R1. Competitive apnea and its impact on the human brain; focus on the redox-regulation of blood-brain barrier permeability and neuronal-parenchymal integrity

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Running title: Redox-regulation of cerebrovascular function

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NON-STANDARD ABBREVIATIONS

$A^{\bullet-}$, ascorbate radical; AH^-, ascorbate monoanion; *a*-v_D, arterial to internal jugular venous concentration difference; gCBF, global cerebral blood flow; HR, heart rate; IJVP, internal jugular venous pressure; LDL low density lipoprotein oxidation; LOOH, lipid hydroperoxides; MAP, mean arterial pressure; MBP, human myelin basic protein; NO_3^-, nitrate; NO, nitric oxide; NO_2^-, nitrite; NSE, neuron-specific enolase; PO_2, partial pressure of oxygen; PCO_2, partial pressure of carbon dioxide; S100*β*, S100Beta; $R^{\bullet}$, free radical; RNS, reactive nitrogen species; ROS, reactive oxygen species; RSNO, *S*-nitrosothiols.

ABSTRACT

Static apnea provides a unique model that combines transient hypertension, hypercapnia, and severe hypoxemia. With apnea durations exceeding five minutes, the purpose of the present study was to determine how this impacts cerebral free radical formation and corresponding implications for brain structure/function. Measurements were obtained before and following a maximal apnea in fourteen divers with trans-cerebral exchange kinetics measured as the product of global cerebral blood flow (Duplex ultrasound) and radial arterial to internal jugular venous concentration differences (a-v_D). Apnea increased the systemic (arterial) and to a greater extent regional (jugular venous) concentration of the ascorbate free radical resulting in a shift from net cerebral uptake to output ($P < 0.05$). Peroxidation (lipid hydroperoxides, low density lipoprotein oxidation), nitric oxide bioactivity (nitrite) and S100β were correspondingly enhanced ($P < 0.05$), the latter interpreted as minor and not pathological disruption of the blood-brain barrier. However, these changes were insufficient to cause neuronal-parenchymal damage confirmed by the lack of change in the a-v_D of neuron-specific enolase and human myelin basic protein ($P > 0.05$). Collectively, these observations suggest that increased cerebral oxidative stress following a prolonged apnea in trained divers may reflect a functional physiologic response rather than a purely maladaptive phenomenon.

Keywords: Hypoxia; hypercapnea; hypertension; cerebral perfusion; free radicals

INTRODUCTION

The human brain functions almost entirely on oxidative metabolism, and requires a disproportionately large ~20% of the basal oxygen (O_2) budget (1, 2) despite occupying only 2 to 3% of the body's mass. A burden of such high oxidative energy demands is the potential for cerebral impairments when O_2 is deprived. The practice of extreme competitive apnea (where breath holds often exceed 5 min) provides a unique model to study the limits of acute hypoxemia in healthy humans, with recorded arterial O_2 saturations as low as ~40% (3-5).

Both hypoxemia and the rapid re-oxygenation during recovery are well-established stimuli for increased free radical and associated reactive oxygen-nitrogen species (ROS-RNS) formation (6, 7). Furthermore, the metabolic acidosis associated with elevations in the partial pressure of arterial carbon dioxide ($PaCO_2$) can further compound intracellular oxidative stress subsequent to formation of the carbonate radical ($CO_3^{\bullet -}$) (8). This has important implications for the brain and underlying cerebrovascular endothelium given its vulnerability to peroxidative stress, the consequence of modest antioxidant defenses, abundance of transition metal ions, auto-oxidizable neurotransmitters and neuronal membrane lipids rich in eicosapentaenoic and docosahexaenoic polyunsaturated fatty acid side chains exposed to a high mass-specific O_2 flux (9).

Emerging evidence suggests the practice of repeated and prolonged apnea might be associated with increased oxidative stress (10-12), transient disruption of the blood-brain barrier (BBB) (13, 14) and neuronal-parenchymal damage (15). However, the acute impacts of a single dry static apnea on cerebral oxidative stress and related BBB opening/neuronal-parenchymal damage remains unexplored. Moreover, most studies have relied on indirect markers confined to the peripheral circulation, thus to what extent the human brain contributes towards the systemic rise in oxidative stress and related damage markers remains unknown.

To address these limitations, the current study combined electron paramagnetic resonance (EPR) spectroscopy and ozone-based chemiluminescence (OBC), the most sensitive, specific and direct molecular techniques for the detection and molecular identification of free radicals and nitric oxide (NO metabolites) (16) with simultaneous arterial-jugular venous sampling. Our primary aim was to document the transcerebral exchange of oxidative-nitrosative stress biomarkers in response to a single prolonged apnea (>5 minutes) in elite competitive divers. Our secondary aim was to determine if cerebral exchange is associated with alterations in nitric oxide (NO) bioactivity and corresponding implications for cerebral perfusion and structural integrity of the BBB given the anticipated elevations in cerebral blood flow (CBF) and arterial pressure (3-5, 17). We hypothesized that apnea would be associated with an increase in the jugular venous outflow and corresponding net cerebral output of free radical-mediated lipid peroxidants and NO metabolites. We also hypothesized that apnea would be associated with a net output of S100β, neuron-specific enolase (NSE) and human myelin basic protein (MBP) that would be collectively interpreted to reflect transient disruption of the BBB and neural injury.

MATERIALS AND METHODS

Ethics

The ethical committees of the University of Split, the University of British Columbia, and the University of South Wales approved the study procedures and experimentation, which conformed to Helsinki Declaration. All participants provided written informed consent before experimentation.

Participants

Fourteen actively competitive breath-hold divers (2 female/12 male) aged 29 (mean) ± 7 (SD) years old with a body mass index of BMI 23.0 ± 2.1 kg/m^2 were recruited from the Croatian national apnea team. Participants were free from any respiratory, cardiometabolic, and cerebrovascular disorders.

Design

Aspects of this study (cardiovascular and CBF data) have previously been published (3). Thus, while the present study adopted an identical design, it represents a separate experimental question with additional measures. Experimentation was completed at the Department of Integrative Physiology, University of Split School of Medicine. Participants arrived at the laboratory following a minimum of 24-hour abstinence from vigorous exercise, alcohol, and caffeine, and following a 12 hour fast. Upon arrival to the laboratory and following initial screening (medical history and standard pulmonary function test), a 20-gauge arterial catheter (Arrow, Markham, Ontario, Canada) was placed in the right radial artery, and a central venous catheter (Edwards PediaSat Oximetry Catheter, California, USA) was placed in the right internal jugular vein and advanced towards the jugular bulb. Cannulation was completed under local anesthesia (1% lidocaine) with ultrasound guidance. Facial vein contamination was ruled

out by assuring that all jugular venous SO_2 recordings were below 75%. The arterial and jugular venous catheters were each attached to an in-line wasteless sampling setup (Edwards Lifesciences VAMP, California, USA) attached to a pressure transducer that was placed at the height of the right atrium (TruWave transducer).

Following instrumentation, but prior to the experimental maximal apnea, subjects completed a preparatory apnea phase, consisting of two sub-maximal apneas. The first preparatory apnea was performed at the end of a normal expiration, and terminated upon reaching six involuntary breathing movements. After a 2-min rest, the second preparatory apnea was performed at total lung capacity, until reaching 10 involuntary breathing movements. These practice apneas were performed as a well-accepted method to optimize the subsequent maximal apnea time. The maximal apnea was performed following a 6 min rest from the end of the second preparatory apnea. Blood was collected immediately prior to the start of the first preparatory apnea (pre-apnea), and within 10 to 60 seconds following termination of the maximal apnea (post-apnea).

Measures

***Cardiovascular*:** Heart rate (HR) was obtained from the R-R intervals measured from a three-lead ECG. Mean arterial blood pressure (MAP) was measured with the pressure transducer connected to the radial catheter. Internal jugular pressure (IJVP) was measured with the pressure transducer connected to the jugular catheter. All cardiovascular measures were integrated into PowerLab® and LabChart® software (ADInstruments) for online monitoring, and saved for offline analysis.

***Cerebrovascular*:** Blood flow in the right internal carotid artery (ICA) and left vertebral artery (VA) was simultaneously measured using duplex vascular ultrasound (Terason 3200, Teratech,

Burlington, MA). The right ICA was insonated an average of 2cm from the carotid bifurcation, while the left VA was insonated at the C5–C6 or C4–C5 space. The steering angle was fixed to 60 °, and the sample volume was placed in the center of the vessel adjusted to cover the entire vascular lumen. All files were screen captured and saved as video files for offline analysis at 30 Hz using custom designed software (18). Simultaneous measures of luminal diameter and velocity over a minimum of 12 cardiac cycles were used to calculate flow. Global cerebral blood flow (gCBF) was subsequently calculated from 2 [ICA + VA].

Blood gases and oximetry: Arterial and jugular venous partial pressures of oxygen (PO_2) and carbon dioxide (PCO_2), oxyhemoglobin saturation (SO_2), and pH were collected at baseline (pre-apnea) and every 30 seconds throughout the maximal apnea, and 2 minutes into recovery following apnea termination. Analysis was performed immediately using a commercially available cassette based analyzer (ABL90 Flex; Radiometer, Copenhagen, Denmark).

Blood collection and storage: At baseline (pre-apnea) and immediately following termination of the maximal apnea (post-apnea), blood samples were drawn simultaneously from the radial artery and jugular vein into Vacutainers® (Becton, Dickinson and Company, Oxford, UK) prior to centrifugation at 600*g* (4°C) for 10 min. Plasma and serum supernatant were decanted into cryogenic vials (Nalgene® Labware, Thermo Fisher Scientific Inc, Waltham, MA, USA) and immediately snap-frozen in liquid nitrogen (N_2) prior to transport to the UK under N_2 gas (Cryopak, Taylor-Wharton, Theodore, AL, USA) for batch analysis after being left to defrost at 21ºC in the dark for 5 min.

Antioxidants

Ascorbic acid: Plasma was stabilized and deproteinated using 10% metaphosphoric acid (Sigma Chemical, Dorset, UK). Ascorbic acid was assayed by fluorimetry based on the condensation of dehydroascorbic acid with 1,2-phenylenediamine (19). Concentrations of all lipid soluble antioxidants (LSA) were determined using an HPLC method (20, 21). The intra and inter-assay CV for all parameters were <5%.

Free radicals and lipid peroxidation

Ascorbate free radical ($A^{\bullet-}$): Plasma (1 mL) was injected into a high-sensitivity multiple-bore sample cell (AquaX, Bruker Daltonics Inc., Billerica, MA, USA) housed within a TM_{110} cavity of an electron paramagnetic resonance (EPR) spectrometer operating at X-band (~9.8 GHz) (22). Samples were recorded by cumulative signal averaging 10 scans using the following instrument parameters: resolution, 1024 points: microwave power, 20.12 mW; modulation amplitude, 2.00 G; modulation frequency, 100 kHz; receiver gain, 2×10^5 arbitrary units (AU); time constant, 40.96 ms; centre field, 3486.00 G; sweep width, 8.00 G and sweep time, 41.9 s. Spectra were filtered identically (moving average, 15 conversion points) using WINEPR (Version 2.11, Bruker, Karlsruhe, Germany) and the double integral of each doublet calculated using Origin software (OriginLab Corps, Massachusetts, USA). The intra and inter-assay CVs were both <5 %.

Lipid hydroperoxides (LOOH): Serum LOOH was determined by the ferric oxidation of xylenol orange version II method (23, 24). The intra/inter-assay CVs were both <5%.

Low-density lipoprotein (LDL) susceptibility to oxidation: Low-density lipoprotein was isolated by rapid ultracentrifugation and purified by size-exclusion chromatography (25). The

protein concentration was standardized to 50 mg/mL and oxidation was initiated following addition of copper II chloride (2 μmol final concentration) at 37° C. Conjugated diene formation was monitored spectrophotometrically in triplicate by the change in absorbance at 234 nm using a 96-well microplate reader (Spectromax 190, Molecular Devices Corp). The time at half maximum absorbance of the propagation phase (time $½_{MAX}$ in min) was taken as a marker of LDL's susceptibility to oxidation. The intra and inter-assay CVs were both <5%.

Nitric oxide (NO) metabolites

Plasma NO metabolites were measured by ozone-based chemiluminescence (OBC Model 280i, NOA®, Sievers, Boulder, CO, USA) (26). Samples (20 μl) were analyzed for the total concentration of plasma NO [nitrate (NO_3^-) + nitrite (NO_2^-) + *S*-nitrosothiols (RSNO)] by vanadium (III) reduction(27). A separate sample (200μL) was injected into tri-iodide reagent for the measurement of NO_2^- + RSNO and 5% acidified sulphanilamide added and left to incubate in the dark at 21º C for 15 min to remove NO_2^- for the measurement of RSNO in a third parallel sample. Plasma NO_3^- was calculated as total NO – (NO_2^-+ RSNO). All calculations were performed using Origin/Peak Analysis software (Northampton, MA, USA). The intra- and inter-assay CVs for all metabolites were ≤ 10%.

Brain-specific proteins

Serum S100*ß* (314701) and neuron specific enolase (NSE, 314561) were quantified via automated chemiluminescence (LIAISON® XL, DiaSorin, Saluggia, Italy). Human myelin basic protein (MBP) concentration was determined by ELISA kit (Elabscience, USA). The intra- and inter-assay CVs for all proteins were <10%.

Trans-cerebral exchange

Net cerebral exchange was calculated as:

$$\text{Cerebral exchange (g/min)} = [\text{gCBF} \times (1\text{-}\text{Hct}_v)] \times (C_A - C_V)$$

whereby $C_A - C_V$ refers to the arterial-jugular venous concentration difference for any given variable of interest and Hct_v is the jugular venous hematocrit. By convention, a positive value indicates net uptake whereas a negative value indicates net output across the brain.

Statistics

All analyzes were performed using the statistical software package SPSS v22 (IBM®). Cardiovascular and cerebrovascular data were averaged over 20-second bins. Tests for normality was confirmed using repeated Shapiro–Wilks W tests. Statistical analyzes of cardiovascular data were performed using one-way repeated measures analyzes of variance (ANOVA). All remaining analyzes were performed using two-way (condition: pre-apnea *vs*. post apnea *x* site: arterial *vs*. venous) repeated measures ANOVA. Following an interaction, post hoc analyzes were performed using repeated measures Student's *t*-tests. Significance was determined at an alpha level of 0.05 with data are presented as mean ± standard deviation (SD).

RESULTS

Apnea duration

Apnea duration ranged from 216 to 446 sec (307 ± 64 sec).

Blood gases

Arterial and internal jugular venous blood gas data (PO_2, PCO_2, SO_2, pH) are presented in Table 1. As expected, the latter stages of the maximal apnea were associated with severe hypoxemia, hypercapnia, and acidosis ($P < 0.05$ *vs*. pre-apnea).

Cardiovascular and cerebrovascular responses

Cardiovascular and cerebrovascular data are presented in Table 1. Apnea increased gCBF by ~70% ($P < 0.05$ *vs*. pre-apnea). There was a transient increase in HR at the beginning of apnea and progressive reduction towards the latter stages ($P<0.05$). Apnea increased MAP and IJVP by ~66% and 162% respectively ($P < 0.05$ *vs*. pre-apnea).

Antioxidants

Apnea failed to alter ascorbate ($P > 0.05$ *vs*. pre-apnea) whereas there was a tendency towards an increase in α-tocopherol ($P = 0.068$) and corresponding elevation in the cumulative concentration of LSA ($P < 0.05$, Table 2). However, these changes failed to translate into any significant differences in net cerebral exchange ($P > 0.05$).

Free radicals and lipid peroxidation

Apnea was associated with an increase in both systemic (arterial) and venous $A^{\bullet-}$ ($P < 0.05$ *vs*. pre-apnea) that was especially pronounced in the venous circulation resulting in an increased a-v_D and corresponding shift from net cerebral uptake to output (Table 2). Typical examples of

the characteristic EPR doublets (a_H^4 = 1.76 G) obtained before and after apnea are illustrated in Figure 1. Likewise, apnea increased LOOH and decreased LDL lag-time at ½ max ($P < 0.05$ *vs*. pre-apnea) and though there was a tendency towards a greater elevation in venous LOOH ($P = 0.063$ *vs*. arterial) this failed to alter net exchange ($P = 0.082$, Table 2). The reduction in PaO_2 at break-point was related to changes (post minus pre-apnea) in the a-v_D of $A^{\bullet-}$ ($r = 0.544$, $P = 0.044$), LDL lag-time at ½ max ($r = -0.663$, $P = 0.010$) but not LOOH ($r = -0.259$, $P = 0.372$).

Nitric oxide metabolites

Apnea failed to alter the arterial or venous concentrations of total NO, NO_3^-, NO_2^- or RSNO ($P > 0.05$ *vs*. pre-apnea, Table 3) whereas venous concentration NO was consistently shown to be lower due primarily to a reduction in NO_3^- (Table 2). In contrast, apnea reversed the a-v_D of NO_2^- resulting in a shift from net cerebral uptake to output ($P < 0.05$).

Brain-specific proteins

Apnea was associated with a general increase in S100*ß* ($P < 0.05$ *vs*. pre-apnea) whereas it failed to alter NSE or MBP ($P > 0.05$, Table 4). Apnea failed to significantly alter a-v_D and corresponding exchange of S100*ß*, NSE, and MBP ($P > 0.05$).

DISCUSSION

Static apnea is a unique model that combines transient hypertension, hypercapnia, and severe hypoxemia and our findings have provided novel insight into the local formation of free radicals and corresponding implications for cerebrovascular structure and function. Consistent with our original hypothesis, apnea was associated with increased systemic and cerebral free radical-mediated lipid peroxidation, as indicated by the combined elevation in $A^{\bullet-}$ (net uptake to output), LOOH and LDL susceptibility to oxidation that prevailed despite improved antioxidant defense and general mobilization of LSA into the circulation. Moreover, apnea enhanced NO bioactivity. However, while circulating S100ß was higher post-apnea, potentially indicating transient (but minor) BBB disruption, contrary to our original expectations, a single dry static apnea failed to cause indications for neuronal damage. Collectively, these observations suggest that increased cerebral oxidative stress in trained divers may reflect a normal physiologic response that may serve to maintain cerebral O_2 homeostasis, as opposed to a purely maladaptive phenomenon.

Free radical-mediated lipid peroxidation

The net cerebral release of $A^{\bullet-}$ including the combined elevation in LOOH and LDL susceptibility to oxidation immediately following the maximal apnea indicates localized increases in free radical formation measurable during a single arterial-venous transit. Systemic and regional peroxidative stress was seen to prevail despite mobilization of LSA into the circulation though we failed to observe any corresponding increases in the primary water/lipid soluble chain-breaking antioxidants, ascorbate and α-tocopherol. This may not necessarily reflect an improvement in antioxidant defense, rather the consequence of increased lipoprotein delivery and available pool for hydrolysis, which is consistent with our previous findings albeit confined to exercising human skeletal muscle (28).

Candidate sources for cerebral free radical formation are numerous (29) however, the brain's almost exclusive reliance on oxidative metabolism at rest and during maximal apnea (3, 4) suggests the mitochondria as a potential source. Indeed, severe hypoxia and re-oxygenation can impair physical properties of both the mitochondrial membrane and components of the electron transfer chain (ubiquinone Q9 and Q10), thereby enhancing free radical leakage (29). Bailey *et al*., (30) provided direct EPR evidence for an increase in the cerebral output of free radicals in healthy men exposed to nine hours of hypoxia (12.9% O_2) that correlated in direct proportion to the reduction in (calculated) mitochondrial PO_2. This concept was supported by findings in the present study, in that the more marked (albeit) systemic arterial hypoxemia observed at break-point was generally associated with the greatest cerebral formation of free radicals and secondary lipid peroxidants. Outside the mitochondria, increased free radical production may also occur from NO synthase activity, and hydrolysis of xanthine dehydrogenase, both of which are up-regulated in hypoxia (31, 32).

While hypoxia/re-oxygenation likely provided the main stimulus for increased cerebral free radical formation, the attending hypercapnia and metabolic acidosis (arterial pH of ~7.42 to 7.34 from pre- to post apnea, respectively) may have also contributed given that acidosis can increase the binding of transition metal ions and promote oxidative catalysis through Fenton and Haber-Weiss reactions compounding superoxide, hydrogen peroxide, and hydroxyl radical formation (29). Furthermore, acidosis has the capacity to enhance O_2 toxicity and increase intracellular oxidation through increased $CO_3^{\bullet -}$ formation (8). In contrast, hypercapnia has been shown to depress the cerebral metabolic rate of oxygen by approximately 25% (3, 4), that has the potential to reduce mitochondrial free radical formation subsequent to reduced electron flux through the electron transport chain. Thus, to what extent hypercapnia *per se* regulates cerebral free radical formation remains unknown.

NO metabolism

The mechanisms underlying endocrine vasoregulation during hypoxemia, one of the primary stimuli encountered during apnea, have been widely contested with *S*-nitrosohemoglobin (SNO-Hb) and NO_2^- identified as principal sources of red blood cell (RBC)-derived vasoactive NO (33). The SNO-Hb hypothesis proposes that NO binds covalently to the highly conserved cysteine 93 residue on the β-chain of Hb, which, upon deoxygenation results in an as of yet unidentified intermediate being exported from the RBC as a low molecular weight RSNO (34-36). In contrast, the NO_2^- reductase hypothesis contends that NO_2^- constitutes the principal vascular endocrine NO reservoir with conversion to NO or dinitrogen trioxide (N_2O_3) catalyzed by deoxyHb-mediated reduction and acidic disproportionation (37-39).

Our findings in competitive breath-hold divers do not appear to support either hypothesis. The SNO-Hb hypothesis was originally predicated on allosterically mediated release of SNO-Hb from *a-v* at rest ($a > v$), resulting in a drop in the RBC and reciprocal rise in the venous delivery of plasma RSNO (40), the opposite of what we observed. Equally, while the reduction in the arterial concentration of NO_2^- following apnea could be taken to reflect systemic consumption subsequent to deoxyHb-mediated reduction (in the RBC) to form NO [NO_2^- + $HbFe^{2+}$(deoxyHb) + H^+ → $HbFe^{3+}$ (metHb) + NO + OH^-: Eq 1] (37), it clearly fails to account for the local (venous) increase that was roughly equivalent to the systemic decrease. Follow-up studies incorporating this unique model defined by physiological extremes of cerebral perfusion may help further distinguish between these two competing hypotheses.

Furthermore, apnea was accompanied by net cerebral NO_2^- formation (and not loss) suggesting that the magnitude of oxidative stress incurred following the apnea was unlikely to have limited local NO bioavailability subsequent to the oxidative inactivation of NO (superoxide/alkoxyl radicals + NO → peroxynitrite) though it would have been interesting to have confirmed this

through the measurement of the primary "reactant", peroxynitrite (16). Therefore, although speculative, the net cerebral NO_2^- formation indicates that shear stress-induced Akt-dependent activation of conduit vessel endothelial NO synthase, (41) and/or RBC released ATP mediated eNOS activation likely contributed to increased cerebral NO formation and bioavailability (42). Indeed, shear mediated NO release would have been favored by the hypoxic/hypercapnic vasodilation and near doubling of gCBF (and thus shear), while RBC mediated ATP release would have been favored by the extreme hypoxia, acidosis, and shear (42-45). Collectively the vasodilatory mechanisms at play led to a preserved cerebral O_2 delivery.

BBB integrity and neuronal-parenchymal damage

Hypoxia and re-oxygenation can disrupt the structure and location of proteins that maintain tight-junctions between endothelial cells of the BBB (46), that accumulating evidence suggests may prove the consequence of increased free radical (47) and NO (48) formation. S100ß is a binding protein that is constitutively secreted by astrocytes (49) where it primarily localizes. Given its size (~10 kDa), S100ß cannot pass an intact BBB (50), thus a systemic increase in S100ß levels indirectly reflect increased BBB permeability (51). In support, systemic concentrations of S100ß correlate directly with the magnitude of BBB opening as shown by contrast-enhanced MRI (52). The ~40% increases in jugular venous S100ß observed in the current study likely indicates that apnea increased the permeability of the BBB, consistent with Andersson et al's interpretation, (53) who found a ~26% increase in peripheral venous S100ß within 10 minutes following a maximal breath-hold in trained apneists. Importantly, however, the concentration increase in S100ß from apnea never reached pathological levels.

The trans-cerebral concentrations of S100ß remained unchanged from pre to post apnea, findings that are consistent with our previous work in non-apneists exposed to poikilocapneic normobaric hypoxia (30, 54). It therefore could be argued that the increase in S100ß might

have originated from extracranial sources, e.g. adipocytes, gastrointestinal apparatus glial cells, supporting adrenal medulla cells, skeletal muscle satellite cells, and arterial smooth muscle cells (49), rather than leaking across a permeated BBB. Alternatively and perhaps more likely is that a transient increase in cerebral S100ß release from the brain was missed using the single arterial-venous measure within a minute following the apnea. That is, the small systemic increase in S100ß may have resulted from minor BBB leakage subsequent to the transient increase in intracranial pressure (evidenced by increased IJVP, Table 1) experienced exclusively *during* the apnea (55, 56). Importantly, intracranial pressure was normalized almost immediately following the apnea breaking point since it occurs primarily from high lung volumes in combination with involuntary breathing movements (55).

There were no detectable changes in the systemic accumulation or cerebral output of NSE or MBP. Although NSE has relatively poor specificity to be an exclusive brain protein biomarker, its high concentration in neurons provides a useful prognostic indicator for traumatic brain injury (57). MBP, in contrast, is highly specific (~99%) to the brain. MBP is major constituent of the myelin sheath in the nervous system (oligodendrocytes and Schwann cells), thereby providing a highly specific biomarker for cerebral axonal damage (58).

Collectively, that both NSE and MBP remained unchanged from pre- to post apnea suggests that the magnitude of cerebral oxidative stress failed to cause structural neuronal damage. Thus, the marked increase in cerebral free radical formation reported here and in other studies published by our group (30) point to a homeostatic role for cerebral free radicals as cell-signaling molecules, rather than existing purely as toxic, damaging “accidents of chemistry”. Though beyond the scope of the current study, cerebral free radical formation may be an important hypoxic sensor in attempt to preserve cellular O_2 homeostasis by, for example, stabilization of the hypoxia-inducible factor-1α (30, 59).

Considerations and implications

A few methodological considerations deserve attention. First, it is important to emphasize that the findings from the present study reflect global cerebral values. Given the large PO_2 gradient and disparate vulnerability for hypoxic injury across separate brain regions (60), regional differences in free radical-mediated lipid peroxidation likely exist. For example, deeper brain regions may become more prone to hypoxic/re-oxygenation stress than the superficial regions (61). Furthermore, peripheral serum levels of NSE peak approximately 12 to 24 hours following cerebral hypoxic insult (62, 63). In keeping with this delayed response, Kjeld et al., (64) found elevated concentrations of serum NSE in breath-hold divers approximately three-hours following apnea competition (with more than a single maximal breath-hold). The lack of any increases in jugular or systemic NSE must therefore be interpreted within the present context (i.e. a single dry static apnea) and thus may not appropriately reflect longer-term cerebral parenchymal damage from repeated dynamic breath-holds.

To what extent our findings can help inform the pathophysiology and treatment of clinical diseases of the circulation characterized by severe hypoxemia-hypercapnea-hypertension remains unclear. There is currently no clear evidence for a clinical link between professional breath-holding and sleep apnea given that cerebrovascular CO_2 reactivity (65) and sympathetic baroreflex gain/respiratory muscle sympathetic nerve activity modulation (66) have been shown to be normal (ie. unimpaired) in apneists unlike adults with obstructive sleep apnea. Conversely, long-term neurological complications have been reported amongst professional breath-holders albeit primarily related to cerebral decompression sickness (14, 67). Thus, we cannot exclude the possibility that repeated exposure to severe intermittent cerebral oxidative stress (described in the present study) may eventually lead to neuronal-parenchymal damage, consistent with pathological sleep apnea. Clearly, longitudinal research is warranted to understand the long-term clinical consequences of professional apnea.

Conclusions

In summary, we show for the first time that a prolonged static breath hold is associated with increased systemic and cerebral free radical-mediated lipid peroxidation and enhanced NO bioactivity. However, apnea failed to cause major disruption of the BBB or structural/neuronal damage. Collectively, these observations suggest that the acute increase in cerebral oxidative stress in trained divers may in part reflect a functional physiologic response as opposed to a purely maladaptive phenomenon.

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AUTHOR CONTRIBUTIONS

AR Bain, PN Ainslie, O Barak, Z Dujic, DB MacLeod and DM Bailey designed research; AR Bain and DM Bailey analyzed data; AR Bain, PN Ainslie, RL Hoiland, OF Barak, I Drvis, M Stembridge, DM MacLeod, Z Dujic, DB MacLeod and DM Bailey performed research; AR Bain and DM Bailey wrote the paper; J McEneny, BS Stacey, E Tuaillon, N Marchi and A Fayd'Herbe De Maudave contributed new reagents or analytic tools; all authors reviewed the final manuscript prior to submission.

COMPETING INTERESTS

None declared

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LEGENDS

Table 1. Blood gas and cerebrovascular data

Values are mean ± SD; PO_2/PCO_2, partial pressure of oxygen/carbon dioxide; SO_2, oxyhemoglobin saturation; gCBF, global cerebral blood flow; HR, heart rate; MAP, mean arterial pressure; IJVP, internal jugular venous pressure. *different between condition ($P < 0.05$).

Table 2. Antioxidant, free radical and lipid peroxidation biomarkers

Values are mean ± SD; cumulative LSA refers to the concentration of α + γ-tocopherol + retinol + lutein + zeaxanthin + β-cryptoxanthin + α + β-carotene + lycopene; $A^{\bullet-}$, ascorbate radical; AU, arbitrary units; LOOH, lipid hydroperoxides; LDL, low density lipoprotein; a-v_D, arterio-jugular venous concentration difference; positive/negative a-v_D/exchange value reflects net cerebral uptake/output; *different between given condition for given site ($P < 0.05$); †different between condition ($P < 0.05$).

Table 3. Nitric oxide metabolites

Values are mean ± SD; NO_3^-, nitrate; NO_2^-, nitrite; RSNO, *S*-nitrosothiols. a-v_D, arterio-jugular venous concentration difference; positive/negative a-v_D/exchange value reflects net cerebral uptake/output; **different between site for given condition ($P < 0.05$); †different between condition ($P < 0.05$).

Table 4. Brain-specific proteins

Values are mean ± SD; NSE, neuron specific enolase; MBP, human myelin basic protein; a-v_D, arterio-jugular venous concentration difference; positive/negative a-v_D/exchange value reflects net cerebral uptake/output; †different between condition ($P < 0.05$).

Figure 1. Direct electron paramagnetic resonance (EPR) spectroscopic detection of the ascorbate radical ($A^{\bullet-}$) in a single participant

Oxidation of the ascorbate monoanion (AH^-) by any free radical ($R^\bullet$) with a one electron reduction potential that exceeds +282 mV will yield $A^{\bullet-}$ (schematic illustrated inset). The unpaired electron is delocalized over a highly conjugated tri-carbonyl π-system rendering it resonance stabilized thereby facilitating direct detection. At the current settings, $A^{\bullet-}$ appears as a doublet with a hydrogen hyperfine coupling constant (a_H^β) of ~1.76 Gauss. Note the general increases observed in the systemic (arterial) and cerebral (jugular venous) signal intensity of $A^{\bullet-}$ during apnea and transition towards a negative arterio-venous concentration difference reflecting a shift from net cerebral uptake to output.