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NOS inhibition increases bubble formation and reduces survival in sedentary but not exercised rats

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Previously we have shown that chronic as well as a single bout of exercise 20 h prior to a simulated dive protects rats from severe decompression illness (DCI) and death. However, the mechanism behind this protection is still not known. The present study determines the effect of inhibiting nitric oxide synthase (NOS) on bubble formation in acutely exercised and sedentary rats exposed to hyperbaric pressure. A total of 45 adult female Sprague-Dawley rats (270–320 g) were randomly assigned into exercise or sedentary control groups, with and without NOS inhibition, using L-NAME (0.05 or 1 mg ml^{-1}) (a nonselective NOS inhibitor). Exercising rats ran intervals on a treadmill for 1.5 h, 20 h prior to the simulated dive. Intervals alternated between 8 min at 85–90 % of maximal oxygen uptake, and 2 min at 50–60 %. Rats were compressed (simulated dive) in a pressure chamber, at a rate of 200 kPa min^{-1} to a pressure of 700 kPa, and maintained for 45 min breathing air. At the end of the exposure period, rats were decompressed linearly to the 'surface' (100 kPa) at a rate of 50 kPa min^{-1}. Immediately after reaching the surface the animals were anaesthetised and the right ventricle was insonated using ultrasound. The study demonstrated that sedentary rats weighing more than 300 g produced a large amount of bubbles, while those weighing less than 300 g produced few bubbles and most survived the protocol. Prior exercise reduced bubble formation and increased survival in rats weighing more than 300 g, confirming the results from the previous study. During NOS inhibition, the simulated dive induced significantly more bubbles in all sedentary rats weighing less than 300 g. However, this effect could be attenuated by a single bout of exercise 20 h before exposure. The present study demonstrates two previously unreported findings: that administration of L-NAME allows substantial bubble formation and decreased survival in sedentary rats, and that a single bout of exercise protects NOS-inhibited rats from severe bubble formation and death. This is the first report to indicate that biochemical processes are involved in bubble formation, and this information may be important in the search for preventive measures for and treatment of DCI.

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Intravascular bubble formation detected in the pulmonary artery has been used as an indicator of decompression stress (Nishi, 1990) and it is generally accepted that bubbles grow from preexisting gas filled precursors (nuclei; Yount & Strauss, 1982). In blood vessels, nuclei can not be found in free flowing blood, but are probably attached to the blood vessel endothelium where they grow into bubbles that are dislodged into the blood stream (Harvey *et al.* 1944; Harvey, 1951). The formation of bubbles is the basis for decompression illness (DCI), which can lead to severe central nervous system injury and permanent disability (Francis & Gorman, 1993). A DCI-protective effect of training has previously been observed. Broome *et al.* (1995) demonstrated that endurance training reduced the incidence of DCI in the pig, and Rattner *et al.* (1979) showed that 14–28 days of treadmill running in mice, 1 h per day, reduced the symptoms of DCI and increased survival after decompression. In a recent study (Wisløff & Brubakk, 2001) we demonstrated that high-intensity aerobic exercise (85–90 % of maximal oxygen uptake) protected rats from severe decompression and death, and that a single bout 20 h prior to a simulated dive was as effective as a prolonged daily training regimen. Thus, the mechanism behind reduced bubble formation in exercised rats is not due to increased aerobic capacity *per se*, but rather is an acute effect most notable 20 h after a single, or the last, exercise bout, with less effect after 48 h (Wisløff & Brubakk, 2001).

Despite evidence of exercise as a DCI-protective factor, the mechanism has not been identified. From previous studies

(Boycott & Damant, 1908; Harvey, 1951; Dempert *et al.* 1984) it is known that there is a relationship between adiposity and the risk of bubble formation and DCI. More nitrogen tended to be found in the tissue due to the relatively high solubility of the gas in this tissue, enhancing the total nitrogen content of the body and increasing the risk of bubble formation.

Rather than altering nitrogen elimination due to increased capillary density, for example, acute exercise may alter the population of nuclei from which bubbles form. Gas nuclei can be stable more or less indefinitely on a hydrophobic surface (Libermann, 1957), such as on the endothelium of veins and in the aorta (Hills, 1992). Therefore, any process that influences the surface properties of the endothelium may affect bubble formation in the vascular system. One potential process that is known to have substantial influence on the endothelial function and structure is nitric oxide (NO) release. It is known that the NO production from endothelial cells increases within hours following an increase in blood flow and shear stress, as experienced during exercise (Buga *et al.* 1991; Roberts *et al.* 1999). Aside from effects on vascular tone, NO has physiological properties that may be anti-atherogenic, including inhibition of smooth muscle cell proliferation, platelet aggregation and adhesion, and leukocyte activation and adhesion (Radomski *et al.* 1991; Bath, 1993; Lefer *et al.* 1993). Thus, it is conceivable that a decrease in NO may change endothelial properties, increasing the adherence of bubble precursors to the blood vessel endothelium.

Therefore the primary hypothesis of the present study was that NOS inhibition would elevate bubble formation in both rested and acutely exercised rats exposed to hyperbaric pressure. A sub-goal was to describe the relationship between adiposity and the risk for bubble formation in rats.

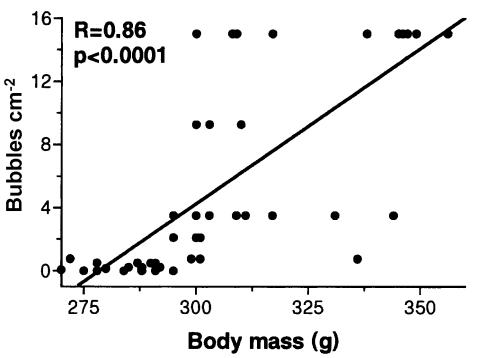

Figure 1. Relationship between body mass and bubble formation in sedentary normal rats

Individual data from 55 sedentary rats exposed to the simulated dive protocol described in the Methods.

METHODS

All experimental procedures conformed with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and the protocol was approved by the Norwegian Council for Animal Research.

Study population and NOS inhibition

A total of 45 adult female 270–320 g Sprague-Dawley rats (Møllegaards, Denmark) were maintained three per cage, and randomly assigned to either training or sedentary control groups. Furthermore, 55 rats with varying body weight were included to establish the relationship between adiposity and the risk of bubble formation. Light was controlled on a 12 h dark/12 h light cycle. Temperature was 21.5 ± 1.5 °C and humidity 55.6 ± 4.0 %. Animals were fed a pellet rodent diet *ad libitum* and had free access to water. The rats were assigned into four groups, as described in Table 1. Pilot studies showed that bubble formation produced by the present simulated dive protocol was highly dependent upon body weight of the rat. In sedentary rats with a body weight above 300 g the protocol produced a lot of bubbles, whereas in those with a body weight below 300 g few bubbles were produced (Fig. 1). To confirm results from a previous study (Wisløff & Brubakk, 2001) and control for possible biases caused by laboratory conditioning (such as small differences in room temperature, light conditioning, timing of anaesthesia, and general handling of the rats) we chose to include a group of rats with a body weight above 300 g (Group I). Group II included only sedentary rats as these were expected to produce few or no bubbles, and exercise could, therefore, not give any further reduction in bubble formation in this group (Fig. 1).

To determine whether NOS inhibition could affect bubble formation, N^{ω}-nitro-L-arginine methyl ester (L-NAME) was administrated in the drinking water (1 mg ml^{-1}, Group III, or 0.05 mg ml^{-1}, Group IV) 6 days prior to the exercise or sedentary protocol (Groups III and IV). The high dose of L-NAME used in the present study was chosen on the basis of previous reports (e.g. Qian *et al.* 2001) to be sure of a high degree of NOS inhibition. This high dose of L-NAME may cause hypertension so to determine whether any effect was nitric oxide mediated *per se*, a group of rats received a lower dose of L-NAME that still provided substantial inhibition of NOS with only moderate pressor effect.

L-NAME was added to fresh water every morning, and the volume of water drunk was monitored. In five control (Group II) and four sedentary high-dose L-NAME rats (Group III) we measured blood pressure in carotid artery. These rats did not undergo the simulated dive procedure. As previously described (Wisløff *et al.* 2002), left ventricular pressures were measured with a micro-tip catheter transducer, (model SPR 407 2F; Millar Instruments, Houston, TX, USA) introduced through the right carotid artery during anaesthesia with Midazolam (Dormicum 'Roche')/Fentanyl/Fluanison (Hypnorm) (0.35 ml 100 g^{-1}, S.C.). End diastolic and peak systolic pressures were calculated as the mean of five consecutive pressure cycles.

Maximal oxygen uptake ($\dot{V}_{O_2,max}$)

Nine days before group assignment, oxygen uptake and respiratory exchange ratio were measured during treadmill running (25 deg inclination, corresponding to ~47 % slope) during the dark cycle in all rats, as previously described (Wisløff *et al.* 2001). The 70-cm-long treadmill had a stainless steel grid at the end of the lane that supplied an electrical stimulus (0.25 mA, one stimulus of 200 ms duration every second) to keep the rats

Table 1. Overview of group assignment, body weight and number of rats in each protocol

Group	Protocol	Body weight	Number of rats
I	Exercised and sedentary	> 300 g	12
II	Sedentary normal rats	< 300 g	11
III	Exercised and sedentary, high dose L-NAME	< 300 g	16
IV	Sedentary, low dose L-NAME	< 300 g	6

Total number of rats is presented. The simulated dive protocol started 20 h after the exercise session.

Table 2. Effects of exercise and NOS inhibition on bubble formation and survival

	Normal rats			NO block		
	Group I		Group II	Group III		Group IV
	Ex	Sed	Sed	Ex	Sed	Sed
Weight (g)	314 ± 8†	311 ± 8†	285 ± 9	280 ± 7	280 ± 11	288 ± 6
Scan grade	1.0 (0–2)*	5.0 (4–5)	0(0–4)*	0 (0–5)*	5.0 (3–5)	5.0 (3–5)
Survival time (min)	> 60 (—)*	20 (4–40)	60 (26–60)*	60 (7–60)*	23 (7–60)	18 (10–60)

*Significant differences between exercised and sedentary counterparts, $P < 0.03$. † Significantly different from Groups II and III, $P < 0.05$. Note that at 60 min after reaching the surface (100 kPa) all surviving rats were killed and, thus, are recorded as having a survival time of 60 min. Body weights are presented as means ± S.D., other data as medians with ranges in brackets. Decompression time was 12 min for all groups. For all groups the compression–decompression protocol started 20 h after the exercise session. Ex, exercised; Sed, sedentary.

running on the lane. In general all rats avoided the electrical grid with 2 ± 1 touches in the exercise session. The treadmill was placed in a metabolic chamber (Sira Engineering, Trondheim, Norway) with a volume of 11 l. Ambient air was pumped through the metabolic chamber at a flow rate of 4.5 l min^{-1}, and samples of extracted air (200 ml min^{-1}) were directed to an oxygen analyser based on a paramagnetic oxygen transducer (Servomex type 1155, Servomex Ltd, UK), and a carbon dioxide analyser (LAIR 12, M&C Instruments, The Netherlands). $\dot{V}_{O_2,max}$ was measured as previously described (Wisløff *et al.* 2001). Briefly, after a 15 min warm-up at 40–50 % of $\dot{V}_{O_2,max}$, treadmill speed was increased by 0.03 m s^{-1} every 2 min until a levelling-off of oxygen uptake was observed despite increased workload. To determine the correct running speed during interval exercise, we established the relationship between running velocity and oxygen uptake for each rat.

Exercise procedure

Interval running exercise (1.5 h total duration) was performed on a treadmill 20 h prior to the simulated dive protocol. Exercise intervals alternated between 8 min at 85–90 % of $\dot{V}_{O_2,max}$ and 2 min at 50–60 %. Before the first interval, each rat performed a 20 min warm-up at 40–50 % of $\dot{V}_{O_2,max}$. Sedentary rats remained in their cages. After the exercise session, each rat was rewarded with 0.5 g chocolate (Crispo, Nidar Bergene, Norway). Sedentary rats were given the same reward. None of the rats were excluded from the study because they avoided running.

Simulated dive protocol and bubble analysis

Rats underwent the compression procedure in threes, one from each group (Groups II and III), or in pairs (Groups I and IV), in a decompression chamber (Sira Engineering) at a rate of 200 kPa min^{-1} to a pressure of 700 kPa, maintained for 45 min breathing air. At the end of the exposure period rats were decompressed linearly to the surface at a rate of 50 kPa min^{-1}. Immediately after surfacing the animals were anaesthetised with Midazolam (Dormicum 'Roche')/Fentanyl/Fluanison (Hypnorm) (0.1 ml 100 g^{-1}, S.C.), and the right ventricle was insonated using a Vivid 5 ultrasonic scanner (GE Vingmed Ultrasound AS, Norway), with a 10 MHz transducer. Bubbles could be seen in the right ventricle and the pulmonary artery as bright spots. Data were stored and played back in slow motion for analysis. If there are few bubbles, they are initially difficult to detect in the images due to the high heart rate in rats, but can be clearly seen on slow motion playback. When many bubbles are present, there is a pressure increase in the right heart giving better images of the right ventricle as it dilates. To facilitate the ultrasonic scanner method, Doppler was used to locate the relatively small vessels and confirm the presence of bubbles. Images were graded according to a previously described method (Eftedal & Brubakk, 1997), with the observer blinded to the group allocation of the rat. This grading system has been used extensively in several animal species as well as in man, and it has been demonstrated that even untrained operators are able to grade the images accurately (Eftedal & Brubakk, 1997). It has also been demonstrated that the grading system for Doppler (Spencer, 1976) coincides with that used for images (Brubakk & Eftedal, 2001). The grading system is non-linear when compared with the actual number of bubbles in the pulmonary artery. To show the relationship between weight and the number of bubbles in the pulmonary artery, the grades were converted to bubbles cm^{-2} (Brubakk & Eftedal, 2001). Surviving rats were killed, by decapitation whilst still under anaesthesia, 60 min after reaching surface pressure (100 kPa). Blood samples were collected immediately after decapitation and haematocrit and haemoglobin analyses were made.

Statistics

Data are expressed as means ± S.D., or as medians and ranges. We chose to use non-parametric tests due to the limited number of rats. A Mann-Whitney *U*-test was used to evaluate differences in bubble formation, whereas the Gehan generalised Wilcoxon test was used to evaluate differences in survival time between groups. $P < 0.05$ was considered statistically significant. Group size and statistical power were estimated using nQuery Advisor software

Table 3. Haemoglobin, haematocrit, respiratory exchange ratio and mean arterial blood pressure in the different groups

	Normal rats			NO block		
	Group I		Group II	Group III		Group IV
	Ex	Sed	Sed	Ex	Sed	Sed
$\dot{V}_{O_2,max}$ (ml kg^{-1} min^{-1})	78.2 ± 3.9	75.5 ± 2.8	74.0 ± 4.9	75.2 ± 2.7	74.0 ± 3.1	74.5 ± 2.1
Hb (g dl$^{-1)}$	14.5 ± 0.3	14.6 ± 0.6	14.1 ± 0.6	15.4 ± 0.4	14.9 ± 0.8	15.2 ± 0.6
Hct (%)	41.0 ± 1.9	40.4 ± 2.1	42.5 ± 1.6	40.5 ± 1.9	42.7 ± 2.3	40.4 ± 1.6
RER	1.08 ± 0.03	1.12 ± 0.01	1.08 ± 0.02	1.14 ± 0.02	1.12 ± 0.02	1.16 ± 0.04
MAP (mmHg)	—	—	72.7 ± 12.4	—	90.7 ± 18.1	—

$\dot{V}_{O_2,max}$, maximal oxygen uptake rate; Hb, haemoglobin; Hct, haematocrit; RER, respiratory exchange ratio; MAP, mean arterial pressure. Blood pressures are calculated from 5 control rats (Group II) and 4 sedentary high dose L-NAME rats (Group III). Data are presented as means ± S.D.

(version 3.0, Statistical Solutions Ltd, Cork, Ireland). Based on cautious estimates from a previous study (Wisløff & Brubakk, 2001) six rats in each group would permit us to detect a 15% difference between groups in bubble grade and survival time ($P = 0.01$, power = 0.80).

RESULTS

Consistent with previous experiments the present exercise protocol reduced bubble formation and increased survival in rats weighing more than 300 g (Table 2, Group I). As expected, the simulated dive protocol induced few bubbles in sedentary rats weighing less than 300 g, and only one died (Table 2, Group II). When using both high (1 mg ml^{-1}) and low dose (0.05 mg ml^{-1}) L-NAME treatment for NOS inhibition the simulated dive protocol induced a lot of bubbles in all sedentary rats, and only one rat in each group survived the protocol (Table 2, Groups III and IV). However, in the exercised and NOS-inhibited groups, bubbles were seen in only one rat, which was the only exercised rat in Group III that did not survive the protocol. No differences in bubble formation or survival time were observed between sedentary rats not receiving L-NAME (Group II) and exercised NOS-inhibited rats (Group III).

The rats drank on average 36 ± 7 ml water each day for the last 5 days before the experimental protocol, with no differences between groups. However, on day 1 the rats receiving water with L-NAME drank only 22 ± 6 ml compared with 35 ± 5 ml in the sedentary groups not receiving the drug. Haematocrit, haemoglobin, maximal oxygen uptake and respiratory exchange ratio measurements were not different between groups (Table 3). There was a trend toward higher blood pressure in the rats receiving high-dose L-NAME compared to controls (Table 3).

DISCUSSION

The present study demonstrates two previously unreported findings: that administration of N$^{\omega}$-nitro-L-arginine methyl ester, a non-selective inhibitor of NO synthase (NOS), allows substantial bubble formation and decreases survival in sedentary rats, and that a single bout of exercise protects NOS-inhibited rats from severe bubble formation and death. The data suggest that the mechanisms behind reduced susceptibility to DCI are multifactorial. Both acute exercise and NOS have effects but they may not be linked. How these factors may contribute to the protective effect is discussed below.

The role of normal NOS in the process protecting against bubble formation in sedentary rats is suggested by the fact that NOS inhibition abolished the resistance to bubble formation in rats weighing less than 300g. Even if L-NAME reduced tissue blood flow as a result of vasoconstriction in sedentary rats, which is likely, the 45 min simulated dive was long enough for saturation of dissolved tissue gas in all well perfused organs. We therefore assume that the tissue gas tensions at the start of decompression was similar between the groups. The delayed washout of gas (due to low tissue blood flow) during the 12 min decompression could cause greater supersaturation and, hence, an increased bubble formation in NOS-inhibited rats compared to that in control rats. If flow limitations are part of the mechanism for increased bubble formation, the data indicate, however, that reduced flow during decompression is of minor importance, as there was no difference in response between the animals receiving high and low doses of L-NAME. The present data suggest that the L-NAME effect is NOS-mediated *per se* and not a consequence of hypertension. Chronic oral administration of L-NAME in the rat is a widely used model of hypertension (reviewed in Zatz & Baylis, 1998). Previous studies have shown that one to six months treatment with L-NAME induces hypertension (e.g. Tibbits *et al.* 1981), while 5 days treatment in the present study was not enough to increase the blood pressure substantially. From this we speculate that NOS inhibition (due to administration of L-NAME) makes the blood vessel endothelium more hydrophobic and increases the adherence of bubble precursors. Furthermore, in acutely exercised rats, we believe that either bubble precursors are washed out,

and/or the exercise stimulus is so strong that it overrides the NOS inhibition, allowing a certain degree of NOS, and thereby making the blood vessel endothelium less hydrophobic, possibly reducing the number of bubble precursors.

Even if bubble nuclei are formed or enlarged by exercise, a recent study indicates that the lifetime of those bubbles are of the order of minutes to a few hours (Dervay *et al.* 2002). It takes 10–100 h to regenerate a depleted nuclei population (Yount & Strauss, 1982). These results are in accordance with our previous study (Wisløff & Brubakk, 2001), in which we observed a most notable reduction in bubble formation 20 h after a single exercise bout, with less effect after 48 h.

It is known that short-term high-intensity exercise (about 90 % of $\dot{V}_{O_2,max}$) can induce hypervolaemia (Richardson *et al.* 1996), and the responses of aldosterone, angiotensin–renin and atrial natriuretic peptide (amongst others) all have significant consequences that last 24–48 h (Richardson *et al.* 1996). Increased plasma volume could increase the functionally active capillary bed (i.e. the number of capillaries and rate of plasma exchange through the muscle bed) which might increase the rate of nitrogen elimination. However, the haematocrit and haemoglobin values were similar in the two groups indicating that difference in plasma volumes was unlikely.

In the search for the mechanism of the exercise effect other possible parameters should also be evaluated. Increased blood flow experienced during exercise increases, amongst other things, the release of platelet derived growth factor β, transforming growth factor β, tissue plasminogen activator, endothelin-1 and prostacyclin, all of which can modulate endothelial structure and function, and thus probably the bubble formation. Prostacyclin, in particular, might be as important a mediator of flow-dependent vasodilation as nitric oxide in skeletal muscle beds (Koller *et al.* 1995), presumably the major site of increased blood flow caused by exercise and probably a significant contributor to the bubbles detected in the central venous blood.

Reduced bubble formation and increased survival after a single bout of exercise in rats weighing more than 300 g is in line with a recent study (Wisløff & Brubakk, 2001). These results confirm previous reports (Boycott & Damant, 1908; Dempert *et al.* 1984) that there is a relationship between adiposity and the risk of DCI and bubble formation. In line with our results Harvey (1951) found a correlation between bubble appearance in the caval vein and fat content in resting cats, but not in cats in which the muscles were electrically stimulated. More nitrogen would tend to be found in the fat tissue due to the relatively high solubility of the gas in this tissue, thus, increasing the total nitrogen content of the body in fat animals compared to lean counterparts. This relationship is indicated in Fig. 1. Our results, as well as those of Harvey (1951), could indicate that other factors than total supersaturation are of importance for bubble formation.

In conclusion, this is the first report to indicate that biochemical processes are involved in bubble formation. This information may be important in the future search for methods of prevention and treatment of DCI. The present study indicates that several independent mechanisms may be involved in the protection against formation of vascular bubbles, as exercise protects even in the presence of NOS inhibition, which promotes bubble formation in sedentary rats. The present data neither answer the question of whether additional NOS induced by NO-releasing agents is more beneficial than the normal basal production of NO before and during the compression–decompression exposure, nor elucidate the volume and timing of exercise required to get a protective effect. This should be addressed in future studies as well as evaluating the importance of these findings in man.

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