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# Nitric oxide increases myocardial efficiency in the hypoxia-tolerant turtle *Trachemys scripta*

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## SUMMARY

**Nitric oxide (NO) may influence cardiac mechanical performance relative to $O_2$ consumption by depressing respiration rate and by affecting the excitation–contraction coupling. Such effects of NO should be particularly important during hypoxia in species such as the hypoxia-tolerant turtle *Trachemys scripta*. In heart ventricle preparations from this species, the ratio of twitch force to $O_2$ consumption increased by approximately 15% during full oxygenation and by approximately 60% during hypoxia in the presence of added L-arginine [the substrate for nitric oxide synthase (NOS)]. This effect was primarily due to a decrease in $O_2$ consumption and may represent an increase in the twitch force obtained per ATP and/or in the ATP obtained per $O_2$. Lactate production during hypoxia did not differ between preparations treated with either L-arginine or asymmetric dimethylarginine (ADMA), an inhibitor of NOS, suggesting that NO does not elicit a compensatory increase in anaerobic metabolism. ADMA did not reverse the effects of L-arginine on $O_2$ consumption significantly, although pre-treatment with ADMA abolished the effect of L-arginine, consistent with the competitive binding of L-arginine and ADMA to NOS. Histochemical studies using the fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2 DA) revealed NO production in the presence of added L-arginine. In conclusion, NO may augment heart contractility obtained per $O_2$ by deceasing $O_2$ consumption without affecting either lactate production or developed force. This effect was particularly pronounced under $O_2$ deficiency and may therefore contribute towards preserving cardiac function and to the overall excellent hypoxic tolerance of the turtle.**

Key words: nitric oxide, heart muscle, freshwater turtle, contractile force, oxygen consumption, hypoxia.

## INTRODUCTION

Nitric oxide (NO) has been recognized as a signal molecule, having autocrine and paracrine actions, and as a key regulator of a number of cellular processes in vertebrates, including ectotherms (Skovgaard et al., 2005; Tota et al., 2005; Imbrogno et al., 2006). Different isoforms of nitric oxide synthase (NOS), which catalyzes the formation of NO from L-arginine (L-Arg), are expressed in the mammalian heart muscle. The extent of this expression varies between and within cells as well as with animal living conditions; however, typically, endothelial NOS is quantitatively the predominant isoform expressed. Although endothelial NOS isoforms from non-mammalian species have not yet been isolated, recent evidence indicates the existence of functional NOS isoforms in the heart and vasculature of dipnoi, teleosts, reptiles and amphibians (e.g. Tota et al., 2005; Amelio et al., 2006; Amelio et al., 2008; Jennings and Donald, 2008). As an important general action, NO downregulates the $O_2$ consumption rate, primarily by inhibiting the binding of $O_2$ to mitochondrial cytochrome *c* oxidase both competitively and uncompetitively (Mason et al., 2006; Erusalimsky and Moncada, 2007; Cooper et al., 2008). Because of its competition with $O_2$, NO inhibits cellular respiration to a larger extent at low $O_2$ tensions and may thus protect cellular functions under hypoxia by extending $O_2$ availability (Hagen et al., 2003). Such effects may also become evident during normal oxygenation. NO has been found to increase efficiency in oxygenated heart muscle from guinea pig, by downregulating cellular respiration relative to both mechanical performance and ATP synthesis rate (Shen et al., 2001). NO has also been found to increase contractile performance relative to $O_2$ consumption in dog cardiac muscle (Setty et al., 2002). A number of NO-dependent mechanisms may be important for myocardial hypoxia. NO may open mitochondrial ATP-sensitive $K^+$ channels ($K_{ATP}$) (Ljubkovic et al., 2007; Jennings and Donald, 2008), which appear to be an element in pre-conditioning (Lebuffe et al., 2003), or may influence the fatty acids to carbohydrates substrate preference in the mammalian heart (Canty 2000; Williams et al., 2008) and consequently change the amount of ATP formed per $O_2$ consumed. Apart from influencing energy metabolism, NO produced by NOS also influences $Ca^{2+}$ regulation in cells and enhances the release of $Ca^{2+}$ from the sarcoplasmatic reticulum (SR) in the mammalian myocardial cell (Xu et al., 1999; Viner et al., 2000; Petroff et al., 2001).

With this background, NO may be implicated in the remarkable hypoxic tolerance of certain ectothermic vertebrates such as freshwater turtles. Some turtle species experience prolonged periods of severe hypoxia, e.g. being submerged in water without access to air during winter hibernation and during dives in the summer (e.g. Shi et al., 1999; Jackson, 2002). Accordingly, heart muscle from turtles (*Trachemys scripta* and *Chrysemys picta*) shows an excellent maintenance of contractility and energy state when subjected to severe hypoxia (Overgaard and Gesser, 2004; Overgaard et al., 2007). The finding that hypoxia, compared with full oxygenation, increased twitch force development relative to the energy liberated, as assessed by $O_2$ consumption and lactate production (Overgaard and Gesser, 2004), is of particular interest in the present study as NO may contribute to this process. For these reasons, we examined the influence of NO on mechanical performance, recorded as twitch force and resting tension, and also on cellular energy liberation, in terms of $O_2$ consumption and lactate production, in turtle ventricular muscle under exposure to full oxygenation and hypoxia.

## MATERIALS AND METHODS

### Experimental animals and myocardial preparations

Freshwater turtles (*Trachemys scripta* Schoepff) were obtained from Lemberger Reptiles (Oshkosh, WI, USA) and kept in water tanks at 20°C in a 12 h:12 h light:dark cycle. They had free access to basking platforms and were fed regularly.

The turtles were captured with a net and decapitated. The plastron was opened with a clinical bone saw, and the heart was quickly transferred to an ice-cold physiological solution containing (mmol $l^{-1}$): NaCl (95), KCl (2.5), $MgSO_4$ (0.94), $NaHCO_3$ (25), $NaH_2PO_4.H_2O$ (1), $CaCl_2$ (1) and glucose (5), equilibrated with 48% $O_2$, 2% $CO_2$ and 50% $N_2$, which resulted in pH 7.7.

Ring-shaped preparations were made from the ventricle. The rings weighed between 18 and 50 mg.

### Experimental setup

The experimental setup has been described previously (Kalinin and Gesser, 2002; Overgaard and Gesser, 2004). Briefly, a single ring-shaped preparation was mounted on two hooks. The lower end of the myocardial preparation was attached to one hook fixed in the measuring chamber whereas the upper end of the preparation was carried by a second hook connected to the force transducer (Fort 10; World Precision Instruments, Sarasota, FL, USA) through a hole in the lid of the measuring chamber. A micrometer screw allowed the force transducer to be moved vertically and the length of the myocardial preparation to be adjusted. The micrometer screw also provided the distance between the two hooks and thus an estimate of the preparation length, which was in the range of 4–10 mm.

Two silver electrodes, coated with silver chloride, were placed on opposite sides of the myocardial preparation and connected to a stimulator (Grass SD 9, Quincy, MA, USA) through two separate holes in the lid of the measuring chamber. The stimulator provided square pulses at a rate of 0.25 Hz and with a polarity that was altered between stimulations. Each stimulation had a duration of 5 ms and a voltage 1.5 times that which was necessary to elicit full twitch force development (e.g. Kalinin and Gesser, 2002).

The physiological solution was recirculated between the measuring chamber (2.56 ml) and the reservoir (20 ml). The solution contained in the reservoir was continuously bubbled with a gas mixture delivered by a precision gas-mixing pump (Wösthoff, Bochum, Germany). The chamber with the $O_2$ electrode and the reservoir were thermostatically controlled to 20°C, and the solution in the chamber was continuously stirred using a glass-covered magnetic stir bar. During measurements of $O_2$ consumption and of the associated twitch force, the recirculation of the solution was stopped and the decrease in $O_2$ tension in the chamber was recorded over time using an $O_2$ electrode (Radiometer E5046, Copenhagen, Denmark).

Values of $O_2$ tension and force were sampled at a rate of 100 $s^{-1}$ using a Biopack MP100 data acquisition system (Biopack Systems, Goleta, CA, USA) connected to a computer using the program Acqknowledge 3.7.0 (Biopack Systems). The rate of $O_2$ tension change was obtained by linear regression analysis, and the twitch force was determined as the difference between the minimal (resting tension) and maximal force values.

The myocardial preparations were stretched to the peak of the force–length relationship. Force development was allowed to stabilize for at least 60 min before experiments began.

### Equipment calibration

The $O_2$ electrode was calibrated each day. A zero value was obtained using a solution of 1.6 mmol $l^{-1}$ $NaSO_3$ in 10 mmol $l^{-1}$ $Na_2B_4O_7$ Borax (sodium tetraborate), and the value of atmospheric $O_2$ was obtained by gassing the solution with air. The physiological solution was equilibrated with 48% $O_2$, 50% $N_2$ and 2% $CO_2$. A 20 min test was performed to assess any background $O_2$ changes without tissue in the chamber and with stimulation switched on. Values below 0.2 μmol $O_2$ $min^{-1}$ were accepted and subtracted from the $O_2$ consumption rate in the presence of myocardial tissue.

### Experimental protocol

The effects of 0.1 mmol $l^{-1}$ L-Arg on $O_2$ consumption and twitch force were examined in an experimental series in which $O_2$ was either high (48% $O_2$, 50% $N_2$, 2% $CO_2$) or low (8% $O_2$, 90% $N_2$, 2% $CO_2$). This concentration of L-Arg was chosen to maximize NO production by saturating NOS with L-Arg, as Michaelis constant, $K_m$, for L-Arg has been assessed to be 0.6 μmol $l^{-1}$ (Matsuoka et al., 1994). Experiments also included the application of 1 mmol $l^{-1}$ asymmetric dimethylarginine (ADMA), which inhibits cellular NOS activity (Kodja and Kottenberg, 1999; Böger et al., 2003). Preliminary experiments using L-NAME, a commonly used NOS inhibitor, have shown no significant effects on the parameters examined in the present study (data not shown), which agrees with a previous report of no effect of L-NAME on the *in vivo* heart function in this species (Crossley et al., 2000). After the initial stabilization, the recirculation of solution between the measuring chamber and the reservoir solution was stopped for 40 min, during which $O_2$ consumption and twitch force were recorded.

At the end of this period, the substance to be tested (either L-Arg or ADMA) was added to the reservoir solution, whereby it reached the myocardial preparation when circulation was resumed. After 40 min, circulation was again stopped and $O_2$ consumption and twitch force were recorded as before in the presence of the substance added. The sequence with open and closed circulation could be repeated, allowing the effects of several substances to be tested in each experiment (Fig. 1).

To examine spontaneous changes in $O_2$ consumption and twitch force development during the experiments, myocardial preparations were exposed to two or three cycles of recordings without any additions.

### Hypoxic lactate production

Tissue lactate production and twitch force development were recorded in a separate experimental series. Two rings were prepared from each heart and mounted around two hooks, in a setup identical to that described above, and immersed in 15 ml of thermostatted physiological solution.

Myocardial ring-shaped preparations were stimulated and stretched to produce maximal twitch force. After stabilization in 48% $O_2$, the physiological solution was exchanged for a new solution equilibrated with 8% $O_2$ (hypoxia). After 30 min under hypoxia, the bath solution for each myocardial preparation was replaced with a new solution, which contained 0.1 mmol $l^{-1}$ L-Arg for one of the two preparations and 1 mmol $l^{-1}$ ADMA for the other preparation to inhibit endogenous NO production (i.e. in the absence of added L-Arg). After a further 60 min, the myocardial preparations were rapidly frozen in liquid $N_2$ and stored at –80°C together with samples of the physiological bath solution. The lactate contained in both the tissue and the bath solution was measured so as to estimate the anaerobic glycolytic rate of the heart preparations treated with either L-Arg or ADMA. To assess tissue lactate production, the frozen heart tissue preparation was homogenized (Ultra-Turrax T25, Jancke and Kunkel, Steufen, Germany) in 3 mol $l^{-1}$ perchloric acid and centrifuged. The lactate contained in the supernatant and in the

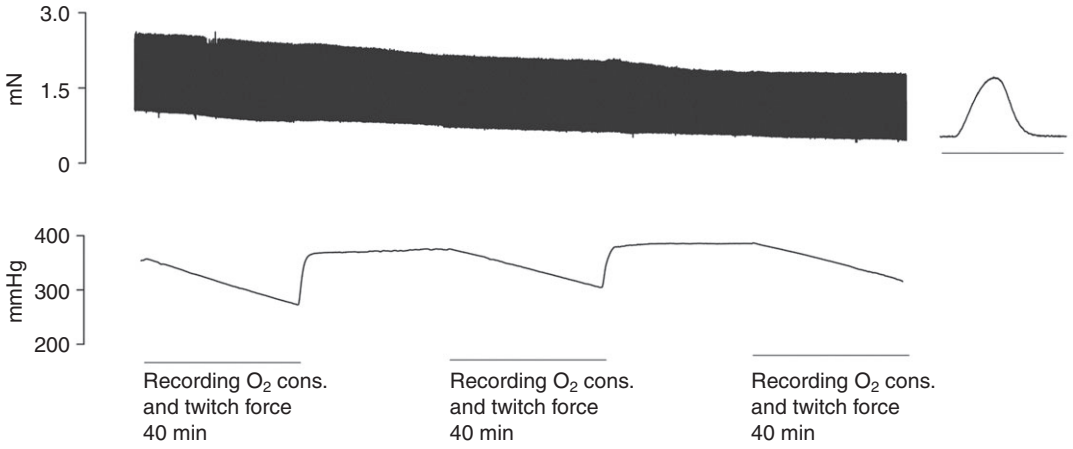


Fig. 1. Representative traces showing three consecutive 40 min recordings of twitch force (upper panel) and $O_2$ consumption (lower panel). (1 mmHg= 0.133 kPa.)

physiological bath solution was determined enzymatically (Lowry and Passonneau, 1972).

### Confocal imaging of tissue NO

Four ring-shaped preparations from each ventricle were stretched and maintained at 48% $O_2$, 50% $N_2$ and 2% $CO_2$ for 40 min. The gas was then switched to 8% $O_2$, 90% $N_2$ and 2% $CO_2$ and, after 30 min, L-Arg and ADMA were added. After 40 min, the presence of NO in the tissue was examined according to the protocol provided by Rodriguez and colleagues (Rodriguez et al., 2005). The cell-permeable fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2 DA, Alexis, Lausanne, Switzerland) was dissolved in dimethylsulphoxide (DMSO) and immediately added to a final concentration of 0.001 mmol $l^{-1}$ in the bath solution. After 60 min, the bath solution was replaced by a solution containing 1 mmol $l^{-1}$ ADMA to stop NO production.

Heart tissue preparations were then mounted on a coverslip to detect fluorescence of DAF-2T, the product of the reaction between NO and DAF-2 DA, using a confocal microscope (Zeiss LSM 5 pascal, Zeiss, Germany) with 488 nm as excitation and 515 nm as emission wavelengths, respectively (Rodriguez et al., 2005).

### Calculations and statistics

During the 40 min with a closed chamber, the mean value of the twitch force tension, sampled every 5 min, was taken to measure the twitch force production associated with $O_2$ consumption.

Myocardial $O_2$ consumption per gram wet mass was calculated according to the following equation:

$$\dot{V}_{O_2} = (k\,\alpha_{O_2}\,v)\,/\,m\,.$$

Here, $\dot{V}_{O_2}$ is the $O_2$ consumption rate (μmol $O_2$ $min^{-1}$ $g^{-1}$), $k$ is $\Delta P_{O_2}$/min, $\alpha_{O_2}$ is the $O_2$ solubility coefficient (12.93 μmol $l^{-1}$ $kPa^{-1}$), $v$ is the volume of the chamber (2.56 ml) and $m$ is the tissue preparation mass (g).

All data are expressed as means ± s.e.m. Fractional changes were tested for significance with Student's $t$-test. A $P$-value of $<0.05$ was considered to indicate significance.

In order to use isometric twitch force as an indicator of mechanical function, the thickness and cross-sectional area of the myocardial preparations were kept as constant as possible so that the twitch force given in mN could be assumed to be proportional to the twitch force related to the cross-sectional area. The relationship between mechanical performance and aerobic metabolism was assessed by the ratio of developed twitch force to $O_2$ consumption rate (mN)/(μmol $O_2$ $min^{-1}$ $g^{-1}$). Changes in this ratio were analyzed by normalizing it to the ratio obtained under control situations.

## RESULTS

### Full oxygenation

The effects of 0.1 mmol $l^{-1}$ L-Arg during full oxygenation (48% $O_2$, 50% $N_2$ and 2% $CO_2$) were examined in the first series of experiments. In the first 40 min run, twitch force and $O_2$ consumption were recorded in the absence of L-Arg and in the second run with 0.1 mmol $l^{-1}$ L-Arg in the bath solution. The addition of L-Arg caused a 12±2% decrease in $O_2$ consumption (Fig. 2A) whereas twitch force did not change significantly (Fig. 2B). The decrease in $O_2$ consumption together with an unchanged twitch force resulted in an increased efficiency (i.e. twitch force to $O_2$ consumption ratio) by 15±3% (Fig. 2C).

The addition of ADMA during full oxygenation did not change the parameters significantly (data not shown). Three preparations subjected to the same experimental time course but without the addition of L-Arg displayed no significant spontaneous changes in the parameters recorded (data not shown).

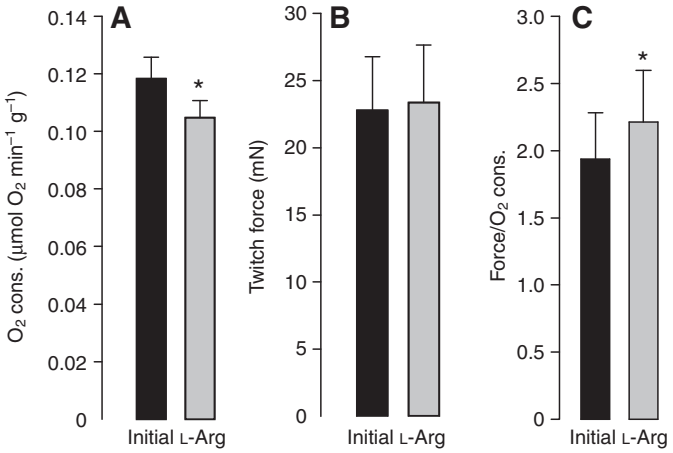


Fig. 2. (A) $O_2$ consumption (μmol $O_2$ $min^{-1}$ $g^{-1}$). (B) Developed twitch force (mN). (C) Ratio of twitch force to $O_2$ consumption recorded before (black bars) and after (gray bars) the addition of 0.1 mmol $l^{-1}$ L-arginine (L-Arg) in full oxygenation ($N$=6). Data are means ± s.e.m. *Significant change from the initial value ($P$<0.05).

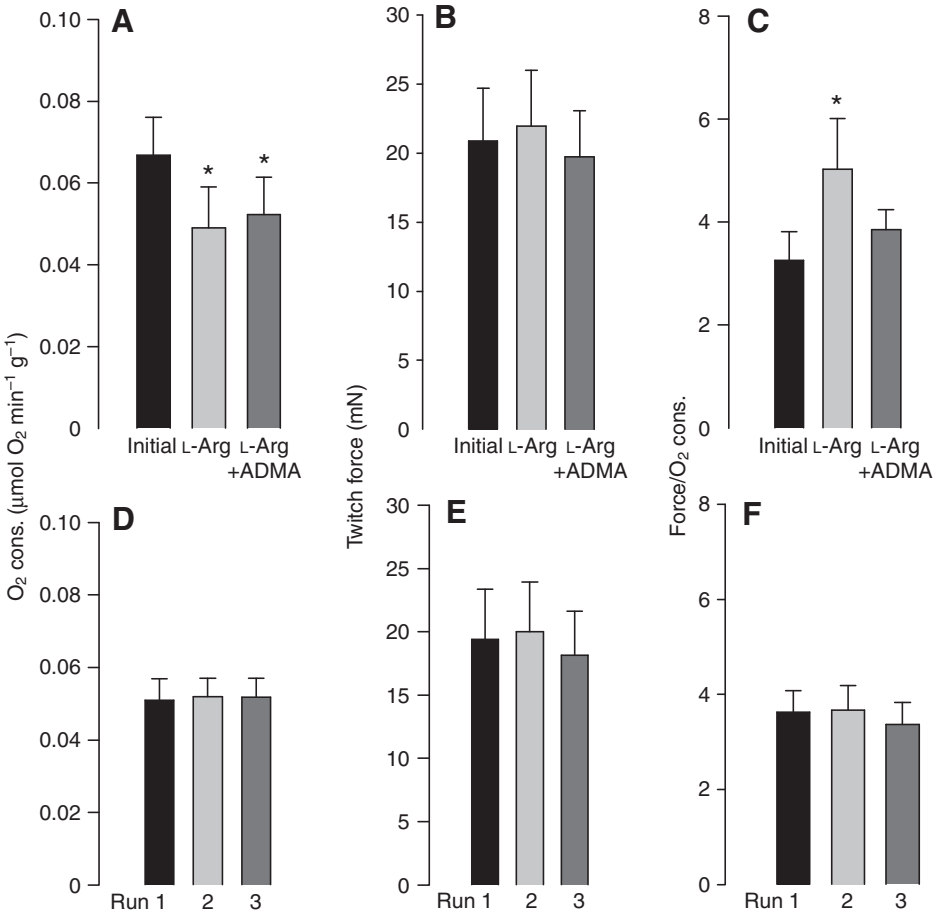


Fig. 3. (A) $O_2$ consumption (μmol $O_2$ $min^{-1}$ $g^{-1}$). (B) Developed twitch force (mN). (C) Ratio of twitch force to $O_2$ consumption recorded for preparations sequentially subjected to no addition, 0.1 mmol $l^{-1}$ L-arginine (L-Arg) and L-Arg+1 mmol $l^{-1}$ asymmetric dimethylarginine (ADMA) (*N*=6), as indicated. As control (D–F) the three parameters were also recorded according to the same time schedule in preparations receiving neither L-Arg nor ADMA (*N*=5). Data are means ± s.e.m. *Significant change from the initial value (*P*<0.05).

### Low $O_2$

After lowering $O_2$ from 48% to 8% (8% $O_2$, 90% $N_2$ and 2% $CO_2$), $O_2$ consumption and twitch force were recorded in three subsequent runs; the first one without any additions, the second run in the presence of 0.1 mmol $l^{-1}$ L-Arg and the third run with L-Arg and 1 mmol $l^{-1}$ ADMA. $O_2$ consumption decreased significantly by 30±4%, following the addition of 0.1 mmol $l^{-1}$ L-Arg, which is significantly larger than the decrease observed at full oxygenation. $O_2$ consumption also remained significantly lower after the addition of ADMA (Fig. 3A).

Neither L-Arg nor ADMA influenced twitch force significantly (Fig. 3B) whereas L-Arg entailed an increase in the ratio of twitch force to $O_2$ consumption by 60±18% (Fig. 3C), which was significantly higher than that of 15±3% recorded at full oxygenation, i.e. with 48% $O_2$ in the gas mixture. ADMA tended to reverse the effects of L-Arg, although not significantly (Fig. 3A,C). The control experiment did not indicate any significant spontaneous changes over the course of the experiment (Fig. 3D–F). In accordance with the result observed at 48% $O_2$, ADMA seemed to be unable to efficiently displace L-Arg from the enzyme active site under the conditions applied. At a reversed order of additions, i.e. when ADMA was added before L-Arg, we did not observe any significant changes in either $O_2$ consumption or twitch force (Fig. 4), indicating that ADMA is an efficient NOS inhibitor in this species.

### Confocal NO detection

In order to verify whether the effects seen with added L-Arg could be ascribed to an increased NOS activity, we examined NO production in hypoxic heart tissue using the fluorescence NO probe DAF-2DA. The L-Arg-dependent production of NO was examined using the procedure described in Table 1. We observed an increase in fluorescence in the presence of L-Arg, indicating a stimulation of NO production, which tended to be only slightly reversed by ADMA (Fig. 5). The basal production of NO in the absence of externally added L-Arg was too low to be detected by this technique.

### Lactate

Anaerobic glycolysis and lactate production are typically enhanced at low $O_2$. Lactate might also have contributed to the higher ratio of twitch force to $O_2$ consumption observed in the presence of L-Arg (Fig. 2C, Fig. 3C). This possibility was, however, not supported as a separate experimental series did not reveal any significant difference in lactate production for hypoxic heart preparations exposed to either L-Arg or ADMA, the values being 0.17±0.04 and 0.19±0.05 μmol $min^{-1}$ $g^{-1}$ lactate, respectively (*N*=7).

## DISCUSSION

A main finding of the present study was that the $O_2$ consumption rate in turtle ventricular preparations stimulated to isometric force development was diminished in the presence of L-Arg and particularly during hypoxia. The fact that the myocardial preparations were stretched to maximize twitch force should stimulate NOS activity according to previous studies, showing that stretch is an important regulator of this activity (e.g. Tota et al., 2005). In our confocal studies, we could not detect a basal NO production from endogenous L-Arg under control conditions, whereas NO production was clearly observed in the presence of L-Arg added as a substrate. Hence, the turtle myocardium seems

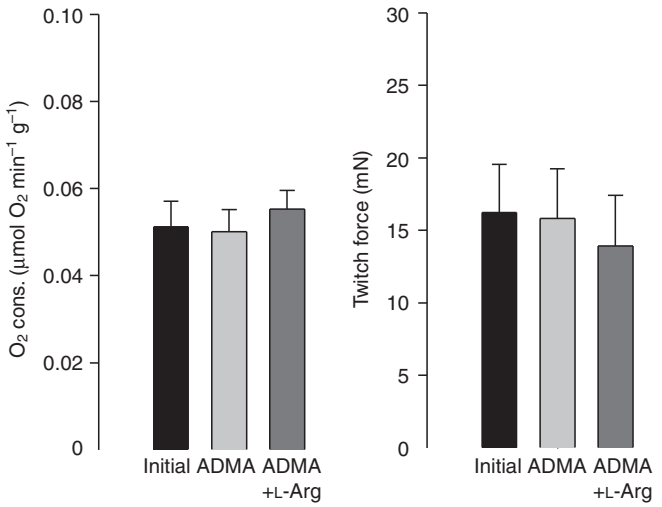


Fig. 4. (A) $O_2$ consumption (μmol $O_2$ $min^{-1}$ $g^{-1}$). (B) Developed twitch force (mN) recorded for preparations sequentially subjected to no addition, 1 mmol $l^{-1}$ asymmetric dimethylarginine (ADMA) and 1 mmol $l^{-1}$ ADMA+0.1 mmol $l^{-1}$ L-arginine (L-Arg), as indicated ($N$=5).

Table 1. Experimental conditions for the samples depicted in Fig. 5

| | Sample 1 | | Sample 2 | | Sample 3 | | Sample 4 | |
|---|---|---|---|---|---|---|---|---|
| Min | L-Arg | ADMA | L-Arg | ADMA | L-Arg | ADMA | L-Arg | ADMA |
| 40 | + | – | – | – | + | – | + | + |
| | DAF-2 DA | | | | | | | |
| 60 | – | | + | | + | | + | |

Sample 1, tissue autofluoroscence in the presence of L-arginine (L-Arg) (0.1 mmol $l^{-1}$) and absence of 4,5-diaminofluorescein diacetate (DAF-2 DA); sample 2, basal nitic oxide (NO) production without L-Arg added; sample 3, NO production with 0.1 mmol $l^{-1}$ L-Arg added; sample 4, effect of asymmetric dimethyl arginine (ADMA) (1 mmol $l^{-1}$) in the presence of 0.1 mmol $l^{-1}$ L-Arg. After 40 min of exposure, samples were incubated for 60 min with 0.001 mmol $l^{-1}$ DAF-2 DA as indicated.

to differ from the mammalian myocardium, in which a basal production of NO was evident in the absence of added L-Arg (Shen et al., 2001; Eu et al., 2003; Palacios-Callander et al., 2004). Although the *in vivo* levels of L-Arg in ectotherms are unknown, it should be noted that the L-Arg concentration (0.1 mmol $l^{-1}$) used in the present study to stimulate NO production is approximately the same as the plasma concentrations recorded in mammals [~0.1 mmol $l^{-1}$ (Morris, 2000)]. It should also be recalled that $K_m$ for L-Arg in mammalian NOS is about 100-fold lower [~0.6 μmol $l^{-1}$ (Matsuoka et al., 1994)].

Despite its importance in the control of cardiovascular function in mammals, endothelial NOS isoforms have not been cloned in non-mammalian animals. The results shown in the current study, and previously by other groups (Tota et al., 2005; Amelio et al., 2006; Amelio et al., 2008; Jennings and Donald, 2008), strongly suggest that functional (possibly non-endothelial) NOS isoforms are expressed in several ectotherm species, belonging to dipnoi, teleosts, amphibians and reptiles, and that NO as a cardiovascular signaling molecule has been conserved in the evolution of vertebrates.

The decrease in $O_2$ consumption rate of the turtle myocardium observed in the present study in the presence of L-Arg may be explained by an inhibition by NO of mitochondrial respiration due largely, but not exclusively, to a competitive inhibition of the binding of $O_2$ to cytochrome *c* oxidase, i.e. the final step in the respiratory chain (Erusalimsky and Moncada, 2007; Cooper et al., 2008). Accordingly, the relative decrease in $O_2$ consumption was significantly more pronounced during hypoxia than during full oxygenation. As a further contribution to this difference, cytochrome *c* oxidase seems

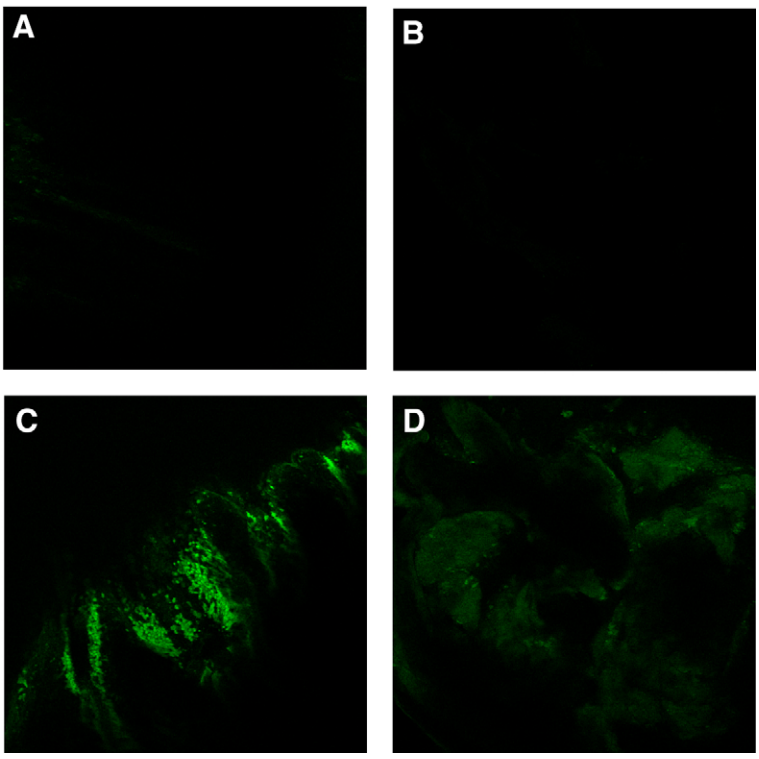


Fig. 5. Results for four ventricular samples treated with DAF-2 DA as described in Table 1. (A, sample 1) Tissue autofluoroscence in the presence of L-arginine (L-Arg) (0.1 mmol $l^{-1}$) and absence of 4,5-diaminofluorescein diacetate (DAF-2 DA). (B, sample 2) Basal nitric oxide (NO) production without L-Arg added. (C, sample 3) NO production with 0.1 mmol $l^{-1}$ L-Arg. (D, sample 4) Effect of asymmetric dimethylarginine (ADMA) (1 mmol $l^{-1}$) in the presence of 0.1 mmol $l^{-1}$ L-Arg.

to remove NO more efficiently at a high oxidation level (Palacios-Callander et al., 2007). Consistent with this, Kojic and colleagues found no effect of NO on $O_2$ consumption in the mouse heart *in vivo*; a result suggested to be due to high tissue oxygenation in combination with a high concentration of the NO scavenger, myoglobin (Kojic et al., 2003). It should be noted that NO may not only act on the respiratory chain but it may also inhibit $O_2$ consumption by inhibiting creatine kinase and, in turn, the sensitivity of mitochondrial respiration to cytosolic ADP (Kaasik et al., 1999).

Besides cytochrome *c* oxidase of mitochondria, another biological target of NO is soluble guanylate cyclase, whose activation by NO generates cGMP, which induces muscle relaxation in the vasculature and influences heart performance (Balligand et al., 2000). Under the conditions used in our present study, an increase in L-Arg levels only affected $O_2$ consumption and not contractility. However, our data do not exclude a physiological role of the NO–cGMP pathway in the regulation of turtle heart contractile function.

In the myocardial tissue of many species, NO affects contractility either positively, as in the icefish (Pellegrino et al., 2003), or negatively, as in the eel (Imbrogno et al., 2001). However, in guinea pig myocardial tissue, Shen and colleagues found that NOS-derived NO depressed $O_2$ consumption, while leaving contractility unchanged (Shen et al., 2001). We obtained similar results with turtle myocardium as L-Arg augmented the ratio of twitch force to $O_2$ consumption by diminishing $O_2$ consumption without affecting twitch force development. This effect was particularly evident under hypoxia but also occurred under full oxygenation. The drop in $O_2$ consumption appeared not to involve any significant compensatory enhancement of anaerobic metabolism in the heart recorded as lactate production. Hence, NO seems to augment either contractility relative to the rate of ATP consumption or alternatively to the ATP produced aerobically relative to $O_2$ consumed. A number of possible mechanisms can be envisaged. In mammals, the inhibition of the cytochrome *c* oxidase activity by NO and a consequent increase in reactive oxygen species (ROS) formation at least in some cell types activates AMP kinase and, in turn, glycolysis (Erusalimski and Moncada, 2007) whereas in ectotherms activation of AMP kinase may inhibit glycolysis (Bickler and Buck, 2007). However, and of relevance to our results with turtle heart, the activity of AMP kinase also entails a decrease in biosynthetic activity (Erusalimsky and Moncada, 2007), which may potentially increase the fraction of ATP available for contractility. Furthermore, during hypoxia the aerobic ATP production may possibly decrease less than the $O_2$ consumption, because of a partial inhibition of the respiratory chain that may reduce the proton gradient and the proton leak across the inner mitochondrial membrane and tighten the coupling between transmembrane proton flux and ATP synthetase; a coupling that has proven to be rather variable (Gnaiger et al., 2000; Brand, 2005). This scenario is compatible with the study of Shen and colleagues (Shen et al., 2001), providing evidence that NO may entail a lowering of myocardial $O_2$ consumption relative to twitch force development without influencing either ATP synthesis rate or the concentrations of phosphocreatine and inorganic phosphate. Alternatively, an increase in ATP production relative to $O_2$ consumption may be due to a shift from fatty acids to carbohydrates (Canty 2000; Williams et al., 2008). In opposition to this, another study has shown an increased $O_2$ consumption relative to mechanical performance upon inhibition of NOS in dog ventricular muscle exposed to noradrenaline (Setty et al., 2002), although NOS inhibition seems to shift substrate selection from fatty acids to glucose in dog heart (Recchia et al., 1999).

In conclusion, the present study suggests that NO generated from L-Arg contributes to the superior hypoxic tolerance of the freshwater turtle and its heart muscle by reducing the $O_2$ consumption needed for the maintenance of a given contractility. This effect may represent an increase in the force obtained per ATP and/or in the number of ATP obtained per $O_2$.

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