Early genetic responses in rat vascular tissue after simulated diving

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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 (PO2) 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 PO2 in the breathing gas, and it has been hypothesized that hypoxia 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 (hypoxia) and low (hypoxia) PO2 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...
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 positive 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 O2 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.

Haldol 0.33 mg/100 g given in one bolus as a subcutaneous injection. Every 5 min during diving. The temperature control rats were euthanized 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 anesthetized with a mixture of midazolam 0.5 mg/100 g, fentanyl 5 μg/100 g, and halothol 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·h−1, fentanyl 3 μg·100 g·h−1, and halothol 0.2 mg/100 g·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 CO2 (P CO2) and O2 (P O2), total hemoglobin (tHb), hemoglobin oxygen saturation (SO2), fraction of oxygenated hemoglobin (O2Hb), and calculation of total blood oxygen (PtO2) 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 RNA later 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 log transformed, and samples were quantile normalized before analysis. Differentially expressed genes were identified using the open source Bioconductor Limma package (http://www.biocductor.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 QuantiTeq rat primer assays with the SYBR Green RT-PCR kit (Qiagen, Valencia, CA). One-step qRT-PCR analysis was performed for the Nrf4a3, Serpin1, Tnfrsf12a, Blih40, and Adm genes with Hpt1 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 epoxide 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 Immunno-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 Imulocene 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 O2 = 149 kPa). One hour after diving, the pH was higher and P CO2 lower in diving rats compared with nondiving controls, but there was no difference in P O2 (Table 1). However, the concentration of total
23 genes were significantly upregulated after respiration rates 1 h after simulated diving. Fig. 1. qRT-PCR analysis of the after diving (34.3 °C in diving rats vs. 34.7 °C in controls). Body temperature was unaffected by diving; it deviated higher heart and respiration rates than controls. Body temperature was unaffected by diving; it deviated higher heart and respiration rates than controls. Body temperature was unaffected by diving; it deviated higher heart and respiration rates than controls. Body temperature was unaffected by diving; it deviated higher heart and respiration rates than controls.

Oxygen status

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\begin{align*}
\text{O}_2, \text{Vol}\% & = 18.8 \pm 1.8 \\
P_S & = 5.7 \pm 0.5 \\
\text{O}_2, \text{Vol}\% & = 15.0 \pm 0.7
\end{align*}
\]

Values are means ± SD. pH, blood gases, 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).

Heart and respiration rates

<table>
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<th>Diving</th>
<th>Control</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>384 ± 30</td>
<td>326 ± 46</td>
<td>0.015*</td>
</tr>
<tr>
<td>Respiration rate, per min</td>
<td>52 ± 8</td>
<td>41 ± 6</td>
<td>0.005*</td>
</tr>
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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 \( Nr4a3, \text{Serpine1, Blhhe40, and Adm} \) (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, and their targets among upregulated genes are shown in Fig. 2A. The listed transcription factors EGR1, HSFl, NF\( \kappa \)B, CEBPB, SP1, cJUN, HIF1-\( \alpha \), 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 \( (n = 4 \text{ in both}) \). 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-\( \alpha \) 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-\( \alpha \) is rapidly degraded by prolyl hydroxylase domain proteins (19), but

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1 The online version of this article contains supplemental material.
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. 4B) compared with the control (Fig. 4A). There was no increase in mRNA transcription of either HIF1 subunit after diving (Fig. 4C), 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.
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 PO2.

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 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...
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 PO2 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 PO2 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 PO2 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 PO2 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.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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