatory reactions could also be attributed to the elevated P_{O_2} in the breathing gas, and it has been hypothesized that hyperoxia is the primary instigator of dysfunction of the vascular endothelium after diving (22). Strict regulation of oxygen supply and use is essential for cellular survival, and both high (hyperoxia) and low (hypoxia) P_{O_2} cause excessive oxidative stress, which activates host defense responses via oxygen-sensitive transcription factors such as hypoxia-inducible factor (HIF1) (34, 39).

The aim of this study was to analyze the genetic reactions in vascular tissue from rats exposed to pressure and breathing gas composition that are comparable to those experienced by human divers. Reduced vascular function in divers manifests itself shortly after diving (7), and the study therefore focuses on immediate early gene expression changes. Data obtained in this study are relevant not only to divers, but also to others who are exposed to extreme changes in ambient pressure and breathing gas oxygen content, such as astronauts and aviators.

METHODS

Ethical approval. The protocol was approved by the local Animal Research Ethics Authority at the Norwegian University of Science and Technology and conforms to the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes.

Animals. We used 42 adult female Sprague-Dawley rats (Taconic, Denmark), 16–18 wk of age and weighing 272 ± 16 g SD. Before

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experiments, rats were kept together three to a cage with a 12 h dark/12 h light cycle, temperatures of 22 ± 1°C, humidity of 55 ± 5%, and free access to water and a pellet diet. All procedures were performed at the same time of day to minimize circadian effects on gene expressions patterns.

Simulated diving. Rats were randomly assigned to a diving or control group on the day of diving. Simulated diving was performed in an air-filled pressure chamber according to a protocol that has previously been shown to generate high bubble loads without compromising postdive survival rates (45). During dives, the rats were breathing air and moving freely in a plastic cage with mesh roofing. Compression was made at a rate of 200 kPa/min to a total pressure of 709 kPa and a P_{O_2} of 149 kPa, corresponding to 60 meters below sea level, and the rats were maintained at that pressure for 50 min. Decompression to one atmosphere (101 kPa) was carried out at a linear rate of 50 kPa/min. Control rats were treated simultaneously and identically to those exposed to simulated diving, with the exception that controls were breathing only normobaric air. Out of a total of 21 diving rats, four died with massive bubble loads within 1 h after decompression. These were excluded from further analysis.

Body temperature during diving. To measure body core temperature in conscious rats during simulated diving, four additional female control rats had wireless thermal sensors (iButton, Maxim Integrated Products) implanted in the abdomen, and the temperature was logged every 5 min during diving. The temperature control rats were euthanized after diving and not used in analyses.

Anesthesia. Immediately after diving, the rats were anaesthetized with a mixture of midazolam 0.5 mg/100 g, fentanyl 5 μg/100 g, and haldol 0.33 mg/100 g given in one bolus as a subcutaneous injection. Rats were observed either for 1 h on a single dose, or for 4 h with regular addition of midazolam 0.3 mg·100 g^{-1}·h^{-1}, fentanyl 3 μg·100 g^{-1}·h^{-1}, and haldol 0.2 mg·100 g^{-1}·h^{-1}.

Postdiving physiological measurements. All postdiving measurements were done while the rats were kept under anesthesia. Circulating bubbles in the pulmonary artery (venous circulation) and the aorta (arterial circulation) were detected 15, 30, and 60 min after completion of decompression using a Vivid 5 ultrasonic scanner (GE Vingmed Ultrasound) with a 10 MHz transducer and graded from 0 (no detectable bubbles) to 5 (massive bubbling) according to the method described by Eftedal and Brubakk (13). For measurement of blood pH, partial pressures of CO_2 (P_{CO_2}) and O_2 (P_{O_2}), total hemoglobin (*c*tHb), hemoglobin oxygen saturation (S_{O_2}), fraction of oxygenated hemoglobin (FO_2Hb), and fraction of deoxyhemoglobin (*F*HHb), and calculation of total blood oxygen (ctO_2) and hemoglobin oxygen affinity (*P*50), capillary blood from a cut toenail was collected in 220 μl heparinized capillary tubes 1 h after diving, and immediately analyzed in a blood gas analyzer (ABL 700; Radiometer, Brønshøj, Denmark). Body temperature was measured with a rectal digital thermal probe, respiration rate was counted manually, and heart rate was recorded by ultrasonic Doppler.

Total RNA preparation. Since veins are exposed to circulating bubbles that may cause mechanical tissue injury, the genetic analyses in this study were performed on arterial tissue, i.e., the abdominal aorta (30). One hour after simulated diving, the aorta was dissected out and rinsed in RNAlater buffer solution (Ambion, Austin, TX), transferred to 1.5 ml fresh RNAlater, and kept for 4 h at room temperature before storage at −80°C until further analysis. For RNA extraction, samples were disrupted using an UltraTurrax rotor/stator (IKA Werke, Staufen, Germany). Total RNA was extracted using Ambion MirVana miRNA kit (Ambion) according to the manufacturer's instruction.

Microarray analysis. Total RNA from the aorta from eight diving rats and nine nondiving control rats was used in microarray analysis, providing adequate sample sizes for analysis of data from two groups (26). RNA concentration and quality were determined on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), and RNA integrity numbers in samples used in microarray analysis ranged from 7.4 to 9.4, indicating good quality RNA. The Illumina TotalPrep RNA amplification Kit (Ambion) was used to amplify RNA for hybridization on Illumina RatRef-12 v1 Expression BeadChips (Illumina, San Diego, CA). Microarray hybridization was performed according to the manufacturer's instruction.

Microarray data processing. Microarray probe measurements were log2 transformed, and samples were quantile normalized before analysis. Differentially expressed genes were identified using the open source Bioconductor Limma package (http://www.bioconductor.org). Transcription factor gene sets were ordered by a z-score based on hypergeometric distribution as implemented in MetaCore (GeneGo, St. Joseph, MI). *P* values were adjusted by controlling the false discovery rate. Microarray data were submitted to the ArrayExpress repository (http://www.ebi.ac.uk/arrayexpress/) according to the minimum information about a microarray experiment (MIAME) recommendations. The ArrayExpress accession code is E-MTAB-933.

qRT-PCR. For validation of microarray results, five genes were analyzed for mRNA expression levels by qRT-PCR using Qiagen QuantiTect rat primer assays with the SYBR Green RT-PCR kit (Qiagen, Valencia, CA). One-step qRT-PCR analysis was performed for the Nr4a3, Serpine1, Tnfrsf12a, Bhlhe40, and Adm genes with Hprt1 as housekeeping mRNA expression standard.

Immunohistochemistry. Small sections (<10 mg) of the abdominal aorta harvested from diving rats and controls ($n = 4$ in both groups) 1 h after decompression were formalin fixed, paraffin embedded, cut into 4-μm cross sections, and mounted on microscope slides. After epitope unmasking, two slides from each rat were stained overnight at 4°C with polyclonal HIF1-α anti-human primary antibody diluted 1:100 (NB100-134; Novus Biologicals, Littleton, CO). Rabbit Immuno-Cruz Staining System (sc-2051; Santa Cruz Biotechnology, Santa Cruz, CA) was used as secondary antibody and normal rabbit IgG as negative control. After hematoxylin counterstaining, results were documented on an Olympus BX51 microscope using Olympus Cell B analysis software version 3.3 (Olympus Soft Imaging Solutions, Hamburg, Germany), with all slides equally exposed.

Plasma plasminogen activator inhibitor 1 protein detection. Arterial blood was collected 1 (diving rats $n = 8$ and controls $n = 9$) and 4 (diving rats $n = 9$ and controls $n = 9$) h after completion of the simulated diving protocol, and plasma was prepared by centrifugation at 10 krpm at 4°C within 30 min of blood collection. Total plasminogen activator inhibitor 1 (PAI1) was measured with Imuclone Rat PAI1 ELISA kit (American Diagnostica, Stamford, CT). Each sample was analyzed in duplicates of 50 μl, with absorbance measured at 450 nm with 620 nm wavelength correction. Protein concentrations were calculated from standard curves by linear regression.

Statistical analysis. We used two-tailed Student's *t*-tests when comparing data from two groups. Values are presented as means ± SD unless otherwise stated. For all analyses, P values < 0.05 defined statistical significance.

RESULTS

Vascular bubbles and physiological status prior to gene expression analysis. The eight diving rats used in gene expression analysis had very different maximum venous bubble loads during the postdive observation: four rats had no observable bubbles at any time point (*grade 0*), whereas the remaining four had high bubble loads in the pulmonary artery (*grade 4*, >10 bubbles per heart cycle). No passing of bubbles into the arterial circulation was observed, and none of the nondiving control rats had bubbles. During diving, the rats experienced a sevenfold increase in ambient oxygen tension ($P_{O_2} = 149$ kPa). One hour after diving, the pH was higher and P_{CO_2} lower in diving rats compared with nondiving controls, but there was no difference in P_{O_2} (Table 1). However, the concentration of total

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Table 1. *pH, blood gasses, oxygen status, and heart and respiration rates 1 h after simulated diving*

	Diving	Control	*P* Value
pH	7.29 ± 0.053	7.22 ± 0.043	0.015*
Blood gasses			
P_{CO_2}, kPa	7.3 ± 0.8	9.0 ± 1.2	0.008*
P_{O_2}, kPa	9.9 ± 1.1	8.9 ± 1.5	0.20
Oxygen status			
ctO_2, Vol%	18.8 ± 1.8	15.6 ± 1.8	0.006*
*P*50, kPa	5.7 ± 0.5	6.4 ± 0.5	0.021*
*c*tHb, g/dl	15.0 ± 0.7	13.7 ± 0.3	0.004*
S_{O_2}, %	89 ± 5	80 ± 9	0.041*
FO_2Hb, %	89 ± 5	80 ± 9	0.038*
*F*HHb, %	11 ± 5	20 ± 9	0.042*
Heart and respiration rates			
Heart rate, beats/min	384 ± 30	326 ± 46	0.015*
Respiration rate, per min	52 ± 8	41 ± 6	0.005*

Values are means ± SD. pH, blood gasses, and oxygen status were obtained from samples of capillary blood, heart rate was measured by Doppler ultrasound, and respiration was counted manually. *Significant difference between the diving and control group ($P < 0.05$).

blood oxygen was higher, as was hemoglobin oxygen saturation, total hemoglobin, and fraction of oxygenated hemoglobin in diving rats, and the fraction of deoxyhemoglobin was lower. Oxygen *P*50 decreased after diving, indicating a left shift of the oxygen dissociation curve. Both diving rats and controls maintained heart rates in the normal range during anesthesia, while respiration rates fell. However, diving rats had significantly higher heart and respiration rates than controls. Body temperature was unaffected by diving; it deviated <0.5°C from what is normal in the rat (38) during dives, and there were no temperature differences between diving rats and controls 1 h after diving (34.3 ± 0.4 °C in diving rats vs. 34.7 ± 1.6 in controls).

Gene expression changes after simulated diving. More than 22.500 transcripts were screened in the microarray analysis; a list of the top 200 differentially expressed mRNAs representing 194 genes is shown in the online data supplement.[1] Of these, 23 genes were significantly upregulated after *P* value adjustment for false discovery. No downregulated genes were observed. Five upregulated genes were chosen for verification of microarray results by qRT-PCR analysis, selected on basis of their involvement in oxidative stress response in vascular cells. These were the HIF1 target genes *Nr4a3*, *Serpine1*, *Bhlhe40,* and *Adm* (6) and the Tweak cytokine receptor *Tnfrsf12a* (44), which all had $P < 10^{-4}$ in the microarray analysis. The qRT-PCR results confirmed increased mRNA expression for all five genes 1 h after simulated diving (Fig. 1).

To identify transcription factors likely to be involved in activation of the observed genetic reactions, the microarray data was analyzed for transcription factor enrichment using MetaCore. The top 9 transcription factors predicted from MetaCore analysis are shown in Fig. 2*B*, and their targets among upregulated genes are shown in Fig. 2*A*. The listed transcription factors EGR1, HSF1, NFκB, CEBPB, SP1, cJUN, HIF1-α, CREB1, and SRF1 are all involved in hyperoxia and/or oxidative stress responses in mammalian cells (1, 4, 8, 9, 17, 18, 23, 37, 47).

Gene expression in relation to vascular bubble loads. In an attempt to identify genetic reactions related to vascular bubble loads, we further divided the diving rats into two subgroups according to presence or absence of bubbles ($n = 4$ in both). Microarray data from each of these subgroups were analyzed against the nondiving controls and against each other. This revealed no additional significantly affected genes in the subgroup with high venous bubble loads, and the differentially expressed genes in the group with no bubbles were the same as those in the complete group of diving rats. However, the results from the two subgroups were not identical: when mRNA levels from significantly upregulated genes were displayed in a heat map (Fig. 3), controls, diving rats with no detectable bubbles and diving rats with high bubble loads clustered into distinct parts of the map even if their mRNA levels where not significantly different in *t*-tests.

HIF1-α protein detection after simulated diving. When vascular cells are exposed to significant changes in oxygen bioavailability, stabilization of hypoxia inducible transcription factors such as HIF1 is an immediate reaction (37). Under normoxic conditions, the HIF1 subunit HIF1-α is rapidly degraded by prolyl hydroxylase domain proteins (19), but

[1] The online version of this article contains supplemental material.

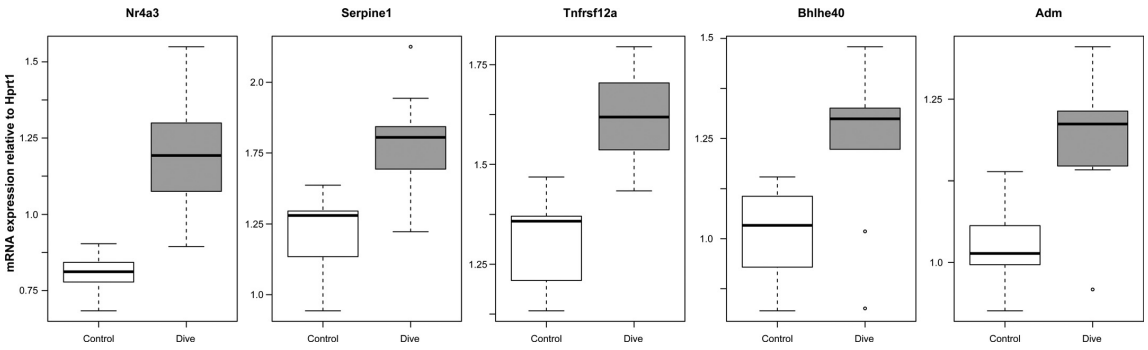

Fig. 1. qRT-PCR analysis of the *Nr4a3*, *Serpine1*, *Tnfrsf12a*, *Bhlhe40,* and *Adm* genes. The 5 selected genes were upregulated in microarray analysis of the aorta 1 h after simulated diving, and the qRT-PCR analysis confirmed elevated expression in the diving rats ($n = 8$) compared with controls ($n = 9$). Normalization was done relative to the *Hprt* gene. Bars and whiskers indicate lower and upper quartiles and min/max values, respectively. Singular dots are outliers deviating >1.5× from interquartile range.

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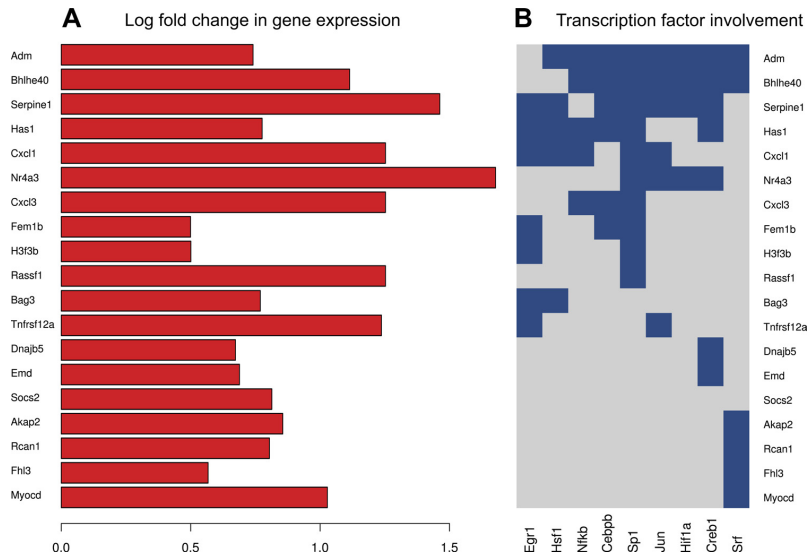

Fig. 2. *A*: log fold change in expression of genes that were significantly upregulated in microarray analysis of mRNA from the aorta 1 h after simulated diving ($P < 0.05$). *B*: the top 9 transcription factors involved in differential gene expression were predicted by GeneGo analysis of the complete set of microarray data. The potential involvement of the transcription factors in *B* in activation of genes in *A* is indicated by blue squares.

altered oxygen levels inhibits this degradation and HIF1-α is relocated to the cell nuclei to form an active transcription factor complex with HIF1-β. Nineteen verified targets for the transcription factor HIF1 were among the top 194 differentially expressed genes after simulated diving (6). The presence of HIF subunit HIF1-α in the aorta was examined by immunohistochemistry (Fig. 4). HIF1-α staining was markedly increased 1 h after simulated diving (Fig. 4*B*) compared with the control (Fig. 4*A*). There was no increase in mRNA transcription of either HIF1 subunit after diving (Fig. 4*C*), indicating

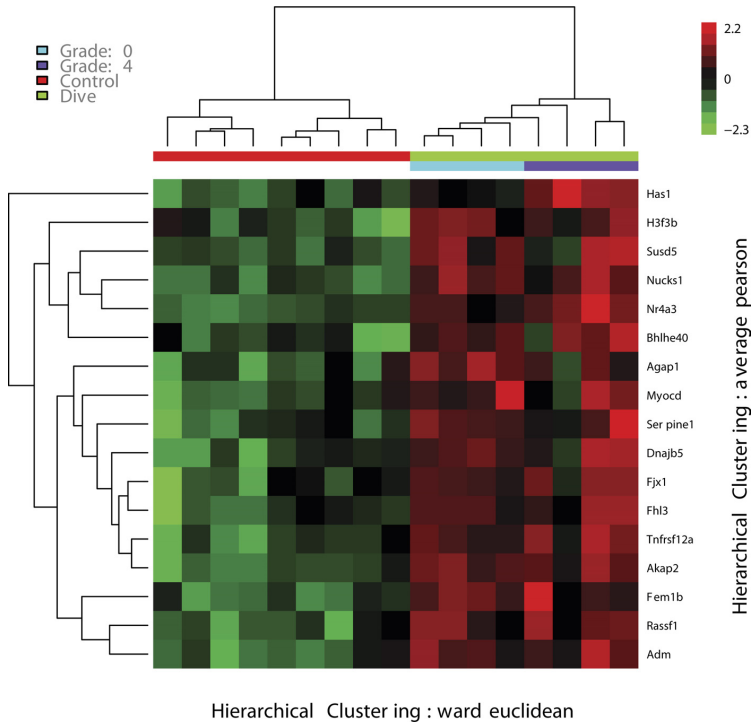

Fig. 3. Heat map displaying hierarchical clustering of the genes with highest expression fold-change in the aorta 1 h after simulated diving (rows) versus relative mRNA levels in each individual rat (columns). The color scale is relative to average mRNA level in each row, with green and red shades indicating lower and higher than average levels as indicated in the scale at *top right*. After cluster analysis, controls ($n = 9$) and diving rats ($n = 8$) fall into 2 distinct groups. Within the diving group the rats cluster in 2 equally large subgroups according to venous bubble loads, but *t*-testing revealed no significant difference in the identity of upregulated genes within these subgroups.

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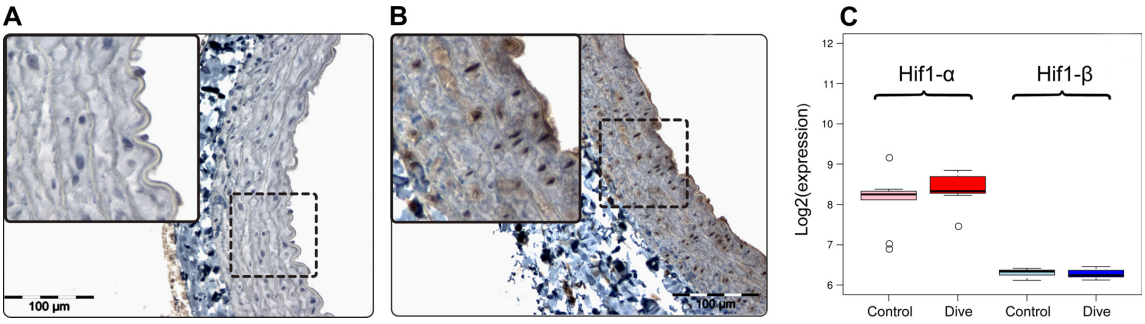

Fig. 4. Representative slides showing HIF1-α immunostaining of aortas harvested 1 h after simulated diving from nondiving control rat, no visible anti-HIF1-α staining (*A*) and rat exposed to simulated diving, displaying anti-HIF1-α staining of smooth muscle cells (brown spots) (*B*). *C*: mRNA expression of HIF1 subunit genes Hif1-α and Hif1-β. No mRNA expression change was observed, indicating that HIF1-α accumulation was caused by protein stabilization rather than by increased transcription.

that the cause of HIF1-α build-up was protein stabilization rather than de novo synthesis.

Total PAI1 in blood plasma after simulated diving. Serpine1 mRNA was significantly upregulated in the aorta after simulated diving (Fig. 1). The gene product of Serpine1 is PAI1, which is the major physiological inhibitor of fibrinolysis in blood and essential for maintaining tissue integrity in the vasculature (12). Elevated PAI1 is involved in the pathogenesis of a number of vascular disorders (16, 42). Levels of total PAI1 in blood plasma after diving were measured by ELISA at two time points: no significant change was seen 1 h after completion of dives, but after 4 h there was a fivefold increase in plasma PAI1 in diving rats compared with controls (Fig. 5).

DISCUSSION

The results from this study indicate that the acute genetic reactions in the arterial vasculature of rats exposed to simulated diving in hyperbaric air are triggered by high P_{O_2}.

The most upregulated transcript observed was that of the *Nr4a3* gene, which codes for transcription factor NOR1. When endothelial cells are activated, NOR1 causes transcriptional activation of chemokines and adhesion molecules that mediate recruitment and adhesion of leukocytes and platelets to the endothelium (25, 48). The protein products and function of other upregulated genes that are involved in endothelial activation included cytokine/cytokine receptor signaling (Tweak-receptor and suppressor of cytokine signaling 2 and Suppressor of cytokine signaling 2 encoded by *Tnfrsf12a* and *Socs2*), activation and fine-tuning of stress responsive transcription (DEC1, Adrenomedullin and Myocardin encoded by *Bhlhe40*, *Adm,* and *Myocd*), and heat shock protein HSP70-associated molecular chaperons (Heat shock protein Hsp40 and BCL2-associated athanogene 3 encoded by *Dnajb5* and *Bag3*).

Endothelial activation is also associated with a procoagulant development, in which platelet-rich clots bind to the endothelial surface. In this study, increased expression of the *Serpine1* gene was observed shortly after diving, followed by highly elevated blood plasma levels of its gene product PAI1. PAI1 expression is stimulated by oxidative stress (20, 21), and elevated plasma PAI1 protein levels are found in conditions such as atherosclerosis and metabolic syndrome and correlate with increased risk of cardiovascular disease (3, 16, 42). The physiological function of PAI1 in blood is to promote clot formation by blocking the active site of plasminogen activator tPA, inhibiting the formation of active plasmin needed for lysis of clots in thrombotic or injured vessels. Divers diagnosed with dysbaric osteonecrosis have been shown to have persistently elevated plasma levels of PAI1, supporting a role of PAI1 in diving-induced pathology (27). This is in apparent conflict with another study in which reduced plasma PAI1 was observed in

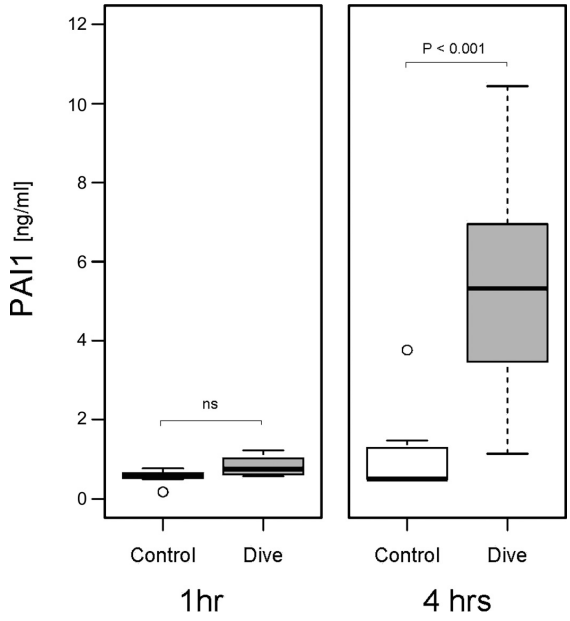

Fig. 5. PAI1 protein levels in blood plasma measured by ELISA 1 and 4 h after simulated diving. After 1 h, there was no significant difference (ns) in plasma PAI1 in diving rats [n = 8, (PAI1) = 1.44 ± 0.96 ng/ml] compared with nondiving controls [n = 9, (PAI1) = 1.08 ± 0.11 ng/ml]. Four hours after diving, plasma PAI1 was increased fivefold in the diving group [n = 9, (PAI1) = 5.18 ± 2.77 ng/ml] compared with nondiving controls [n = 9, (PAI1) = 1.06 ± 1.09 ng/ml], $P < 0.001$. Values are $\log_{10}$ transformed. Bars and whiskers indicate lower and upper quartiles and min/max values, respectively. Singular dots are outliers deviating >1.5× from interquartile range.

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human divers after exposure to hyperbaric air at 400 or 700 kPa for 30 min (36). In the latter study, intersample variation in PAI1 concentration before exposure was large relative to postexposure changes, and proteins were measured 15 min after the exposure, which is likely to be too early to detect PAI1 induction by ELISA-based assays. Expression of PAI1 in humans is variable and displays strong circadian regulation with plasma levels peaking in the morning, which may limit its usefulness as a biomarker for acute effects of diving (32). PAI1 does, however, appear to be involved in the etiology of diving-induced pathology, and we have shown that a single bout of hyperbaric exposure is sufficient to significantly increase its expression. Based on this, we suggest that individuals with prior vascular disease associated with high plasma PAI1 levels may be predisposed to developing DCS when PAI1 is further elevated after diving.

During diving and decompression, the diving rats maintained higher blood oxygen content compared with controls, and prolonged hyperoxia activates genetic pathways sensitive to oxidative stress (46). Increased levels of oxidative stress markers in rats exposed to similar P_{O_2} levels (33) as well as improved endothelial function after antioxidant intake prior to diving have been reported (31), supporting the involvement of oxidative stress in diving-induced reduction of vascular function. Several genetic pathways, such as those of HIF1 and NF-κB, are shared between responses to oxidative stress secondary to hyperoxia or to inflammatory reactions (28), and the accumulation of HIF1-α in the aortic vessel wall after diving indicates that the oxygen-sensitive transcription factor HIF1 is involved in the observed genetic responses to diving.

Limitations to interpretation of the gene expression data. In the design of this study, we took care to use rats that were similar and handled equally before, during, and after diving, and all samples were treated identically. However, the outcome with respect to venous bubbles after diving was binary, large venous bubble loads vs. no detectable bubbles, and it is reasonable to assume that high bubble loads represent a more complex exposure than bubble-free diving. Since the bubble-free rats had upregulation of the same genes as those observed for the complete group of diving rats, it may be concluded that high P_{O_2} was the primary instigator of the genetic reactions identified in this study. Interestingly in that respect, a recent human study concluded that vascular function after diving was affected by P_{O_2} but not by venous bubbles after identical dives performed with different breathing gas compositions (24). However, even if the current study did not identify bubble-dependent genetic reactions, a larger group of animals would be required to conclude whether vascular bubbles affect the expression of genes not identified in this study or cause further disturbance of genetic pathways already activated by hyperoxia.

Conclusions

In conclusion, differential gene expression in the rat aorta after simulated diving is characteristic of cellular responses to oxidative stress, of which high breathing gas P_{O_2} is likely to be a major contributor. The presence of increased amounts of circulating PAI1 after transcriptional activation of its coding gene *Serpine1* indicates a genetic link between diving and procoagulant development that may be involved in the etiology of diving-induced pathology.

ACKNOWLEDGMENTS

Arnfinn Sira, Marianne B. Havnes, Astrid Hjelde, and Svein Erik Gaustad assisted in simulated diving experiments. The Norwegian University of Science and Technology (NTNU) Microarray Core Facility performed microarray analyses, and the NTNU Animal Department provided animal care. The Funksjonell genomforskning (FUGE) Writer's Club and Lesley Blogg participated in manuscript preparation. All above are affiliated with the NTNU.

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GRANTS

This study was supported by Samarbeidsorganet HMN-NTNU, Kontaktutvalget St. Olavs Hospital University Hospital/NTNU, and FUGE Midt-Norge, Norway.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: I.E., A.J., A.F., and A.O.B. conception and design of research; I.E., A.J., and R.R. performed experiments; I.E., A.J., R.R., and A.F. analyzed data; I.E. and A.J. interpreted results of experiments; I.E. prepared figures; I.E., A.J., and R.R. drafted manuscript; I.E., A.J., R.R., A.F., and A.O.B. edited and revised manuscript; I.E., A.J., R.R., A.F., and A.O.B. approved final version of manuscript.

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Differential mRNA expression in the rat abdominal aorta after simulated diving

ProbeID	TargetID	Log Fold Change	Log Average Expression	T-score	P-Value	adjusted P-Value	Log Odds Ratio
1230128	Nr4a3	1,677982	8,220998801	7,6090012	5,7053E-09	4,8558E-05	9,698434953
2690528	Akap2	0,855798	7,99512279	7,17143279	2,0879E-08	8,885E-05	8,606919488
7040112	Tnfrsf12a	1,236998	10,00369264	6,50999796	1,5312E-07	0,0004344	6,910414118
4060600	H3f3b	0,500689	11,84899839	5,49990635	3,3507E-06	0,00588911	4,243837906
5390040	Serpine1	1,294005	9,678981857	5,48945143	3,4597E-06	0,00588911	4,215979756
2630575	Nucks	0,389313	8,691799211	5,31672203	5,8689E-06	0,00832504	3,755552276
3520435	Myocd	1,028143	7,857833266	5,06569956	1,2627E-05	0,01488871	3,086705998
4920719	Serpine1	1,462376	9,785028041	5,03191636	1,3995E-05	0,01488871	2,996802946
1780176	Has1	0,776011	6,727962282	4,90899318	2,0335E-05	0,01820335	2,670071702
2570551	Fjx1	0,997662	8,841365982	4,88697856	2,1739E-05	0,01820335	2,611633566
430239	Fhl3	0,56722	9,422645472	4,86091827	2,3527E-05	0,01820335	2,542491183
1990279	Dnajb5	0,673096	7,626941276	4,75425469	3,2491E-05	0,02038238	2,259941316
6900411	Adm	0,741026	8,64785459	4,75045851	3,2866E-05	0,02038238	2,249899871
1990253	Bhlhb2	1,114234	8,616853374	4,72834692	3,5136E-05	0,02038238	2,191433359
4730446	Rassf1	0,391155	8,379394609	4,72101025	3,5922E-05	0,02038238	2,172042404
50047	Socs2	0,81365	6,702111203	4,61629812	4,9246E-05	0,02619601	1,895779985
2350196	Fem1b	0,499465	7,88090017	4,58783482	5,3644E-05	0,02685694	1,820857917
130673	Centg2	0,393777	9,467937449	4,51868097	6,601E-05	0,03121158	1,639171299
4200452	Cnnm2	0,826081	8,232901275	4,42926299	8,6236E-05	0,03862921	1,4050343
4480093	Dscr1	0,80428	10,47520978	4,3834025	9,8864E-05	0,04151489	1,285329997
730020	Emd	0,688578	9,775500704	4,37148098	0,00010243	0,04151489	1,254257549
4920113	Cxcl1	1,252665	7,62869134	4,30574621	0,00012451	0,04817018	1,083275674
3390433	Bag3	0,769	11,54682839	4,2841393	0,00013275	0,04912189	1,027209056
60685	Zfand2a	0,567525	8,852693068	4,15912595	0,00019196	0,06807376	0,704240509
4280593	Rgd1359509	0,628354	10,78489768	4,12906007	0,00020968	0,06831037	0,626952136
580537	Gclc	0,548276	9,527324397	4,12441701	0,00021256	0,06831037	0,615030647
3440619	P2ry2	0,685993	7,816812347	4,11782291	0,00021671	0,06831037	0,598106245
6550400	Junb	1,008351	11,29836653	4,05909753	0,00025734	0,06942424	0,447730875
5570369	Ccl2	0,951248	7,673641738	4,05560437	0,00025997	0,06942424	0,438806344
2350193	Rgd1565319	0,807536	9,76607966	4,04909695	0,00026496	0,06942424	0,422186971
4280142	Nsun6	0,724199	8,571175125	4,03956683	0,00027243	0,06942424	0,397862543
6450056	Tpm1	0,627517	8,656955783	4,03633185	0,00027501	0,06942424	0,389609654
2370053	Snf1lk	0,901119	9,698813277	4,03513579	0,00027597	0,06942424	0,386558836
2480121	Rnf12	0,367782	6,935035146	4,03344404	0,00027734	0,06942424	0,382244145
3780438	Lmcd1	0,689702	11,07036457	3,92870802	0,00037594	0,09141887	0,116241774
6620446	Atf3	1,15273	7,77074126	3,90108067	0,00040719	0,09626748	0,046458485
6380465	Egr2	0,563253	8,956968236	3,85974937	0,00045871	0,10267949	-0,057624782
1050736	Klf4	0,584809	10,89647457	3,85553168	0,00046431	0,10267949	-0,068224381
5360324	Btg3	0,450512	9,026772427	3,85092412	0,00047051	0,10267949	-0,079799122
1740112	Verge	0,455842	7,252605777	3,81172476	0,00052656	0,11203819	-0,178073448
4200463	Cryab	0,864666	12,01311753	3,73317707	0,00065902	0,1336329	-0,373884642
6550138	Myc	0,602169	8,908085732	3,73295001	0,00065945	0,1336329	-0,374448452
2760673	Bmp2	0,547446	7,043972098	3,71990082	0,0006844	0,13546266	-0,406829116
6100242	Tgfb1i1	0,835849	8,006230496	3,69715354	0,00073008	0,14122116	-0,463170314
7050373	Egfl7	0,601506	8,130108269	3,65354718	0,00082605	0,14611149	-0,570796287
870487	Spsb2	0,439486	8,34366554	3,6455569	0,00084491	0,14611149	-0,590462116
5550270	Filip1	0,587073	8,361860403	3,64080216	0,00085633	0,14611149	-0,602156367
4760687	Niban	0,486462	6,967022426	3,63967952	0,00085905	0,14611149	-0,604916603
1050717	Hspb6	0,580304	10,65212094	3,63895411	0,00086081	0,14611149	-0,606699962
4060458	Ier5l	0,708744	7,995501605	3,63586209	0,00086835	0,14611149	-0,614299895
1940692	Dnajb1	0,444309	10,41062009	3,62492087	0,00089557	0,14611149	-0,641171431
4210059	Fbxo8	0,317589	8,321041453	3,61646038	0,00091717	0,14611149	-0,661927678
4590280	Prph1	-1,81774	10,23841162	-3,6156115	0,00091937	0,14611149	-0,664009223
1770619	Th	-1,38437	8,144646898	-3,61266	0,00092704	0,14611149	-0,671244708
5690403	Rit2	-1,21222	7,740794531	-3,5874225	0,00099521	0,15400485	-0,733014708
6550020	Rtn1	-1,01184	10,78967905	-3,577661	0,00102286	0,15545644	-0,756858093

ProbeID	TargetID	Log Fold Change	Log Average Expression	T-score	P-Value	adjusted P-Value	Log Odds Ratio
2510711	Anapc7	0,296034	6,578999885	3,57040955	0,00104387	0,15586698	-0,774552702
5860402	Siah2	0,451404	7,508518982	3,54711692	0,00111423	0,15874623	-0,83128767
7000075	Adamts1	0,875525	10,30659391	3,54261614	0,00112834	0,15874623	-0,842232214
2350452	Loc246187	0,434945	8,523360091	3,54050502	0,00113502	0,15874623	-0,847363773
2510300	Rgd1566420	-0,41981	6,989743938	-3,5286566	0,00117321	0,15874623	-0,876139609
6100017	Tgfb3	0,805018	9,248776405	3,51158422	0,00123043	0,15874623	-0,917529522
3360167	Slc35f5	0,480886	8,443486601	3,50814879	0,00124226	0,15874623	-0,925847734
6520338	Rgd1307524	0,576718	7,906556537	3,50668959	0,00124732	0,15874623	-0,929379831
6860735	Tmem5	0,33859	8,923933585	3,49967439	0,00127192	0,15874623	-0,946351599
1240538	Nfil3	0,62901	9,105300805	3,49863584	0,0012756	0,15874623	-0,948862886
1850053	Rgd1308165	0,390331	7,98025205	3,49747452	0,00127973	0,15874623	-0,951670666
6770037	Errfi1	0,908559	10,97597507	3,48879732	0,00131099	0,15874623	-0,972636862
2760017	Cyb561	-1,12876	9,482862392	-3,4887769	0,00131107	0,15874623	-0,972686186
1690239	Enah	0,416747	8,214912827	3,48304863	0,0013321	0,15874623	-0,986514401
5340279	Loc295062	0,37376	6,701693826	3,48254435	0,00133397	0,15874623	-0,987731285
3290021	Syt1	-1,74263	8,704485969	-3,4766468	0,001356	0,15874623	-1,001956698
3170167	Dbh	-1,9561	9,894095578	-3,4751655	0,00136159	0,15874623	-1,005528128
2760619	Stmn2	-1,63735	8,998481914	-3,4589162	0,00142435	0,16228615	-1,044659904
3140438	Abl1	0,418185	10,37886397	3,45563976	0,00143734	0,16228615	-1,052540404
1990132	Egr1	0,716783	10,97652682	3,45098716	0,00145598	0,16228615	-1,063724997
6420739	Ndrg4	-1,19418	9,830904805	-3,4479618	0,00146822	0,16228615	-1,070994114
1570538	Sfrs3	0,376161	7,643060775	3,4399208	0,00150125	0,16233934	-1,090300565
1500121	Rwdd1	0,375816	8,78293832	3,43830879	0,00150795	0,16233934	-1,094168543
5670044	Sfrs9	0,248541	7,941532267	3,43402226	0,00152592	0,16233934	-1,10444995
730358	Snai1	0,633746	8,96805436	3,40423901	0,00165659	0,17091296	-1,175724229
580170	Ibrdc1	0,404971	8,658003003	3,39902032	0,00168057	0,17091296	-1,188183651
2690093	Tubb6	0,582674	9,217794867	3,3968297	0,00169073	0,17091296	-1,193411022
1410746	Tnfrsf1a	0,356938	8,947833814	3,39025741	0,00172157	0,17091296	-1,209084806
5670114	Gap43	-1,48032	9,831104017	-3,379355	0,00177393	0,17091296	-1,235053909
2810451	Cd24	-1,16611	8,665867804	-3,378847	0,00177641	0,17091296	-1,236263007
3870538	Nefl	-1,69209	9,074582888	-3,3759027	0,00179082	0,17091296	-1,243268976
2470427	Arf4	0,315336	11,8442041	3,37208874	0,00180967	0,17091296	-1,252340222
780239	Sertad1	0,399481	7,534242134	3,37204283	0,0018099	0,17091296	-1,25244937
1990142	Tubb3	-1,17524	7,961338277	-3,3702998	0,00181857	0,17091296	-1,25659338
3130059	Tef	0,323094	6,801241638	3,36853268	0,00182741	0,17091296	-1,260793632
6590717	Loc367314	0,580025	10,80805805	3,34697001	0,0019386	0,17843205	-1,311961753
940600	Stmn3	-1,37636	8,768652424	-3,3448763	0,00194973	0,17843205	-1,316921842
1230332	Loc303332	-0,32354	8,462143706	-3,3352654	0,00200164	0,18066003	-1,339671174
6100040	Cenpb	0,32109	10,07485554	3,32928573	0,00203459	0,18066003	-1,353809664
6100725	Loc301509	0,793603	9,912248916	3,32871598	0,00203776	0,18066003	-1,355156132
730273	Zfp297	0,248577	9,291173179	3,32490651	0,00205906	0,18066619	-1,364156183
2680446	Rgd1561967	0,448397	9,972010843	3,30517397	0,00217282	0,18830282	-1,410695468
5670524	Atp1b1	-0,90711	8,973304501	-3,3022231	0,00219034	0,18830282	-1,417643648
770687	Pde5a	0,381138	7,044087456	3,29332243	0,00224401	0,19079998	-1,43858266
3190110	Pthlh	0,558592	7,893667229	3,28958284	0,00226693	0,19079998	-1,447371932
3610541	Wdfy1	0,624551	9,063566484	3,28238384	0,00231168	0,19079998	-1,46427816
1740092	Igsf4a	-0,46174	6,955762477	-3,2804197	0,00232404	0,19079998	-1,468887612
360156	Clk1	-0,54951	10,05295797	-3,2792421	0,00233148	0,19079998	-1,471650487
5220253	Lphn2	0,562811	9,835220281	3,27069844	0,00238613	0,19322004	-1,491681471
540687	Leng8	0,307878	7,446891367	3,26681842	0,00241135	0,19322004	-1,50076977
1050215	Klf9	0,501381	12,13128892	3,26410224	0,00242916	0,19322004	-1,507128811
2480402	Pdlim5	0,807154	9,090306736	3,2561946	0,00248171	0,19557281	-1,525626945
770398	Fstl3	0,846645	10,32497518	3,24398233	0,00256502	0,19716995	-1,554150846
6660112	Klf10	0,492417	8,074565346	3,2407929	0,00258721	0,19716995	-1,56159146
7100671	Eef1a2	-1,24704	8,165998828	-3,2371708	0,00261263	0,19716995	-1,570037045
2760022	Loc311772	0,569337	8,918708049	3,23409171	0,00263443	0,19716995	-1,577212735
5130594	Loc302022	0,3699	6,555519483	3,2313696	0,00265385	0,19716995	-1,583553646
4570156	Epb4.1l3	-1,14402	8,96063734	-3,230408	0,00266074	0,19716995	-1,585792971
6200053	Thrap1	0,226071	6,982808983	3,22788488	0,0026789	0,19716995	-1,591667064
5890484	Slc6a2	-1,5129	8,919903302	-3,2253587	0,0026972	0,19716995	-1,597545897

ProbeID	TargetID	Log Fold Change	Log Average Expression	T-score	P-Value	adjusted P-Value	Log Odds Ratio
4120142	Mrvi1	0,501685	6,998215021	3,22353656	0,00271048	0,19716995	-1,601784972
4010537	Pdcl3	0,357948	9,518939558	3,21516916	0,00277225	0,19995408	-1,621235263
6650014	Actg2	0,46413	13,74696465	3,20725525	0,0028319	0,20254003	-1,639607779
2260136	Xbp1	0,436011	10,52382275	3,19989269	0,00288848	0,2048658	-1,656679559
3850364	Esm1	0,599316	7,82668996	3,18906586	0,00297366	0,20916367	-1,681747503
5220079	Chrna3	-1,25725	8,108925891	-3,1822753	0,00302829	0,20951229	-1,697447791
2510280	Rasd1	0,615241	10,41594838	3,18082425	0,00304009	0,20951229	-1,700800466
6220377	Acta1	0,844485	8,987285923	3,17832331	0,00306052	0,20951229	-1,706577119
2230184	Mgc94288	0,3413	8,712633122	3,17630879	0,00307708	0,20951229	-1,71122852
630592	Samd4	0,469608	6,991907805	3,168647	0,00314083	0,21215553	-1,728905195
2810059	Syp	-1,30185	8,134346185	-3,1639062	0,0031809	0,21317042	-1,739831751
1500010	Rgd1565350	0,256601	7,671954506	3,16087842	0,00320675	0,21322353	-1,74680559
6020037	Dctn3	0,395628	8,358889124	3,15346846	0,00327084	0,21579948	-1,763858318
4060609	Lsamp	0,507076	7,710936369	3,14843072	0,00331511	0,21703773	-1,775439834
2640528	Fkbp7	0,424859	9,080639296	3,1347069	0,00343861	0,22340465	-1,806940882
6400278	Pdzk3	0,231636	6,517598716	3,12823903	0,00349832	0,22556183	-1,821761797
6860484	Tead3	0,510803	10,13014861	3,11736039	0,00360096	0,22843493	-1,846653251
630148	Slc35e4	0,271772	7,216649113	3,11596141	0,00361436	0,22843493	-1,849850941
4760066	Rgd1311824	-0,25151	7,125427213	-3,1132534	0,00364045	0,22843493	-1,856038495
3120364	Rgd1563141	0,721468	6,77873442	3,10993058	0,0036727	0,22843493	-1,863626946
780673	Ier2	0,503439	8,216640849	3,10785073	0,00369302	0,22843493	-1,86837457
5050204	Loc690038	0,476756	6,703392359	3,10411935	0,00372975	0,22843493	-1,876887869
5420427	Loc303471	-0,6238	7,707496126	-3,1040174	0,00373075	0,22843493	-1,877120361
3190086	Tnpo2	0,32832	6,799639746	3,09063315	0,0038654	0,22960099	-1,907611442
6620008	Rgd1559787	0,385638	10,76498752	3,08973137	0,00387463	0,22960099	-1,90966328
6040010	Loc304280	-0,99189	7,780114986	-3,0895781	0,0038762	0,22960099	-1,91001206
2340575	Ap4b1	-0,41542	8,046796094	-3,086735	0,00390547	0,22960099	-1,916478571
3780682	Vegfc	0,421162	8,211033968	3,08647565	0,00390815	0,22960099	-1,917068361
2260021	Rock2	0,420152	7,931703671	3,08613583	0,00391166	0,22960099	-1,917841027
6770273	Efhd2	0,482519	7,894579573	3,0826107	0,00394829	0,23016369	-1,925853658
70609	Cspg4	0,735829	10,98075794	3,07906734	0,00398544	0,23074863	-1,93390272
1740725	Ier3	0,700078	9,411364228	3,07557078	0,00402242	0,23131608	-1,941840579
4280048	Rgd1305302	0,225242	6,759099296	3,07080804	0,00407331	0,23144203	-1,952645031
60671	Loc499856	0,640271	9,677212651	3,07027961	0,00407899	0,23144203	-1,953843237
2350672	Rgd1564797	0,661791	7,735065724	3,06095253	0,00418056	0,23563413	-1,974973753
6980519	Resp18	-0,94267	8,305002017	-3,0571449	0,00422271	0,23604805	-1,983589749
7100079	Loc363498	-0,90471	7,151850477	-3,0507442	0,00429446	0,23604805	-1,998060435
6040746	Rgd1564516	0,597561	9,102152979	3,04942984	0,00430934	0,23604805	-2,001029947
6130484	Rgd1565472	0,276502	7,734354999	3,04939706	0,00430971	0,23604805	-2,001103986
2060739	Gaa	-0,36193	6,901698049	-3,0479124	0,00432658	0,23604805	-2,004457306
940273	Sat	0,359134	10,6281317	3,03725775	0,00444945	0,24120528	-2,028496094
6180279	Wsb1	0,425661	9,304548002	3,03059612	0,00452793	0,24311104	-2,043502352
1340435	Chgb	-1,06049	7,899863578	-3,0294364	0,00454173	0,24311104	-2,046112829
2350215	Dnaja1	0,453322	11,53303656	3,02634076	0,00457875	0,24329224	-2,05307861
940333	B4galt3	-0,2693	7,933220825	-3,0211202	0,00464183	0,24329224	-2,064816804
1450403	Rgd1560268	0,577134	10,17619102	3,02063216	0,00464777	0,24329224	-2,06591355
580112	Loc497806	-0,86985	7,204650851	-3,0192549	0,00466457	0,24329224	-2,069008037
4060161	Slc9a3r1	-0,51361	7,683623601	-3,0152986	0,00471314	0,24329224	-2,077893129
2650170	Dicer1	0,288186	8,540150774	3,01501626	0,00471663	0,24329224	-2,07852694
6020044	Mgc94190	0,315465	10,14924161	3,00932556	0,00478738	0,24545426	-2,091294987
2120450	Arhgef2	0,437426	10,64365765	3,0012755	0,00488918	0,24800846	-2,109333701
4590112	Pdlim7	0,547405	11,8498621	3,00078272	0,00489548	0,24800846	-2,110437051
5890025	Rgd1562228	-0,21739	7,715381367	-2,9957825	0,00495981	0,24978089	-2,121627084
2060102	Loc362919	-0,25955	8,373753429	-2,9818663	0,00514308	0,25633353	-2,152714722
2680333	Gmpr	-1,45897	9,859148684	-2,9803222	0,0051638	0,25633353	-2,156159141
450114	Agtr1b	0,693379	7,817070839	2,97707033	0,0052077	0,25633353	-2,163409585
4050593	Cnn1	0,571161	10,03413188	2,97283084	0,00526547	0,25633353	-2,172855475
510435	Pvr	0,502159	7,869925058	2,97221536	0,0052739	0,25633353	-2,174226153
5340072	Rab3b	-0,51005	6,78441217	-2,9709144	0,00529178	0,25633353	-2,177123021
6380059	Dync1i1	-1,07244	8,23878829	-2,9702625	0,00530075	0,25633353	-2,17857414

ProbeID	TargetID	Log Fold Change	Log Average Expression	T-score	P-Value	adjusted P-Value	Log Odds Ratio
2640215	Gadd45b	0,307564	7,021158793	2,96671961	0,00534979	0,25724342	-2,186458281
2370670	Crk	0,357994	6,752734595	2,96377038	0,00539094	0,25776571	-2,193017198
6400176	Ucp1	-1,73334	12,90979673	-2,9528422	0,00554602	0,26229339	-2,217288421
4850390	Zbtb7a	0,300895	9,585434555	2,95275553	0,00554727	0,26229339	-2,21748077
1400369	Bxdc5	0,193031	7,030654031	2,943515	0,00568169	0,26716519	-2,237963494
7100632	Ing1l	0,343309	8,335189112	2,93855878	0,00575505	0,26766986	-2,248934375
2030600	Stmn1	-0,6419	7,811973841	-2,9385402	0,00575533	0,26766986	-2,248975522
110050	Loc294560	0,280712	6,623353322	2,92500253	0,00596026	0,27569457	-2,278887593
5570132	Ccnh	0,265491	9,957704272	2,9192658	0,00604916	0,27829397	-2,291539088
4050576	Jun	0,493285	9,036937703	2,9113869	0,00617328	0,28185142	-2,308891393
770465	Ngfr	-0,77613	7,871210407	-2,9074399	0,00623635	0,28185142	-2,317573886
1240438	Jund	0,395686	12,77650316	2,90592798	0,00626067	0,28185142	-2,320898007
3520048	Pck1	-0,85809	12,97550207	-2,9023442	0,00631868	0,28185142	-2,328773231
3450088	Rgd1306433	-0,33021	7,788610762	-2,9008079	0,00634369	0,28185142	-2,332147483
130632	Foxp1	0,386863	8,99126351	2,90009559	0,00635533	0,28185142	-2,33371153
2940450	Hemk2	0,281967	7,886294757	2,89991378	0,0063583	0,28185142	-2,334110727
5910347	Metrnl	0,371135	9,983634037	2,89250615	0,00648051	0,28578023	-2,350362741
2650438	Mark1	0,466	8,548970038	2,88815819	0,00655326	0,28749889	-2,359890646
430100	Rgd1311381	0,498263	9,780096516	2,88352337	0,00663165	0,2885599	-2,370037893
4570114	Ccndbp1	-0,34746	9,874520871	-2,882548	0,00664826	0,2885599	-2,372172072
580286	Grn	-0,42005	9,931556478	-2,876007	0,00676066	0,2885599	-2,386473505
5910725	Ndn	-0,94894	9,941724943	-2,8751196	0,00677604	0,2885599	-2,388412288
520564	Rgd1562438	0,208002	6,787230251	2,87360064	0,00680245	0,2885599	-2,391730011
6040427	Amigo2	0,525725	9,697545827	2,87221285	0,00682667	0,2885599	-2,394760356

Paper III

Effects of hyperbaric oxygen preconditioning on cardiac stress markers after simulated diving.

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Physiol Rep. 2013 Nov;1(6):e00169. doi: 10.1002/phy2.169. Epub 2013 Nov 24.

Effects of hyperbaric oxygen preconditioning on cardiac stress-markers after simulated diving

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Running head: Effects of HBO-PC on cardiac stress-markers after diving

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Funding Information: This research was supported by grants obtained from the Fulbright Foundation, Statoil, ExxonMobil and Gassco under the Competence Program Diving, Samarbeidsorganet (HMN-NTNU), and the Norwegian Air Ambulance Foundation

ABSTRACT

Hyperbaric oxygen preconditioning (HBO-PC) can protect the heart from injury during subsequent ischemia. The presence of high loads of venous gas emboli (VGE) induced by a rapid ambient pressure reduction on ascent from diving may cause ischemia and acute heart failure. The aim of this study was to investigate the effect of diving-induced VGE formation on cardiac stress-marker levels and the cardioprotective effect of HBO-PC. To induce high loads of VGE, 63 female Sprague-Dawley rats were subjected to a rapid ambient pressure reduction from a simulated saturation dive (50 min at 709 kPa) in a pressure chamber. VGE loads were measured for 60 min in anesthetized animals by the use of ultrasonography. The animals were divided into five groups. Three groups were exposed to either diving or to HBO-PC (100% oxygen, 38 min at 303 kPa) with a 45 or 180 min interval between HBO-PC and diving. Two additional groups were used as baseline controls for the measurements; one group was exposed to equal handling except for HBO-PC and diving, and the other group was completely unexposed. Diving caused high loads of VGE, as well as elevated levels of the cardiac stress-markers, cTnT, *Nppb* and αB-crystallin, in blood and cardiac tissue. There were strong positive correlations between VGE loads and stress-marker levels after diving, and HBO-PC appeared to have a cardioprotective effect, as indicated by the lower levels of stress-marker expression after diving-induced VGE formation.

Keywords: cardioprotection; decompression illness; diving; gas embolism; hyperbaric oxygen preconditioning

INTRODUCTION

The formation of gas emboli as a result of a reduction in ambient pressure (decompression) is a major cause of injury associated with diving (Vann et al., 2011). High loads of decompression-induced venous gas emboli (VGE) may result in cardiorespiratory decompression illness (DCI) with cough, dyspnea, pulmonary edema, shock and in the most severe cases, fatal outcome. Circulating VGE are effectively trapped in the lungs, and may cause increased pulmonary artery pressure, cardiac overload and heart failure (Muth and Shank, 2000). Moreover, blood perfusion and pulmonary gas exchange are impaired, and the arterial partial pressure of oxygen decreases relative to the increased number of VGE, resulting in hypoxia with subsequent cardiac ischemia and cell death (Butler and Hills, 1985, Vik et al., 1990).

The phenomenon of preconditioning, in which a period of sub-lethal cardiac stress can protect the heart against injury during a subsequent ischemic insult, has been the subject of intense research over the last two decades (Yellon and Downey, 2003). Hyperbaric oxygen (HBO), which has been used as a preconditioning stimulus prior to ischemia, has been shown to provide wide-scale cardioprotective effects (Cabigas et al., 2006, Yogaratnam et al., 2010). HBO-preconditioning (HBO-PC) in rats exposed to simulated diving has recently shown promising results in reducing the incidence, severity, and complications of DCI (Martin and Thom, 2002, Butler et al., 2006, Katsenelson et al., 2009, Ni et al., 2013, Fan et al., 2010). However, the potential cardioprotective effects of HBO-PC in relationship to gas emboli formation have not yet been investigated.

In this study, rats were exposed to a simulated dive followed by severe decompression stress inducing high loads of VGE. First, we aimed to investigate whether a simulated dive with subsequent VGE formation would lead to increased levels of cardiac stress-markers indicating cardiac stress and injury, and second, investigate the effect of HBO-PC on these markers. Three

different cardiac stress-markers in rat serum and cardiac tissue were selected; serum cardiac troponin T (cTnT), a biomarker of cardiac injury (Thygesen et al., 2012); cardiac gene expression of the natriuretic peptide precursor B (*Nppb*), which is a biomarker of acute heart failure (Braunwald, 2008, Nakagawa et al., 1995); and the cardiac gene and protein expression of αB-crystallin, a small heat shock protein with a key role in protecting the heart from injury (Latchman, 2001, Whittaker et al., 2009, Christians et al., 2012). We hypothesized that high loads of VGE from simulated diving would result in an elevation of these cardiac stress-markers (cTnT, *Nppb* and αB-crystallin). We further hypothesized that HBO-PC would protect the heart from diving-induced VGE formation, resulting in lower levels of these stress-markers.

MATERIAL AND METHODS

Ethical approval. The experimental protocols were approved by the Norwegian Committee for Animal Experiments, and were performed according to the Guide for the Care and Use of Laboratory Animals published by the Directive 2010/63/EU of the European Parliament.

Experimental animals. A total of 63 adult female Sprague-Dawley rats (Taconic, Denmark), 285 g ± 17 SD, were randomly assigned to five groups:

I) Simulated diving, $n = 12$

II) Hyperbaric oxygen preconditioning (HBO-PC) followed by a 45 min normobaric air interval between HBO-PC and simulated diving (HBO45), $n = 12$

III) HBO-PC followed by a 180 min normobaric air interval between HBO-PC and simulated diving (HBO180), $n = 12$

IV) No diving, $n = 18$

V) Unexposed, $n = 9$

Groups I to IV were observed in anesthesia for 60 min after diving (gr. I-III) or no diving (gr. IV). Groups IV and V served as two different control groups. Group IV assessed the potential effect of anesthesia and handling without diving, and Group V was not exposed to anything (i.e., diving, chamber exposure, handling or anesthesia). All of the animals were housed in groups of three per cage in an animal facility. Light was controlled on a 12:12-h light-dark cycle at a room temperature of 21.0ºC ± 0.9 SD and humidity 51% ± 9 SD. The animals had free access to water and were placed on a pellet rodent diet.

HBO preconditioning. Animals in Groups II (HBO45) and III (HBO180) were exposed to 100% oxygen for five min at normobaric pressure (101 kPa) in a pressure chamber, followed by an increase in ambient pressure (compression) at a rate of 200 kPa·min^{-1} to 303 kPa. The animals were kept at that pressure for 38 min while breathing 100% oxygen. Because HBO exposure results in elimination of nitrogen gas (N_2) from tissues (Foster and Butler, 2009), the animals were exposed to air at the same ambient pressure (303 kPa) for seven min immediately after the HBO exposure. According to the exponential model proposed by Foster *et al.* (1998), and using a critical tissue half-time (whole rat) of 10 min (Lillo and Parker, 2000), this would cause N_2 tissue tensions to differ $\leq$ 0.7 kPa between the groups prior to the dive. The rats were then decompressed at a rate of 200 kPa·min^{-1} back to 101 kPa. The animals in the HBO45 and HBO180 groups were allowed to rest in their cages, breathing normobaric air, for 45 and 180 min, respectively, before simulated diving. The diving (gr. I) and non-diving (gr. IV) groups were exposed to normobaric air in a similar chamber at the same time while the HBO45 and HBO180 animals were exposed to HBO.

Simulated diving and VGE detection. The animals were compressed with air in a pressure chamber at a rate of 200 kPa·min^{-1} from 101 to 709 kPa, breathing hyperbaric air for 50 min to obtain tissue saturation (Lillo and Parker, 2000), and then decompressed linearly back to 101 kPa

at a rate of 50 kPa·min^{-1}. Immediately after diving, the animals were anesthetized with a mixture of; midazolam 0.5 mg·100 g^{-1}, fentanyl 5 µg·100 g^{-1} and haloperidol 0.33 mg·100 g^{-1}, which was administered as one bolus subcutaneous injection. The pulmonary artery and ascending aorta were insonated for 60 min using a 10 MHz transducer connected to a GE Vingmed Vivid 5 scanner. Gas emboli appeared in the pulmonary artery and aorta as bright spots and were recorded for one min at discrete time points (15, 30 and 60 min). The data were stored and played back in slow motion for analysis, in which the images were then graded (scan grade 0-5) according to a previously described method by an observer blinded to the experimental condition of the rats (Eftedal and Brubakk, 1997). Scan grades were converted to the number of emboli·cm^{2}·heart cycle^{-1} as previously described by Nishi *et al.* (2003). Animals that did not survive the 60 min post-dive observation period due to severe DCI were excluded from further analysis. Animals in Groups I-IV were handled equally except for the differences in pressure profiles and breathing gas compositions.

Serum cardiac Troponin T analysis. After the 60 min post-dive observation period, the abdomen was opened and blood from the abdominal aorta was collected into serum tubes. The serum used for the cTnT measurements was prepared by centrifugation at 10k rpm at 4ºC after blood collection. A high-sensitivity cTnT assay (hs-cTnT, Roche Modular System E, Roche Diagnostics GmbH, Mannheim, Germany) was used to detect an elevation in cTnT (Giannitsis et al., 2010). This assay permitted the measurement of concentrations ≥ 10 ng·L^{-1} (Omland et al., 2009).

Preparation of myocardial tissue for mRNA and protein analysis. Immediately after blood sampling, the thoracic cavity was opened and approximately 50 mg of myocardial tissue sections of the right and left ventricle were rapidly excised and rinsed in RNAlater buffer solution (Ambion Inc., Austin, TX, USA). The tissue was then transferred to 1.5 mL fresh RNAlater and

kept at room temperature for up to four hours before storage at -80°C. To prepare the lysates for mRNA and protein analysis, the myocardial tissue was thawed at room temperature, weighed and then transferred into 5 mL round-bottom polystyrene tubes containing 10 volumes per tissue weight ($\mu L \cdot mg^{-1}$) of RNeasy Fibrous Tissue lysis buffer (Qiagen, Valencia, CA, USA) and was mechanically disrupted using an UltraTurrax rotor/stator (IKA Werke GmbH & Co. Staufen, Germany) until completely homogenized. The lysate was split into two equal volumes: one part was used for qTR-PCR analysis, where the total RNA was extracted on a Qiacube nucleic acid extractor using the RNeasy Fibrous Tissue mini kit (Qiagen) according to the manufacturer's recommendations; and the other part was used for western blotting analysis, where 1% protease inhibitor solution (Qiagen) was added into the lysate, and the sample was precipitated by adding an equal volume of 10% ice cold TCA followed by incubation on ice for 20 min. After centrifugation (16000·*g*) the protein pellet was washed with 100% ethanol and then resuspended in 150 µL loading buffer (Invitrogen, Carlsbad, CA, USA).

Nppb and αB-crystallin gene expression. Prior to the analysis, the total RNA concentration and purity was determined using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) (Schroeder et al., 2006). The mRNA expression levels of the genes encoding for the natriuretic peptide, BNP (*Nppb*) as well as the small heat shock protein, αB-crystallin, were analyzed in the left and right cardiac ventricles for each of the five treatment groups (n = 6-8 from each group by random selection) with qRT-PCR using Qiagen QuantiFast FAM-labeled target probe assays with the QuantiFast Probe RT-PCR Plus kit (Qiagen) in a one-step qRT-PCR normalized against MAX-labeled *Hprt*. The PCR was run on a C1000 thermal cycler (Bio-Rad, Pleasanton, CA, USA) with a CFX96 Optical Reaction Module and analyzed on the CFX Manager Software version 2.0 using the $\Delta\Delta C_T$ method in which the

relative quantity of the target genes was normalized against the relative quantity of the control across the samples (Livak and Schmittgen, 2001).

αB-crystallin protein expression. Resuspended protein lysates from each of the five treatment groups (n = 5 from each group by random selection) were analyzed using 1D PAGE in 10% NuPage Novex Bis-Tris gels (Invitrogen) in one MOPS electrophoresis buffer. The gels were run at the same time before they were electroblotted onto nitrocellulose membranes and blocked for one hour in 5% fat-free dry milk diluted in PBS + 0.1% Tween (PBST). The membranes were first incubated together with primary antibody against αB-crystallin (ADI-SPA-222, Enzo Life Sciences, Farmingdale, NY, USA) for one hour in blocking buffer, washed for 3 x 10 min in PBST, further incubated for one hour in a secondary IRDye-conjugated antibody in PBST and finally washed 3 x 10 min in PBST and 1 x 10 min in PBS. As a loading control, the membranes were then similarly treated with an antibody against β-tubulin (AB6046, AbCam, Cambridge, MA, USA). The fluorescence signals were detected using an Odyssey scanner (Li-Cor Biosciences, Lincoln, NE, USA) and the signal intensities and relative protein quantification were calculated using Image Studio 2.0 (Li-Cor Biosciences).

Statistical analysis. The data were expressed as the median with ranges or as the mean ± SEM. We employed non-parametric tests due to the limited number of rats. The Mann-Whitney U-test and Kruskal Wallis test were used to evaluate the differences in VGE loads and the cardiac stress-markers, cTnT, *Nppb*, and αB-crystallin, between the groups. Fisher's exact test was used to evaluate the ratio of animals in each group, and the ratio of animals with low (grade 0-3) or high (grade 4-5) bubble grades, that had cTnT values above the detection limit. Selected bivariate relationships were examined using Spearman's rank correlation test. $P < 0.05$ was considered statistically significant. On the basis of the estimates obtained from previous studies (Wisloff et

al., 2004, Wisloff and Brubakk, 2001), 12 rats in each of the three diving groups would provide a power of 0.86.

RESULTS

VGE loads detected by ultrasonography. The diving protocol resulted in a 25% mortality rate in all of the animal groups during the first hour after diving. The animals that died had massive amounts of venous gas emboli (VGE) with a scan grade 5 (on a scale from 0-5) or approximately 10 emboli·cm^{2}·heart cycle^{-1}. HBO-PC had no effect on VGE formation, which was measured as the maximum amount of emboli·cm^{2}·heart cycle^{-1} (Dive: 3.9, HBO45: 4.1, HBO180: 4.4, P = 0.92, n = 12 in each group, Fig. 1). No gas emboli were detected in the ascending aorta of any of the animals that survived the observation period.

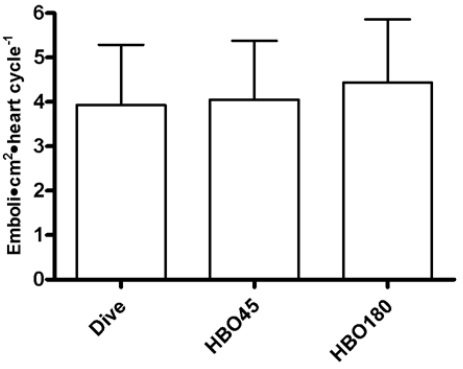

Figure. 1. The maximum number of venous gas emboli (VGE) in the pulmonary artery after diving, which was measured as emboli·cm^{2}·heart cycle^{-1}, did not differ between the three groups of diving rats (n = 12 in each group). HBO45/HBO180: hyperbaric oxygen preconditioning (HBO-PC) followed by a 45 or 180 min rest interval between HBO-PC and diving. The data are presented as the means ± SEM.

Serum cTnT levels. Animals exposed to simulated diving (gr. I) demonstrated significantly higher levels of serum cTnT compared to the non-diving (gr. IV, $P = 0.02$), unexposed (gr. V, $P = 0.0007$) and HBO180 (gr. III, $P = 0.01$) animals but were not different from the HBO45 (gr. II) animals (Fig. 2). A significantly higher percentage (80%) of non-preconditioned diving animals (gr. I) showed elevated cTnT levels (above the detection limit) compared to the preconditioned HBO180 (13%, $P = 0.02$) and unexposed animals (0%, $P = 0.0007$). There was a positive correlation between the amount of cTnT and VGE in all of the diving animals ($r_s = 0.66$, $P = 0.0002$).

Preconditioned animals appeared to tolerate higher VGE loads compared to non-preconditioned animals. For example, in animals with a scan grade of less than 4 ($n = 18$), elevated cTnT levels were found in only 1/12 of the preconditioned animals in contrast to 4/6 of the non-preconditioned animals ($P = 0.02$). All of the animals with a scan grade ≥ 4 ($n = 9$) demonstrated elevated levels of cTnT.

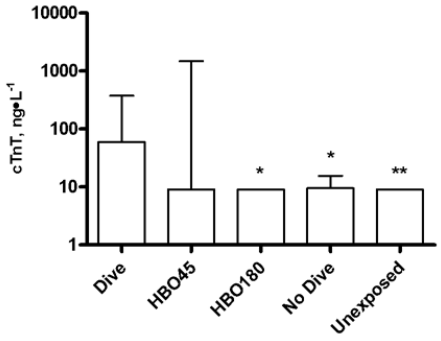

Figure. 2. Hyperbaric oxygen preconditioning resulted in reduced post-diving serum cardiac troponin T levels. Serum cardiac troponin T (cTnT) levels (ng·L^{-1}) were higher in the diving (n = 10) compared to the HBO180 (n = 7), non-diving (n = 18) and unexposed (n = 9) animals but were not different from the HBO45 (n = 9) animals. The cTnT level in the animals with values below the detection limit (10 ng·L^{-1}) was established at 9 ng·L^{-1}. The data are presented as the median ± interquartile range. All of the animals were handled equally and differed only in the pressure and breathing gas exposures, except for the unexposed animals, which were kept shielded in their cages until further blood sampling. HBO45/HBO180: hyperbaric oxygen preconditioning (HBO-PC) followed by a 45 or 180 min rest interval between HBO-PC and diving. *P < 0.05, **P < 0.001 significantly different from the diving animals.

Cardiac Nppb gene expression. The diving animals (gr. I), HBO45 (gr. II) and HBO180 (gr. III) showed a 2.0-fold (P = 0.02), 1.7-fold (P = 0.01) and 1.5-fold (P = 0.03) increase in *Nppb* expression in the left ventricle compared to the unexposed animals (gr. V), respectively (Fig. 3). No differences in *Nppb* expression were observed between the non-diving (gr. IV) and unexposed animals. *Nppb* expression levels were positively correlated with cTnT (left ventricle: r_s = 0.65, P = 0.00001, right ventricle: r_s = 0.38, P = 0.02) and VGE (left ventricle: r_s = 0.44, P = 0.04, right ventricle: r_s = 0.47, P = 0.02).

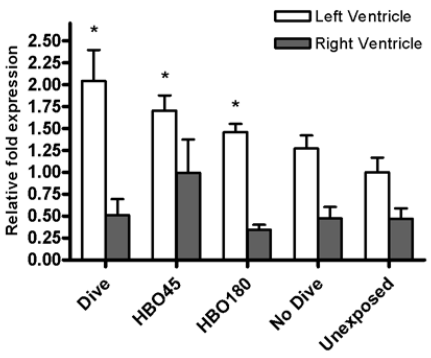

Figure. 3. Cardiac tissue level of brain natriuretic peptide precursor (*Nppb*) was increased after simulated diving. *Nppb* mRNA expression was increased in the left cardiac ventricle in all of the diving animal groups compared to unexposed animals. Differences were shown as the relative fold expression compared to the control gene *Hprt*. All of the animals were handled equally and differed only in the pressure and breathing gas exposures, except for the unexposed animals, which were kept shielded in their cages until further tissue sampling. HBO45/HBO180: hyperbaric oxygen preconditioning (HBO-PC) followed by a 45 or 180 min rest interval between HBO-PC and diving. Values were expressed as the means ± SEM, n = 6-8 in all groups. *$P < 0.05$ significantly different from the unexposed animals.

αB-crystallin gene and protein expression. None of the groups showed altered levels of αB-crystallin mRNA expression in cardiac tissue after simulated diving. However, the relative protein level of αB-crystallin in the non-preconditioned diving animals (gr. I) was increased by 4.0-fold, 6.9-fold and 12.6-fold in the right ventricle compared to the HBO180 preconditioning (gr. III, $P = 0.02$), non-diving (gr. IV, $P < 0.01$) and unexposed animals (gr. V, $P < 0.01$, Fig. 4). In addition, αB-crystallin in the right ventricle was positively correlated with cTnT ($r_s = 0.72$, $P = 0.00005$).

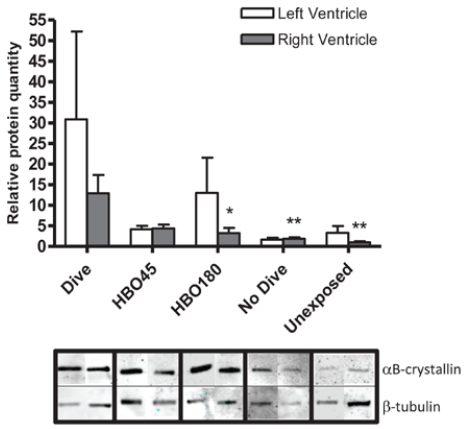

Figure. 4. Hyperbaric oxygen preconditioning resulted in reduced post-diving cardiac αB-crystallin protein levels. Relative protein levels of αB-crystallin in the right cardiac ventricle of diving animals was increased compared to HBO180, non-diving and unexposed animals. The differences were shown as the relative protein quantity compared to the β-tubulin control. For each group, a representative western blot from one animal is shown. All of the animals were handled equally and differed only in the pressure and breathing gas exposures, except for the unexposed animals, which were kept shielded in their cages until further tissue sampling. HBO45/HBO180: hyperbaric oxygen preconditioning (HBO-PC) followed by a 45 or 180 min rest interval between HBO-PC and diving. Values were expressed as the means ± SEM, n = 5 in all groups. *$P < 0.05$, **$P < 0.01$ significantly different from the diving group.

DISCUSSION

The primary findings of this study were that strenuous simulated diving with subsequent venous gas emboli (VGE) formation resulted in increased levels of cardiac stress-marker expression (cTnT, *Nppb* and αB-crystallin) in rat serum and cardiac tissue. Moreover, HBO-PC prior to the dive appeared to provide cardioprotection, as indicated by the lower expression levels

of these stress-markers. The HBO-PC effect was more pronounced when there was a longer (180 min compared to 45 min) interval between HBO-PC and diving. In addition, a strong positive correlation was found between the amount of VGE and stress-marker levels in the serum and cardiac tissue.

Elevated serum levels of cTnT induced by simulated diving were positively correlated with VGE loads. Cardiac troponins are components of the contractile apparatus in cardiomyocytes and demonstrate nearly absolute cardiac tissue specificity and high clinical sensitivity (Omland et al., 2009, Thygesen et al., 2012), and are the preferred biomarkers for the diagnosis of cardiac injury. Thus, our findings indicated that VGE formation after diving induced cardiac injury. The diving protocol resulted in a 25% mortality rate due to the massive amounts of VGE (scan grade 5), and all of the surviving animals with a scan grade ≥ 4 showed elevated cTnT levels. Experiments by Butler and Hills (1985) and Vik et al. (1990) demonstrated a proportional relationship between VGE loads and impeded gas exchange with systemic hypoxia and cardiac overload. These previous findings and the increase in cardiac stress-markers in the present study, indicate that the diving protocol induced severe decompression stress with cardiorespiratory DCI. However, in the rats that died, there may of course have been injuries to other organ systems (e.g., brain and spinal cord) that contributed to the cause of death. However, in surviving rats no gas emboli were detected in the systemic circulation; injuries to other organ systems than the cardiopulmonary are therefore less likely. Cardiorespiratory manifestations of DCI are rare and have been reported to occur in approximately 2-6% of recreational diving accidents (Francis and Mitchell, 2003a, Vann et al., 2011). Such manifestations only occur after highly provocative dives and represent a lethal form of DCI. If not treated immediately, acute heart failure may progress into cardiorespiratory collapse and death (Francis and Mitchell, 2003a). Currently, there have been no published controlled diving studies of troponin release in

animals or humans, but elevated troponins due to diving has been previously described in a case report (Chenaitia et al., 2010). Thus, this study is the first study to demonstrate an association between gas emboli formation and troponin release after diving.

HBO-PC 180 min prior to simulated diving resulted in lower cTnT levels compared to non-preconditioned diving animals (Fig. 2) despite no differences observed in the VGE loads (Fig. 1). Thus, it appears that HBO-PC protects the heart against injury from decompression-induced VGE. This novel observation was consistent with the findings obtained by Martin and Thom (2002) and Butler *et al.* (2006), who demonstrated that similar HBO-PC protocols prior to simulated diving protected rats against severe decompression stress without reducing gas emboli formation. Martin and Thom found that HBO-PC reduced DCI manifestations from the central nervous system, and Butler *et al.* showed that HBO-PC resulted in less overall signs of DCI compared to control animals, and demonstrated lower levels of inflammatory markers in the blood, lungs and urine after the dive.

In this study, two of the animals in the HBO45 group exhibited very high VGE loads (scan grade 4 and 5) throughout the entire 60 min post-diving observation period. These two rats had the highest cTnT levels measured, which may explain why the HBO45 group did not result in statistically significant lower levels of serum cTnT compared to the diving group. In the animals with low-to-moderate VGE loads (scan grade 0-3), significantly more animals showed elevated cTnT levels in the non-preconditioned group (67%) compared to the preconditioned groups (8%). However, all of the animals with high VGE loads (scan grade 4 or 5) exhibited elevated cTnT levels. Thus, HBO exposure appeared to protect the heart against low-to-moderate loads of VGE; however, this protective effect was not evident when the VGE loads were high.

Nppb expression in the left ventricle was increased in all of the diving groups compared to control animals (Fig. 3), and this increase was associated with increased cTnT and VGE levels.

Nppb mRNA encode for brain natriuretic peptide (BNP) and are a well established biomarker of acute heart failure (Braunwald, 2008). This natriuretic peptide is synthesized and released by cardiomyocytes in response to hemodynamic stress when the ventricles are subject to increased wall tension. The action of this peptide functions to oppose the physiological abnormalities that occur during cardiac overload and acute heart failure. It is shown that the expression of *Nppb* is increased within one hour in response to cardiac overload (Nakagawa et al., 1995). Thus, the increase in *Nppb* expression in the left cardiac wall after simulated diving in this study indicated that the heart was exposed to increased wall tension and cardiac overload. During and after decompression, VGE is thought to form or grow on the endothelial surface in peripheral tissues until they are swept away by the bloodstream and trapped in the small vessels of the pulmonary circulation (Stepanek and Webb, 2008). In animal studies, VGE trapped in the lungs caused increased pulmonary artery pressure, which may result in cardiac overload and heart failure (Vik et al., 1990, Bove et al., 1974). Although, this phenomenon has not been shown in human studies (Valic et al., 2005), observations after highly provocative dives with severe cardiorespiratory DCI indicated that heart failure and cardiac arrest may occur due to a massive VGE load in the pulmonary vascular bed (Muth and Shank, 2000, Francis and Mitchell, 2003b).

The elevation of cardiac stress-markers in this study reflected VGE-induced cardiac stress and injury but provided no indication of the mechanisms behind this elevation. However, it is well known that cardiac ischemia, pulmonary embolism and acute heart failure may all result in elevated cTnT and BNP levels (Giannitsis et al., 2000, Giannitsis et al., 2010, Thygesen et al., 2012). Additional measurements of cardiopulmonary hemodynamics (e.g., pulmonary artery pressure and arterial oxygenation) in the present study, could have added further information about the mechanisms.

The concept of cardiac preconditioning has been extensively studied (Hausenloy and Yellon, 2011), and HBO-PC is one strategy used to induce cardiac protection. Exposure to HBO is thought to induce a cardiac stress response of a reduced intensity that initiates cardioprotective responses, thereby reducing the damage caused by subsequent, more severe stress. The functional basis for the protective effects of HBO preconditioning is only partially understood. Protective mechanisms of HBO involve increased oxidative stress, which induces heat shock proteins, nitric oxide production, antioxidant enzymes and modulates inflammatory responses (Thom, 2009, Martin and Thom, 2002, Nishizawa et al., 1999, Cabigas et al., 2006, Christians et al., 2012). In the present study we investigated the effect of HBO preconditioning on the small heat shock protein αB-crystallin, which interacts with cardiac proteins and is likely to play a key role in protecting the heart from cardiac overload and ischemia (Christians et al., 2012, Kumarapeli et al., 2008, Martin et al., 1997, Latchman, 2001). αB-crystallin can protect the heart against various stresses by binding to and stabilizing cytoskeletal structures. In addition, αB-crystallin exhibits anti-apoptotic and immunomodulatory properties, and administration of αB-crystallin is likely to diminish the extent and severity of ischemic lesions, including cardiac infarction, stroke and arterial occlusion (Ousman et al., 2007, Arac et al., 2011). We found that αB-crystallin protein levels was increased in cardiac tissue after diving, and that HBO-PC rats displayed lower αB-crystallin levels compared to non-preconditioned rats. Thus, the high αB-crystallin levels in the heart after diving, may reflect that the heart have been exposed to a high level of diving-induced stress. Furthermore, the significantly lower αB-crystallin levels after HBO-PC and diving may reflect that HBO-PC induced a mild stress to the heart activating the cardioprotective properties of αB-crystallin. The activation of αB-crystallin prior to the dive could probably have prevented a subsequent higher increase in αB-crystallin levels after diving, because the heart was already preconditioned against the stress from diving. However, αB-crystallin mRNA expression levels

were not significantly affected by HBO-PC or simulated diving. Therefore, the increased levels of αB-crystallin in response to diving are most likely initiated by protein stabilization and/or activation rather than by de novo transcription. On the basis of previous and present findings, it is likely that αB-crystallin plays a central role in protecting the heart against the injury from diving-induced VGE.

In the present study, cardiac stress-markers were positively correlated with VGE. However, a recent study has shown that even presumably safe dives with few VGE and no DCI symptoms were associated with significant cardiac strain and increased levels of BNP (Marinovic et al., 2010). Grassi et al. (2009) showed that a water dive that was considered safe resulted in increased plasma BNP levels and that the same dive simulated in a dry hyperbaric chamber did not affect BNP levels. While underwater, divers are exposed to immersion resulting in significant hemodynamic changes (Pendergast and Lundgren, 2009). When investigating how decompression-induced VGE formation affects the cardiovascular system, factors known to affect hemodynamics must be controlled. Simulated diving in a dry pressure chamber, such as the one used in the present study, will eliminate the hemodynamic effects of immersion, enabling the differentiation between the effects of immersion and VGE on the cardiovascular system. However, a limitation of chamber diving is that dry diving is not the same as water diving with regards to cardiorespiratory stress.

HBO exposure is the main treatment of gas embolism and DCI after diving (Vann et al.); however, vascular gas embolization can also result from a reduction in ambient pressure in caisson work, aviation, extravehicular activity during spaceflight or escape from pressurized vessels (Vann et al., 2011, Gennser and Blogg, 2008), as well as from gas entry into the vasculature during inhospital procedures, e.g., cardiac surgery with extracorporeal bypass and through central venous and hemodialysis catheters (Muth and Shank, 2000, Tibbles and

Edelsberg, 1996). Thus, the implication of the present study is that HBO may not only be used in the treatment of DCI after diving but also in a prophylactic manner to prevent DCI and/or injury due to vascular gas embolization during inhospital procedures. However, whether these novel findings can be translated to humans requires further investigation. A better understanding of HBO-PC mechanisms is important because it will facilitate preventive measures that will increase the safety of persons in risk of vascular gas embolization.

In conclusion, we found that the cardiac stress-markers, cTnT, *Nppb* and αB-crystallin, are elevated in rat serum and cardiac tissue after inducing high loads of VGE from simulated diving, and that there is a strong positive correlation between stress-marker levels and post-dive VGE loads. We have further shown that HBO-PC may prevent cardiac injury induced by gas embolism, as indicated by the reduced levels of these cardiac stress-markers.

ACKNOWLEDGEMENTS

We thank the Proteomics and Metabolomics Core Facility (PROMEC) at NTNU, which is supported in part by the Faculty of Medicine and the Central Norway Regional Health Authority, the Department of Clinical Chemistry at St. Olavs University Hospital. We also thank Marianne B. Havnes, Toril Rolfseng and Arnfinn Sira for their excellent technical assistance.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.J., P.P.F., A.O.B. and I.E. conception and design of research; A.J. and I.E. collection, analysis and interpretation of data; A.J., P.P.F., A.O.B. and I.E. drafting manuscript and revising it critically, and approved final version of manuscript.

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