Antioxidants, endothelial dysfunction, and DCS: in vitro and in vivo study

Qiong Wang,†*  Aleksandra Mazur,†*  François Guerrero,†  Kate Lambrechts,†  Peter Buzzacott,†  Marc Belhomme,†  and Michaël Theron†

†Laboratory ORPHY, Department of UFR Sciences and Technologies, European University of Bretagne, University of Brest, Brest, France

Submitted 26 February 2015; accepted in final form 8 October 2015

Wang Q, Mazur A, Guerrero F, Lambrechts K, Buzzacott P, Belhomme M, Theron M. Antioxidants, endothelial dysfunction, and DCS: in vitro and in vivo study. J Appl Physiol 119: 1355–1362, 2015. First published October 15, 2015; doi:10.1152/japplphysiol.00167.2015.—Reactive oxygen species (ROS) production is a well-known effect in individuals after an undersea dive. This study aimed to delineate the links between ROS, endothelial dysfunction, and decompression sickness (DCS) through the use of antioxidants in vitro and in vivo. The effect of N-acetylcysteine (NAC) on superoxide and peroxynitrite, nitric oxide (NO) generation, and cell viability during in vitro diving simulation were analyzed. Also analyzed was the effect of vitamin C and NAC on plasma glutathione thiol and thiobarbituric acid reactive substances (TBARS), plasma angiotensin-converting enzyme (ACE) activity, and angiotensin-II and DCS morbidity during in vivo diving simulation. During an in vitro diving simulation, vascular endothelial cells showed overproduction of superoxide and peroxynitrite, obvious attenuation of NO generation, and promotion of cell death, all of which were reversed by NAC treatment. After in vivo diving simulation, plasma ACE activity and angiotensin-II level were not affected. The plasma level of glutathione thiol was downregulated after the dive, which was attenuated partially by NAC treatment. Plasma TBARS level was upregulated; however, either NAC or vitamin C treatment failed to prevent DCS morbidity. During in vitro simulation, endothelial superoxide and peroxynitrite-mediated oxidative stress were involved in the attenuation of NO availability and cell death. This study is the first attempt to link oxidative stress and DCS occurrence, and the link could not be confirmed in vivo. Even in the presence of antioxidants, ROS and bubbles generated during diving and/or decompression might lead to embolic or biochemical stress and DCS. Diving-induced oxidative stress might not be the only trigger of DCS morbidity.

voxidative stress; nitric oxide; vasodilation; endothelial dysfunction; decompression sickness; SCUBA diving

DECOMPRESSION SICKNESS (DCS) occurs after a rapid drop in ambient pressure and is typically observed after subaquatic diving, when tissue gas supersaturation leads to bubble formation (33). Circulating bubbles are considered to be a trigger for DCS because they are known to induce ischemic stresses through venous gas emboli (4), but bubble scores in the blood stream nevertheless appear to be a poor predictor of DCS occurrence (8). This could be explained by the effects of self-contained underwater breathing apparatus (SCUBA) diving on arterial walls as has been demonstrated by Brubakk et al. (3), Lambrechts et al. (15), Marinovic et al. (19), and Obad et al. (23) because vascular endothelium is an autocrine and paracrine organ that plays a central role in the control of vasomobility and other regulation processes such as hemostasis or vessel wall permeability (4). In those studies, the decreased vasodilation observed after SCUBA diving could be attributed to endothelial dysfunction and the effects of diving on the endothelium could be further demonstrated by a postive increase in endothelial microparticles—small membrane vesicles shed from the endothelium and used as an in vivo marker of endothelial dysfunction (6, 32).

Oxidative stress is one of the key biochemical factors in the pathogenesis of many diseases and its involvement in SCUBA diving effects is also well documented. In fact, during air diving, divers are exposed to multiple oxidative insults that include increased oxygen partial pressure and exercise that may promote the production of reactive oxygen species (ROS) (7). The activities of catalase and superoxide dismutase in plasma have been shown to be upregulated immediately after SCUBA diving, confirming diving-derived oxidative damage (31). A previous study (23) also reported that successive deep dives initiated acute impairment of endothelial function in large-conduit arteries and ROS augmentation, with both endothelial function and plasma pro-oxidant and antioxidant activity failing to return to baseline, further indicating the possible cumulative and long-lasting detrimental role of oxidative stress in postive endothelial dysfunction. Acute or long-term pre-dive supplementation of antioxidants has been presented as protection against abnormal reduction in vascular endothelial function in divers after air diving (24, 25). The postdive decrease in flow mediated dilation (FMD) was partially prevented not only by a 4-wk treatment with vitamins C and E until 3 h before their dives, but also by acute oral administration of an antioxidant 2 h before the dives. In addition, vascular nitric oxide (NO) bioavailability may be affected by oxidative stress during exposure to hyperoxia (21) or to heat stress (9), which might indicate that vasodilation could be affected during a dive probably through the hyperoxia-induced NO availability reduction. ROS, including superoxide anion (O$_2^-$), are known to play a critical role in endothelial dysfunction (11). Peroxynitrite, another potent oxidant formed by the reaction of nitric oxide (NO) and superoxide dismutase (SOD), may act as a vasoconstrictor, and to make matters worse, as a cytotoxic molecule that irreversibly inhibits multiple complexes of the mitochondrial respiratory chain including dismutase enzymes, leading to increased oxidative stress. Peroxynitrite can also induce the uncoupling of endothelial NO synthase (eNOS) through mediating the degradation of its cofactor tetrahydrobiopin (BH4), which limits the bioavailability of endothelium-derived NO. In the absence of its coenzyme BH4, eNOS reduces molecular oxygen rather than L-arginine, resulting in the production of superoxide, which in a positive feedback manner, provokes even more formations of peroxynitrite, superoxide, and other forms of ROS and reactive nitrogen species (RNS) (14), drastically enhancing cellular
oxidative stress and eventually threatening endothelial cell survival and proliferation as well as vascular relaxation (17).

However, even if the implication of ROS in endothelial dysfunction during diving at-depth seems to be confirmed in vitro (37), the link between ROS and DCS morbidity has not yet been established. Therefore, this study aimed to investigate the role of ROS in DCS occurrence and the protection of N-acetylcysteine (NAC), a ROS scavenger, against diving induced-endothelial dysfunction and DCS morbidity, to explore the potential interest of antioxidants for DCS prevention. The study was conducted both in vitro and in vivo. It first included the examinations of NAC effect on ROS and RNS production, NO availability, and cell viability during in vitro diving simulation with endothelial cells. Second, it focused on the protection of NAC against plasma oxidative stress [glutathione-thiol level and thiobarbituric acid reactive substances (TBARS)], plasma levels of vasoactive substances [angiotensin converting enzyme (ACE) and angiotensin-II], and DCS morbidity after in vivo diving simulation with rats.

**METHODS**

**Ethical Aspects**

This study was approved by the Ethics Committee of European University of Bretagne-University of Brest (approval R-2011-FG-01). Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23, revised 1996). The study complied with recognized ethical standards and French and European laws.

**In Vitro Approach**

Cell culture and installation. Tissue from the aorta of a deceased calf was obtained from a commercial slaughterhouse. The experiments were performed on bovine aortic endothelial cells that were isolated and cultured as described previously (34). Briefly, after six passages, the cells were dispersed using a 0.25% trypsin solution and cultured onto sapphire windows (diameter 12.75 ± 0.15 mm, thickness 1.00 ± 0.10 mm; Edmund Optics). Before confluence, the cell covered-sapphire windows were transferred into an in vitro diving simulation system (34) that allows real-time monitoring under fluorescent microscopy during cell diving simulations.

In vitro diving simulation protocol. To examine the effect of NAC treatment on ROS and RNS production, NO availability, and cell viability during simulated air dive, the in vitro simulation was conducted in three groups: air diving, air diving with NAC (Sigma-Aldrich), which is living cell membrane-impermeant. During diving simulation, vascular endothelial cells were constantly exposed to 10 μg/ml of PI at 37°C to indicate cellular death in real time. The images of PI staining were collected with an excitation/emission maxima of 495/515 nm.

Fluorescence measurements. Measurement of mitochondrial ROS production was performed with the fluorescent dye MitoSOX Red (Invitrogen, Saint Aubin, France). Before the simulation, cells were exposed to MitoSOX Red (1 μM) at 37°C for 10 min, and then washed by systemic medium flow. Subsequently, the simulation was started and the images were collected with an excitation/emission maxima of 510/580 nm.

Measurement of peroxynitrite production was performed with the fluorescent dye DAF-FM diacetate (Invitrogen). Before the simulation, cells were exposed to APF (5 μM) at 37°C for 20 min. Then the simulation was started and the images were collected with an excitation/emission maxima of 409/515 nm.

Measurements of endothelial cell death were performed on cells loaded with the fluorescent dye PI (Sigma-Aldrich), which is living cell membrane-impermeant. Measurement of NO generation was performed with the fluorescent dye ANO (Invitrogen). Before the simulation, cells were exposed to DAF-FM diacetate (5 μM) at 37°C for 30 min. Then the simulation was started and cells were collected with an excitation/emission maxima of 495/515 nm.

Measurement of NO generation was performed with the fluorescent dye DAF-FM diacetate (Invitrogen). Before the simulation, cells were exposed to DAF-FM diacetate (5 μM) at 37°C for 30 min. Then the simulation was started and the images were collected with an excitation/emission maxima of 515/515 nm.

Measurements of endothelial cell death were performed on cells loaded with the fluorescent dye PI (Sigma-Aldrich), which is living cell membrane-impermeant. Measurement of NO generation was performed with the fluorescent dye DAF-FM diacetate (Invitrogen). Before the simulation, cells were exposed to DAF-FM diacetate (5 μM) at 37°C for 30 min. Then the simulation was started and the images were collected with an excitation/emission maxima of 495/515 nm.

For all measurements, a fluorescence inverted microscope (Zeiss Axio observer D-1, Jena, Germany) with an A-plan ×10 objective (NA 0.25, WD 4.5 mm) was used to record the images in real time.

**In Vivo Approach**

Animals. Seventy-two Sprague-Dawley rats 12 wk old on the day of the experiment were obtained from Janvier SAS (Le Genest St Isle, France). Animals were housed one per cage in an environmentally controlled room (temperature 21 ± 1°C, 12-h:12-h light:dark cycle) and were fed with 20–25 g of standard rat chow and 50 ml of water per day. The animals were randomly assigned into groups of 13 to 15. The first three groups were identical to the groups defined for the in vitro experiments: air diving (the animals were exposed to the simulated dive without treatment), air diving with NAC treatment (at a dose of 100 mg/kg by ip injection 24 h, 12 h, and 30 min before the simulation) and nondiving. The fourth group was a negative control for NAC treatment (with an equivalent ip injection of saline solutions); the last group was exposed to the simulated dive after vitamin C treatment (at a dose of 200 mg–liter⁻¹ day⁻¹ in drinking water for 2 wk before the simulation) to compare the effects of NAC treatment in vivo with another antioxidant drug.

In vivo diving simulation protocols. According to a previous protocol inducing 70% of morbidity of DCS (20), each rat was positioned in a 130-liter steel hyperbaric chamber, always at the same hour to avoid interference with biological rhythm. Air was used as a breathing mixture. The groups of rats exposed to a simulated dive were given compressed air at a rate of 101 kPa/min up to 1,010 kPa at 202 kPa (10 msw), and the pressure was then kept constant for 45 min. Thereafter, decompression was performed at a rate of 101 kPa/min with three stops: 5 min at 202 kPa (10 msw), 5 min at 160 kPa (6 msw), and 10 min at 130 kPa (3 msw). The duration of the entire simulation was 83 min. Following decompression the rats were observed for 2 h for the appearance of four standard DCS symptoms: 1) respiratory distress, 2) difficulty walking, 3) paralysis, and 4) convulsions. When an animal developed one or more of the four symptoms it was scored as having DCS. A trinary classification...
was applied differentiating: 1) animals with no DCS; 2) animals with mild DCS (exhibiting one or more of the four previously defined symptoms but without death); and 3) animals with severe DCS (defined by death occurrence within 2 h of observation). The nondiving rats were similarly confined but without exposure to pressure and were observed for 1 h before physiological investigation.

Assay of plasma biochemical indexes. After diving simulation and subsequent observation, the animals were euthanized with ketamine at 100 mg/kg and xylazine at 10 mg/kg via ip injection. Blood was collected via cardiac puncture into 2-ml Eppendorf tubes pretreated with 30 µl of 7.5% EDTA, centrifuged at 1,000 g at 4°C for 15 min, aliquoted, and stored at −80°C until analyses.

Plasma glutathione thiol level, TBARS level, ACE activity, and angiotensin-II level were examined with, respectively, a Thioll Detection Assay Kit (Cayman Chemical, Ann Arbor, MI), Thiobarbituric Acid Reactive Substances Assay Kit (Cayman Chemical), Angiotensin Converting Enzyme Assay Kit (RayBiotech, Norcross, GA), and Angiotensin-II Assay Kit (RayBiotech).

Statistical Analysis

Fluorescent image stacks were analyzed using Image J software (National Institutes of Health, Bethesda, MD). The signals of MitoSOX Red, APF, and DAF-FM diacetate were analyzed using a cellular region of interest (ROI) (30): the modification of fluorescence at each ROI and each time point of image collection was computed as the mean of fluorescent intensity (gray) of 30 randomly sampled cells from three different dives. The signal intensity of the background (region without cells) in each image was subtracted from the fluorescent intensity of cellular ROI in the same image. For PI fluorescence analysis, the percent of cell death at each time point of image collection was computed and mean cell death percent was analyzed. Differences between data were analyzed with two-way ANOVA and considered significant when $P < 0.01$. Results are reported as means ± SE for the indicated number (n) of cells.

The data of plasma glutathione thiol level accorded with Gaussian distribution and were analyzed with two-way ANOVA. These results were considered significant when $P < 0.05$ and reported as means ± SE. The data of the levels of plasma TBARS, ACE activity, and angiotensin II did not accord with Gaussian distribution and hence were analyzed with a Kruskal-Wallis test; they were considered significant when $P < 0.05$. Differences between the ratios of DCS morbidity were analyzed with a $\chi^2$ test and considered significant when $P < 0.05$.

RESULTS

In Vitro Approach

Mitochondrial ROS in vascular endothelial cells. During in vitro air diving simulation, vascular endothelial cells exhibited a clear increase in mitochondrial ROS production (compared with nondiving cells $P < 0.01$) (Fig. 1). No excess of mitochondrial ROS was observed after NAC treatment ($P < 0.01$). The cells with NAC treatment during the simulation showed no significant difference in ROS production from the nondiving cells ($P = 0.27$).

Peroxynitrite production in vascular endothelial cells. During in vitro simulation, endothelial cells showed an evident increase in peroxynitrite production (compared with nondiving cells $P < 0.01$), which was attenuated by NAC treatment ($P < 0.01$) (Fig. 2). No significant difference was observed in peroxynitrite production between the cells exposed to simulation with NAC treatment and the nondiving cells ($P = 0.39$).

NO availability in vascular endothelial cells. Endothelial cells presented a sharp decrease in NO availability during in vitro simulation (compared with nondiving cells $P < 0.001$) (Fig. 3). NO availability in cells that underwent simulation was reduced to 26% of the relevant availability in nondiving cell. NAC treatment prevented impairment of NO availability ($P < 0.01$). The endothelial cells that underwent diving simulation with NAC treatment showed no significant difference in NO availability from nondiving cells ($P = 0.07$).

Viability of vascular endothelial cells. During in vitro simulation, the percentage of endothelial cell death presented an evident increase (compared with nondiving cells $P < 0.001$) (Fig. 4). NAC treatment provided a clear protection against the effects of the simulated dive ($P < 0.01$). No significant difference was observed between the NAC air diving group and the nondiving group ($P = 0.42$).

In Vivo Approach

Glutathione thiol. After in vivo air diving simulation, the rats with saline injection showed an obvious decrease in
plasma glutathione thiol level (reduced to 79.5% of the concentration in nondiving rat plasma, \( P = 0.001 \) when compared with nondiving animals) (Fig. 5A). This effect was significantly attenuated by NAC treatment: the rat plasma concentration of glutathione thiol after the diving simulation was 89.0% of the nondiving control value and was different both from the saline diving group (\( P = 0.02 \)) and the nondiving group (\( P = 0.01 \)).

TBARS. After in vivo diving simulation, the rats showed an obvious increase in plasma TBARS level (compared with nondiving rats \( P = 0.005 \)) (Fig. 5B and C). Diving rats that were treated with either NAC or vitamin C presented no significant difference in plasma TBARS level from the diving rats without treatment (\( P = 1.00 \) for either NAC or vitamin C treatment).

ACE activity and angiotensin-II. After the in vivo simulation, the rats presented no significant difference in either plasma ACE activity or angiotensin-II level (compared with nondiving rats \( P = 0.158 \) and \( P = 0.191 \), respectively) (Fig. 5D–G). Simulation with NAC or vitamin C treatment also showed no significant difference in either plasma ACE activity or angiotensin-II level compared with the simulation without treatment (for NAC treatment \( P = 1.00 \) and \( P = 1.00 \), respectively; for vitamin C treatment \( P = 0.39 \) and \( P = 0.28 \), respectively).

DCS morbidity after in vivo simulated dives. The percentage of rats exhibiting either no DCS symptoms, mild DCS, or severe DCS are shown in Figure 6. No statistical difference between the groups could be discerned. In particular, the rats treated with NAC or vitamin C did not show a significant difference in either mild DCS or severe DCS when compared with the rats without treatment during the simulation (for NAC treatment \( P = 0.42 \) and \( P = 0.11 \), respectively; for vitamin C treatment \( P = 0.39 \) and \( P = 0.28 \), respectively).

Fig. 2. Production of peroxynitrite in vascular endothelial cells without treatment or with NAC treatment during in vitro diving simulation. A: production of peroxynitrite was indicated with 3′-(p-aminophenyl) fluorescein (APF). Scale bar = 25 μm. B: kinetics of peroxynitrite generation during compression and dive phases at 9, 24, 39, and 54 min of simulated diving. *Significant statistical difference of APF fluorescence intensity between cells without treatment and cells with NAC treatment during simulation, \( P < 0.01 \).

Fig. 3. Nitric oxide (NO) availability in vascular endothelial cells without treatment or with NAC treatment during in vitro diving simulation. A: NO was indicated with 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM). Scale bar = 25 μm. B: kinetics of NO availability during compression and dive phases at 9, 24, 39, and 54 min of simulated diving. *Significant statistical difference of DAF-FM fluorescence intensity between cells without treatment and cells with NAC treatment during simulation, \( P < 0.01 \).
This work focused on DCS [one of the main diving-induced casualties (2, 5, 33)] in an attempt to clarify its pathophysiological mechanism and explore a potential clinical treatment. The goal of this study was to analyze the link between dive-induced ROS, endothelial function, and DCS. An original in vitro approach allowing assessment in real time of ROS and RNS production, NO availability, and cell viability to confirm the effect of diving on endothelial cells and the preventive role of antioxidants in endothelial dysfunction. In a complementary in vivo approach, the interest of antioxidants in DCS prevention was assessed in rats.

In Vitro Approach

Productions of ROS and RNS. In this study during in vitro air diving simulation, vascular endothelial cells presented evident excess mitochondrial superoxide and peroxynitrite. The peroxynitrite formed during oxidative stress has been shown to affect eNOS activity through BH₄ oxidation and to inhibit the respiratory chain complex and therefore may promote superoxide production (22). However, NAC treatment showed an obvious suppressive effect on the productions of mitochondrial superoxide and peroxynitrite. Actually, NAC can act directly as a free radical scavenger and an antioxidant. Moreover, it is a precursor of the amino acid L-cysteine and a source of sulfhydryl group because it is capable of stimulating glutathione synthesis, which has been reported both in vivo and in vitro (10, 12). Therefore, NAC has been applied clinically for detoxification for many years.

Nitric oxide generation. The concentration of exhaled NO is regarded as a useful index of decompression severity in human divers (26). In exposure to hyperoxia (21) or heat stress (9), vascular NO bioavailability can be affected by the oxidative stress in the circulation. In this study the endothelial cells during in vitro air diving simulation showed a sharp reduction in NO availability, which might be due to the reaction between superoxide and NO to form peroxynitrite, or peroxynitrite-induced eNOS deactivation, or both (22). This result may explain the dysfunction of vascular endothelium during in vivo diving simulation (19).

However, NAC treatment reversed the impairment of NO availability in endothelial cells during the simulation. We may therefore speculate that through scavenging the diving-induced ROS, NAC may suppress peroxynitrite production, therefore contributing to the maintenance of the activity of BH₄, the coenzyme of eNOS, and the recovery of NO availability. Moreover, NAC might also save NO by blocking the reaction between superoxide and NO because NAC can scavenge superoxide. Thus NAC may maintain NO bioavailability and restore the physiological function and activity of vascular endothelial cell by the detoxification of the effects of superoxide and peroxynitrite.

Viability of endothelial cells. In addition to functioning as a source of sulfhydryl group and free radical scavenger, NAC also presents its protective effect on various intoxications such as acetaminophen-induced hepatotoxicity, doxorubicin-induced cardiotoxicity, chemotherapy-induced toxicity, and heavy metal toxicity (29). In this study, the endothelial cells showed an evident increase in cell death ratio during in vitro simulation. However, the cells undergoing diving simulation with NAC treatment were protected, showing a clear attenuation of cell death. Together with the result that excess mitochondrial ROS and peroxynitrite during the simulation were both evidently suppressed by NAC treatment, this result may implicate that during in vitro simulation the outbursts of ROS and/or RNS could be one of the causes triggering cell death, and the detoxification of NAC on diving simulation-induced cell death may be associated with its protection against oxidative stress. This observation is consistent with the work by Obad et al. (23–25) who studied the link between ROS and endothelial dysfunction during human dives with SCUBA equipment.

In Vivo Approach

Oxidative stress. Oxidative stress during SCUBA diving has been demonstrated by the concomitant increase in plasma malondialdehyde level and decrease in plasma antioxidant...
Fig. 5. Assay of plasma biochemical indexes. A: glutathione thiol level in rat plasma after in vivo simulated dives. *Significant statistical difference in concentration of plasma glutathione thiol after simulation between each given condition, \( P < 0.05 \). B and C: levels of thiobarbituric acid reactive substances (TBARS) in rat plasma after in vivo simulated dives. *Significant statistical difference in plasma TBARS level after simulation between dive group and nondive group, \( P < 0.05 \). D and E: angiotensin-converting enzyme (ACE) activity in rat plasma after in vivo simulated dives. F and G: angiotensin-II (AII) level in rat plasma after in vivo simulated dives.
capacity (23). Furthermore, the activities of the antioxidant enzymes have been shown to be upregulated immediately after SCUBA diving, confirming the diving-derived oxidative stress (31). Consistently, in this study the rats showed an obvious increase in plasma TBARS level and a decrease in plasma glutathione thiol level after in vivo air-diving simulation. Together with the results from in vitro superoxide and peroxynitrite examinations, this may further confirm the possibility of air-diving-induced excesses of ROS or RNS in vascular endothelium.

In vivo simulation with NAC treatment obviously prevented the drop in plasma glutathione thiol level, which is logical because as was previously explained, NAC is an acetylated precursor of the amino acid L-cysteine and can be converted into metabolites capable of stimulating glutathione (GSH) synthesis in the body. Therefore, the results from both in vitro and in vivo simulations verify the antioxidant effect of NAC and its capability of repairing the in vivo antioxidative system, which accords with its protective effects on many clinical illnesses characterized by decreased GSH or oxidative stress.

Previous studies have shown that vitamin C has (as a superoxide scavenger) has the capacity of reversing endothelial dysfunction caused by oxidative stress (18). However, in this experiment, antioxidant preconditioning with either vitamin C or NAC before the in vivo simulation failed to help return the plasma level of TBARS to baseline in rats. This result may indicate that in our study the antioxidant NAC through in vivo administration (100 mg/kg ip) is probably unable to elicit complete protection against diving derived-plasma lipid peroxidation because it can suppress the productions of ROS and RNS through in vitro administration. Interestingly, the in vivo protective efficacies of NAC at a similar dose via the same drug administration have been demonstrated by previous studies of hypoxic ischemia induced-brain infarct in rat (13) and acute or chronic inflammation in mice (27, 1). This observation could perhaps suggest a higher ROS production during diving. Regardless, the in vivo potential protection of NAC against oxidative stress-induced damage during air diving still needs further evaluation.

**Plasma ACE activity and angiotensin-II level.** It has been previously shown that after successive deep trimix dives, acute endothelial dysfunction has been triggered in large-conduit arteries and kept unrecovered during the course of repetitive dives (23). Antioxidant therapy was shown to be able to attenuate the reduction in postdive FMD (24, 25), suggesting the positive effect of antioxidant on diving-induced-arterial endothelial dysfunction. In this study, after in vivo simulation the rats did not present any significant variations in plasma ACE activity or angiotensin-II level. The preconditioning with NAC or vitamin C did not show any effect on either ACE activity or angiotensin-II level. These results may only confirm that plasma ACE activity and angiotensin-II level may be not affected during diving.

**Morbidity of DCS.** This study showed no protective effect of either NAC or vitamin C treatment on mild or severe DCS occurrence after in vivo air diving simulation in rats, making it possible to conclude the absence of a direct link between oxidative stress or ROS excess and DCS incidence. In fact, the effects of NAC have been shown to differ in vitro and in vivo (28) and, even if similar doses of NAC appeared to affect hypoxic ischemia-induced brain infarct or acute inflammation (1, 13, 28), the absence of the effect of in vivo NAC treatment on DCS morbidity could possibly be because the dose of antioxidant was too low.

**Conclusion**

Oxidative stress is known to be involved in the postdive reduction in FMD (16), whereas bubbles that form during decompression are considered a major causative element of DCS (4). But bubbles are nevertheless poor predictors DCS occurrence (8), and the endothelial dysfunction hypothesis states that diving-induced endothelial dysfunction, leading to reduction of vasodilation, could be one of the major causes of DCS (16).

This study is the first to investigate the protective mechanism of NAC, a source of sulfhydryl group, on diving-induced endothelial ROS and RNS excesses, NO loss, cellular damage, oxidative stress, and DCS from both in vitro and in vivo perspectives, to explore the potential clinical interest of antioxidants for DCS prevention and treatment. As demonstrated here, NAC had an obvious antioxidant effect both in vitro by suppressing the excesses of mitochondrial superoxide and peroxynitrite, and in vivo by preventing the reduction in plasma glutathione thiol. These results confirm the previously demonstrated diving-induced oxidative damages (19, 23, 24, 25); they are also the first to directly demonstrate a link between ROS outburst during diving at-depth and endothelial cell dysfunction (i.e., the reduction in NO availability and cell death), because when measurements are performed after diving, it is impossible to clearly differentiate the effect of ROS and bubbles on endothelium.

The protection of NAC is thus verified in vivo acting as an antioxidant and in vitro at a cellular level. Because DCS has a multifactorial origin, these findings suggest that oxidative stress could be a contributor to DCS without being the only mechanism or the central mechanism.

These elements also highlight the potential interest of using antioxidants in clinical practice in a hyperbaric or diving context. Due to the technical limitations of an in vitro diving simulation system, the protective effect of NAC on cellular activity was not evaluated in the decompression phase. Therefore, more details on the NAC effect are needed. Nevertheless,
the protective effects of NAC during diving remain, holding new implications for the pharmacology of diving-derived endothelial dysfunction.

ACKNOWLEDGMENTS

We thank Patrick Calves and Stella Roy for their contributions.

GRANTS

Support for this study was provided by the People Program (Marie Curie Actions) of the European Union’s Seventh Framework Program FRP/2007-2013 under REA Grant Agreement 264816.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Q.W., F.G., and M.T. conception and design of research; Q.W., A.M., and M.B. performed experiments; Q.W., A.M., K.L., P.B., and M.T. analyzed data; Q.W., A.M., F.G., and P.B. interpreted results of experiments; Q.W., A.M., and M.T. approved final version of manuscript; M.T. prepared figures; Q.W., F.G., and M.T. drafted manuscript; Q.W., F.G., and M.T. edited and revised manuscript; F.G., Q.W., A.M., F.G., K.L., and P.B. interpreted results of experiments; Q.W., A.M., F.G., K.L., P.B., and M.T. performed experiments; Q.W., A.M., K.L., P.B., and M.T. conceived and designed research; Q.W., A.M., and M.T. acquired funding; Q.W., A.M., F.G., and M.T. conceived and designed research; Q.W., A.M., and M.T. approved final version of manuscript; M.T. prepared figures.

REFERENCES