THE CARDIOVASCULAR RESPONSES OF THE RED-EARED SLIDER (TRACHEMYS SCRIPTA) ACCLIMATED TO EITHER 22 OR 5 °C

II. EFFECTS OF ANOXIA ON ADRENERGIC AND CHOLINERGIC CONTROL

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Summary

Cardiovascular control in cold-acclimated freshwater turtles during chronic anoxic exposure is not well understood. We tested the hypothesis that the observed bradycardia in Trachemys scripta results from increased cholinergic inhibitory tone and reduced sympathetic activity. Cardiovascular status was measured in vivo in turtles acclimated to either 22 °C or 5 °C and either acutely exposed (6 h) to anoxia at 22 °C or chronically exposed (22 days) to anoxia at 5 °C. In 22 °C-acclimated turtles, injection of the cholinergic antagonist atropine induced a significant tachycardia under both normoxic and anoxic conditions. However, in 5 °C-acclimated turtles, atropine injection had little effect on heart rate. Therefore, cholinergic control of heart rate was suppressed during cold acclimation; instead, temperature effects are more important in bringing about bradycardia, while the intrinsic effects of anoxia and acidosis are probably important during chronic anoxia. Injection of adrenaline caused a pressor response through increased systemic resistance at both acclimation temperatures. This response was blunted by acute and chronic anoxic exposure, suggesting that systemic vasomotor control was altered independently of acclimation temperature. This anoxic blunting may be related in part to the anoxia-induced increase in systemic resistance. Injection of nadolol after atropine decreased systemic cardiac output. The tonic β-adrenergic cardiac stimulation was attenuated by acute and chronic anoxic exposure. Some of this attenuation of β-adrenergic control could be attributed to the 39–40 % reduction in cell surface β-adrenoreceptor density in the ventricles of these turtles that accompanied acute and chronic anoxic exposure. In conclusion and contrary to our original hypothesis, cholinergic and adrenergic control of the cardiovascular system in turtles was attenuated under cold anoxic conditions, perhaps assuring in the depressed physiological state of these animals.

Key words: turtle, Trachemys scripta, heart, cardiovascular control, β-adrenoreceptor, atropine, adrenaline, nadolol, temperature, anoxia.

Introduction

Cholinergic and adrenergic regulation of the cardiovascular system has been studied in a variety of lower vertebrates, including turtles (Farrell, 1991; Hicks, 1994). The turtle heart, like that of most other ectothermic vertebrates, is under powerful inhibitory cholinergic control and much weaker stimulatory adrenergic control (White, 1976; Burggren, 1987). Apnoeic diving in turtles results in bradycardia (White and Ross, 1966) mediated through cholinergic mechanisms (Hicks and Wang, 1998). In addition, chronic exposure to anoxia in cold-acclimated turtles results in profound decreases in heart rate that dramatically reduce cardiovascular performance (Herbert and Jackson, 1985b) such that systemic cardiac power output can decrease by 330-fold compared with that of normoxic turtles at 22 °C (Hicks and Farrell, 2000). However, the underlying mechanisms responsible for the cardiovascular changes associated with chronic anoxia in cold-acclimated turtles are not well understood, even though we do know that the cholinergic inhibitory control of the heart is preserved in warm-acclimated turtles during a brief (2 h) anoxic exposure (Hicks and Wang, 1998).

Maximum cardiac work measured in heart preparations in situ following short-term exposure to low temperature and anoxia (Farrell et al., 1994) can be substantially greater than routine cardiac work in vivo during acute and chronic anoxic exposures (Herbert and Jackson, 1985b; Hicks and Wang, 1998; Hicks and Farrell, 2000). These differences may mean that in vivo depression of cardiac activity in cold-acclimated anoxic turtles could involve extrinsic autonomic regulatory mechanisms. Alternatively, these differences could arise from the intrinsic effects of anoxia and the accompanying acidosis. In vitro studies have shown a 53 % decrease in maximum force (Jackson, 1987) and a reduced rate of spontaneous contractions
(Bing et al., 1972; Jackson, 1987) in turtle ventricular strips as a result of anoxic exposure. Acute anoxic exposure also had a weak negative chronotropic effect at both 5°C and 15°C on a turtle heart in situ (Farrell et al., 1994). Nevertheless, anoxia is also associated with an increase in plasma catecholamine levels (Wasser and Jackson, 1991), which should stimulate the heart. Hicks and Wang (1998) found that the cardiovascular response to an adrenaline injection was blunted by acute anoxia in turtles held at 22°C. This blunted response during anoxia could reflect either the already high levels of circulating catecholamine in anoxic turtles or a decrease in the sensitivity of the myocardium to catecholamines, perhaps through a decrease in the density of cell-surface β-adrenoreceptors. For example, hypoxic exposure of mammalian cardiomyocytes caused a 35-65% reduction in cell-surface β-adrenoreceptor density (Rocha-Singh et al., 1991; Bernstein et al., 1990, 1992; Voelkel et al., 1981).

The purpose of the present study was to examine cholinergic and adrenergic cardiovascular regulation in red-eared sliders Trachemys scripta acclimated to either 22°C or 5°C and either acutely exposed (6 h) to anoxia at 22°C or chronically exposed (22 days) to anoxia at 5°C. Our approach was to inject cardiovascular agonists and antagonists while measuring cardiovascular status. In addition, cardiac β-adrenoreceptor density was measured using a radioligand assay. The drug infusion study was performed at the same time as the study described by Hicks and Farrell (2000), but the receptor density and blood chemistry studies were conducted on a separate group of turtles.

Materials and methods

Experimental animals and acclimation procedure

Red-eared sliders (Trachemys scripta Gray) (body mass 761±59 g, mean ± s.e.m., N=24) were used in this study. Details of animal maintenance, surgical procedures and post-surgery recovery and acclimation protocols are reported in Hicks and Farrell (2000). During experiments, individual turtles were housed in a glass chamber (30 cm x 30 cm x 60 cm), covered with black plastic to minimize visual disturbance, and were allowed to move freely within the chamber. Four groups of turtles were used for the drug trials. Experiments were performed on 22°C-acclimated turtles immediately after the 8-day post-surgery recovery period. One group was maintained under normoxia while the other was acutely exposed (6 h) to anoxia at 22°C. Another group was maintained at 5°C for 6 days under normoxic conditions before being switched to anoxic conditions, as described above, for 22 days.

Cardiovascular status was regularly monitored without the experimenter present during these recovery, acclimation and exposure periods, as described by Hicks and Farrell (2000).

Experimental protocol

In vivo study

Systemic cardiac output ($Q_{sys}$), heart rate ($f_h$), cardiac stroke volume ($V_{sys}$), systemic arterial blood pressure ($P_{sys}$), systemic cardiac power output ($P_{0sys}$) and systemic resistance ($R_{sys}$) were measured in turtles as described by Hicks and Farrell (2000). Drug trials were timed in the following way. For 22°C-acclimated turtles, the drug trial for normoxic animals began after routine cardiovascular status had been measured for the last time, while that for anoxic animals began after routine cardiovascular status had been measured at the end of the 6 h anoxic period. For 5°C-acclimated turtles, the drug trial for normoxic animals began after routine cardiovascular status had been measured for the last time on day 35 of the acclimation period, while that for anoxic animals began after routine cardiovascular status had been measured for the last time on day 22 of the chronic anoxic exposure. This timing protocol ensured that the measurements of routine cardiovascular status, as reported in Hicks and Farrell (2000), for the four test conditions, were not compromised and that a steady cardiovascular status existed for the start of the drug trial.

The following series of 0.5 ml intra-arterial injections was used for all four experimental conditions: a 0.5 ml control saline injection, 10 μg·kg⁻¹ adrenaline (α- and β-adrenoregic agonist), 1.5 mg·kg⁻¹ atropine (cholinergic, muscarinic antagonist), 10 μg·kg⁻¹ adrenaline, 2.0 mg·kg⁻¹ nadolol (β-adrenoregic antagonist) and 10 μg·kg⁻¹ adrenaline. Preliminary experiments had established that 10 μg·kg⁻¹ was the minimum adrenaline dose required to achieve a sizeable, but not maximal, pressor response in turtles held at 22°C in normoxia. Atropine and nadolol doses were based on previous experiments on T. scripta (Hicks and Wang, 1998; T. Wang, personal communication). A suitable recovery period (30–90 min) was allowed between each injection to permit cardiovascular variables to return to resting values. Cardiovascular variables were monitored continuously and determined by averaging three random 2 min sections from a 10 min recording either immediately prior to an injection or from a period of maximal response.

All drugs were obtained from Sigma Chemical Co. (St Louis, MO, USA) and were dissolved in turtle saline (in mmol·L⁻¹): NaCl, 80; KCl, 2.7; CaCl₂, 2; MgSO₄, 1.4; NaHCO₃, 40; NaH₂PO₄, 2.2; Na₂HPO₄, 0.2; pH 7.8.

In vitro study

A second group of 24 turtles was acclimated to the four test conditions as described previously (three male and three female turtles for each condition). Following the temperature acclimation and anoxic exposure regimes outlined above, turtles were removed from their holding tank and decapitated, and a piece of umbilical tape was quickly secured around the
severed blood vessels to prevent excessive blood loss prior to blood sampling. An electric bone saw (Mopec, Detroit, MI, USA) was used to remove a 4 cm x 5 cm piece of the plastron, exposing the heart and systemic output vessels. A heparinized syringe was used to withdraw 1.5 ml of blood from the left aorta. The blood sample was analyzed for pH, haemoglobin and haematocrit to assess the haematological changes associated with anoxia. A blood pH analyzer (Radiometer, Copenhagen, Denmark) was used for pH measurements, while haemoglobin content was determined spectrophotometrically using a total haemoglobin kit (Sigma Chemical Co., St Louis, MO, USA). Haematocrit was measured on 20 μl blood samples centrifuged at 10,000 g for 2 min. The ventricle was excised, washed with turtle saline, weighed and frozen in liquid nitrogen. The tissue was stored at −70 °C for no longer than 5 months prior to the β-adrenoreceptor assay. All procedures were in accordance with Simon Fraser University Animal Care Guidelines.

Cardiac β-adrenoreceptors

Cell-surface β-adrenoreceptor density (B_max) and binding affinity (K_D) were determined using cardiac tissue punches incubated with a tritiated ligand. The technique, originally used for mammalian hearts (Wilkinson et al., 1991), has been modified for fish hearts (Gamperl et al., 1994), and these modifications were applied here for turtle hearts. Briefly, tissue punches (2 mm diameter x 350 μm thick) were obtained from both the dorsal and ventral ventricular sections and were incubated with various concentrations of the hydrophilic β-adrenoreceptor ligand [3H]CGP-12177 (CGP) for 2 h. Some of the tissue punches at each concentration were incubated with the competitive β-adrenoreceptor antagonist Timolol (1 μmol l⁻¹) to calculate non-specific binding. Following removal of the incubation medium and two washes in turtle saline, the tissue punches were placed into scintillation vials containing 4 ml of Ecolite scintillation fluid (ICN Biomedical, Costa Mesa, CA, USA) and counted in a liquid scintillation counter (LS 6500, Beckmann). Specific binding was calculated by subtracting the radioactivity measured in punches incubated with CGP and Timolol from the activity in punches incubated with CGP alone. Non-specific binding was less than 23 % of specific binding. Saturation binding curves were analyzed using the method of Zivin and Waud (1982) to determine B_max and K_D. The total protein content of representative punches was measured spectrophotometrically using a Bradford protein assay (Bradford, 1976) so that B_max could be expressed as fmol mg⁻¹ protein. Punches were taken from the dorsal and ventral aspects of the ventricle and incubated separately to determine whether the binding curves were different between these two portions of the heart because adrenergic sensitivity is reported to vary regionally (Ball and Hicks, 1996).

Statistical analyses

In most cases, mean values ± S.E.M. for six animals are presented (N=5 for the 5 °C anoxic drug infusion group). Differences between means of experimental groups were determined using one-way and two-way analyses of variance (ANOVAs) for repeated measures, while multiple comparisons were performed using Student–Newman–Keuls tests. Within-group comparisons used a repeated-measures ANOVA. Where two variables were compared, a Student’s t-test was employed. P<0.05 was taken as the level of significance.

Results

Haematological variables

Haematological variables are summarized in Table 1. The blood pH of anoxic turtles was significantly lower than that of normoxic turtles at both acclimation temperatures. Interestingly, the extracellular acidosis resulting from a 6 h anoxic exposure at 22 °C (pH 7.01 versus pH 7.86) was greater (P<0.05) than after a 22-day anoxic exposure at 5 °C (pH 7.17 versus pH 7.76). Haematocrit decreased significantly with anoxia by approximately 25 % at both acclimation temperatures (Table 1). Mean cell haemoglobin content was not significantly affected by cold acclimation or anoxic exposure (Table 1).

Cardiovascular responses to drug infusion

Saline injections resulted in no significant changes in cardiovascular variables (Table 2). Fig. 1 illustrates the cardiovascular status under normoxic conditions at 22 °C and anoxic conditions at 5 °C.

Effects of atropine

In 22 °C-acclimated turtles, atropine injection produced pronounced cardiovascular changes. While these changes were qualitatively quite similar for normoxic and anoxic conditions (Table 2), the quantitative differences suggested different levels of cholinergic tone for anoxic and normoxic turtles. Under normoxia, atropine injection significantly increased f_H by 44 %, Q_SYs by 61 % and P_O_SYs by 92 % after 1 h. Under anoxic conditions, the cardiovascular changes were proportionately larger. Atropine significantly increased f_H by

| Table 1. Body mass, blood pH, haematocrit and haemoglobin content for the four experimental groups of turtles used for the β-adrenoreceptor density determinations |
|---------------------------------|-----------------|-----------------|--------------------|
| Group             | Body mass (g) | Blood pH         | Haematocrit (%)    | Mean cell haemoglobin content (g d⁻¹ %⁻¹) |
| 22 °C normoxia | 666±74.4       | 7.86±0.060       | 22.9±0.81          | 3.18±0.48                   |
| 22 °C anoxia       | 841±39.4       | 7.01±0.032       | 16.6±1.61          | 3.89±0.56                   |
| 5 °C normoxia      | 707±63.2       | 7.76±0.034       | 21.8±0.99          | 4.6±0.86                    |
| 5 °C anoxia        | 832±60.7       | 7.17±0.034       | 17.2±2.28          | 3.9±0.45                    |

Values are mean ± S.E.M.; for all experimental groups, N=6. Dissimilar letters indicate significant differences between groups (P<0.05).
Table 2. Summary of cardiovascular status in 22 °C-acclimated turtles before and after injection of various antagonist drugs

<table>
<thead>
<tr>
<th>Drug injection</th>
<th>Heart rate (beats min⁻¹)</th>
<th>Systemic stroke volume (ml kg⁻¹)</th>
<th>Systemic cardiac output (ml min⁻¹ kg⁻¹)</th>
<th>Mean arterial pressure (kPa)</th>
<th>Systemic power output (mW g⁻¹)</th>
<th>Systemic resistance (kPa ml⁻¹ min kg⁻¹)</th>
</tr>
</thead>
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<td></td>
<td>Normoxia</td>
<td>Anoxia</td>
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<td>Anoxia</td>
<td>Normoxia</td>
<td>Anoxia</td>
</tr>
<tr>
<td>Routine</td>
<td>24.2±2.3</td>
<td>10.3±0.9</td>
<td>1.49±0.15</td>
<td>0.78±0.10</td>
<td>36.0±2.5</td>
<td>8.03±1.2</td>
</tr>
<tr>
<td>Saline¹</td>
<td>23.0±1.9</td>
<td>9.95±0.9</td>
<td>1.30±0.09</td>
<td>0.72±0.06</td>
<td>29.9±3.2</td>
<td>7.16±0.89</td>
</tr>
<tr>
<td>Pre-atropine</td>
<td>22.6±2.3</td>
<td>9.93±1.0</td>
<td>1.46±0.11</td>
<td>0.74±0.05</td>
<td>33.0±2.0</td>
<td>7.35±0.82</td>
</tr>
<tr>
<td>Atropine after 1 h²</td>
<td>32.6±2.8*</td>
<td>16.7±1.3*</td>
<td>1.63±0.15</td>
<td>0.99±0.09*</td>
<td>53.1±4.8*</td>
<td>16.5±1.4*</td>
</tr>
<tr>
<td>Pre-nadolol</td>
<td>30.8±2.0</td>
<td>12.7±1.2</td>
<td>1.48±0.12</td>
<td>0.94±0.09</td>
<td>45.6±3.2</td>
<td>11.9±0.93</td>
</tr>
<tr>
<td>Nadolol³</td>
<td>25.7±1.1*</td>
<td>10.9±1.0</td>
<td>0.96±0.10*</td>
<td>0.84±0.06</td>
<td>24.7±2.8*</td>
<td>9.16±0.80*</td>
</tr>
</tbody>
</table>

Separate experiments were performed under normoxic conditions and after an acute, 6 h anoxic exposure.
Values are means ± 1 S.E.M. (N=6 for all experimental groups).
Significant differences (P<0.05) from pre-injection values are indicated by an asterisk. All comparable normoxic and anoxic values were significantly different.
¹Saline was injected immediately after routine values were measured. No response was found, but values reported here are 30 min after the routine measurement.
²Approximately 60 min after saline injection, pre-atropine values were measured; the atropine response was then measured 1 h after atropine injection.
³Pre-nadolol values were measured approximately 2 h after atropine injection without full recovery from atropine; the nadolol response was measured approximately 1 h later.

Table 3. Summary of cardiovascular status in 5 °C-acclimated turtles before and after injection of various antagonist drugs

<table>
<thead>
<tr>
<th>Drug injection</th>
<th>Heart rate (beats min⁻¹)</th>
<th>Systemic stroke volume (ml kg⁻¹)</th>
<th>Systemic cardiac output (ml min⁻¹ kg⁻¹)</th>
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<tr>
<td></td>
<td>Normoxia</td>
<td>Anoxia</td>
<td>Normoxia</td>
<td>Anoxia</td>
<td>Normoxia</td>
<td>Anoxia</td>
</tr>
<tr>
<td>Routine</td>
<td>5.20±0.52</td>
<td>1.00±0.24</td>
<td>0.77±0.13</td>
<td>0.26±0.057</td>
<td>4.04±0.89</td>
<td>0.26±0.089</td>
</tr>
<tr>
<td>Saline¹</td>
<td>5.58±0.39</td>
<td>0.98±0.17</td>
<td>0.77±0.11</td>
<td>0.28±0.037</td>
<td>4.30±0.88</td>
<td>0.27±0.026</td>
</tr>
<tr>
<td>Pre-atropine</td>
<td>5.50±0.76</td>
<td>1.05±0.21</td>
<td>0.72±0.07</td>
<td>0.26±0.050</td>
<td>3.96±0.32</td>
<td>0.27±0.043</td>
</tr>
<tr>
<td>Atropine after 1 h²</td>
<td>6.63±0.43</td>
<td>1.24±0.27</td>
<td>0.52±0.07</td>
<td>0.31±0.063</td>
<td>3.45±0.61</td>
<td>0.38±0.061</td>
</tr>
<tr>
<td>Pre-nadolol</td>
<td>6.50±0.58</td>
<td>1.00±0.16</td>
<td>0.51±0.07</td>
<td>0.32±0.047</td>
<td>3.32±0.68</td>
<td>0.32±0.051</td>
</tr>
<tr>
<td>Nadolol³</td>
<td>4.96±0.69</td>
<td>0.67±0.10</td>
<td>0.25±0.03*</td>
<td>0.28±0.040</td>
<td>1.24±0.24*</td>
<td>0.19±0.033</td>
</tr>
</tbody>
</table>

Separate experiments were performed under normoxic conditions and after a chronic, 22-day anoxic exposure.
Values are means ± 1 S.E.M. (N=6 for the normoxic group and N=5 for the anoxic group).
Significant differences (P<0.05) from pre-injection values are indicated by an asterisk. All comparable normoxic and anoxic values were significantly different.
¹Saline was injected immediately after routine values were measured. No response was found, but values reported here are 30 min after the routine measurement.
²Approximately 60 min after saline injection, pre-atropine values were measured; the atropine response was then measured 1 h after atropine injection.
³Pre-nadolol values were measured approximately 2 h after atropine injection without full recovery from atropine; the nadolol response was measured approximately 1 h later.
Cardiovascular control of cold-acclimated anoxic turtles

68%, $V_{s,sys}$ by 34%, $Q_{sys}$ by 124%, $P_{sys}$ by 44% and $PO_{sys}$ by 221%, while $R_{sys}$ decreased by 37% after 1 h. Cardiovascular variables remained quantitatively similar over the 90 min recording period after atropine injection (Table 2). Cardiac cholinergic tone, as estimated from the percentage change in heart rate, was 50% greater during the anoxic exposure compared with normoxia. Clearly, an acute anoxic exposure at 22 °C did not impair cholinergic control, a finding that is consistent with that of Hicks and Wang (1998) for a shorter anoxic exposure at 25 °C in the same species.

In 5 °C-acclimated turtles, most of the cardiovascular variables did not change significantly following atropine injection (Table 3). Therefore, cholinergic control was greatly diminished compared with 22 °C-acclimated turtles. In chronically anoxic 5 °C-acclimated turtles, atropine injection increased only $PO_{sys}$ significantly. Consequently, there was little cholinergic tone in cold anoxic turtles, but this was probably a consequence of cold acclimation rather than the chronic anoxic exposure.

Effects of nadolol after atropine

Immediately prior to a nadolol injection in 22 °C-acclimated turtles, $f_H$, $Q_{sys}$ and $PO_{sys}$ remained elevated and $R_{sys}$ reduced as a result of the preceding atropine infusion. Under normoxic conditions, nadolol depressed cardiovascular status (Table 2) by significantly decreasing $f_H$ by 17%, $V_{s,sys}$ by 35% and $Q_{sys}$ by 46% and by significantly increasing $P_{sys}$ by 61% and $R_{sys}$ by 206% relative to the pre-nadolol values measured. As a result, $PO_{sys}$ did not change significantly. Acute anoxic exposure blunted the response to nadolol in 22 °C-acclimated turtles (Table 2), and the only significant effect of nadolol was a 23% decrease in $Q_{sys}$.

In 5 °C-acclimated normoxic turtles, nadolol significantly decreased $V_{s,sys}$ by 51% and $Q_{sys}$ by 63% and significantly

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Fig. 1. Examples of systemic blood pressure ($P_{sys}$) and blood flow recordings for turtles (A) acclimated to 22 °C normoxia and (B) acclimated to 5 °C anoxia for 3 weeks. $f_H$, heart rate.

Fig. 2. Examples of systemic blood pressure ($P_{sys}$) and blood flow recordings for a turtle acclimated to 5 °C anoxia for 3 weeks (A) before and (B) 1 h after nadolol infusion. The turtle had been injected with atropine 2 h prior to the injection of nadolol and, thus, the pre-injection cardiovascular variables in A show elevated heart ($f_H$) and blood flow rates relative to control values (compare with Fig. 1B).
Table 4. Summary of cardiovascular status in 22 °C-acclimated turtles before and after injection of adrenaline (10 μg kg⁻¹ body mass) with and without cholinergic and adrenergic blockade

<table>
<thead>
<tr>
<th>Drug injection</th>
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<th>Systemic stroke volume (ml kg⁻¹)</th>
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<td>29.4±3.2</td>
<td>7.2±0.9</td>
</tr>
<tr>
<td>Adrenaline¹</td>
<td>28.1±2.1</td>
<td>11.2±1.0</td>
<td>0.78±0.07*</td>
<td>0.69±0.07</td>
<td>21.9±3.5</td>
<td>7.7±0.9</td>
</tr>
<tr>
<td>Post-atropine</td>
<td>34.7±2.6</td>
<td>13.1±1.0</td>
<td>1.17±0.14</td>
<td>0.92±0.08</td>
<td>40.6±2.7</td>
<td>12.1±1.1</td>
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<td>Adrenaline² after atropine</td>
<td>35.4±4.3</td>
<td>12.7±1.4</td>
<td>0.87±0.07*</td>
<td>0.92±0.09</td>
<td>30.8±3.2*</td>
<td>11.7±1.0</td>
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<tr>
<td>Post-nadolol</td>
<td>22.4±2.1</td>
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<td>1.31±0.16</td>
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<td>7.3±0.6</td>
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<td>Adrenaline³ after nadolol</td>
<td>25.9±2.2</td>
<td>11.4±1.8</td>
<td>1.09±0.14</td>
<td>0.73±0.09</td>
<td>28.2±4.1</td>
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Separate experiments were performed under normoxic conditions and after an acute, 6 h anoxic exposure. Values are means ± 1 s.e.m. (N=6 for all experimental groups). Significant differences (P<0.05) from pre-injection values are indicated by an asterisk. All comparable normoxic and anoxic values were significantly different.

¹ Adrenaline was injected 30 min after the saline injection. The peak adrenaline response occurred approximately 10 min after the injection.

² Approximately 1.5 h after atropine injection, post-atropine values were recorded, and this was followed immediately by a second adrenaline injection without full recovery from atropine. Peak adrenaline response occurred approximately 10 min after the injection.

³ Approximately 1 h after nadolol injection, post-nadolol values were recorded, and this was followed immediately by a third adrenaline injection. Peak adrenaline response occurred approximately 10 min after injection.

Table 5. Summary of cardiovascular status in 5 °C-acclimated turtles before and after injection of adrenaline (10 μg kg⁻¹ body mass) with and without cholinergic and adrenergic blockade

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<td>0.77±0.11</td>
<td>0.28±0.037</td>
<td>4.30±0.88</td>
<td>0.27±0.026</td>
</tr>
<tr>
<td>Adrenaline¹</td>
<td>6.46±0.76</td>
<td>1.10±0.24</td>
<td>0.37±0.04*</td>
<td>0.28±0.047</td>
<td>2.39±0.35*</td>
<td>0.31±0.036</td>
</tr>
<tr>
<td>Post-atropine</td>
<td>6.54±0.46</td>
<td>1.04±0.24</td>
<td>0.59±0.06</td>
<td>0.32±0.060</td>
<td>3.86±0.61</td>
<td>0.33±0.049</td>
</tr>
<tr>
<td>Adrenaline² after atropine</td>
<td>6.54±0.41</td>
<td>1.10±0.25</td>
<td>0.52±0.07</td>
<td>0.31±0.053</td>
<td>3.40±0.51</td>
<td>0.34±0.051</td>
</tr>
<tr>
<td>Post-nadolol</td>
<td>4.87±0.64</td>
<td>0.70±0.10</td>
<td>0.16±0.03</td>
<td>0.27±0.036</td>
<td>0.78±0.10</td>
<td>0.189±0.025</td>
</tr>
<tr>
<td>Adrenaline³ after nadolol</td>
<td>4.8±0.61</td>
<td>0.75±0.09</td>
<td>0.12±0.03</td>
<td>0.21±0.032</td>
<td>0.58±0.09</td>
<td>0.159±0.025</td>
</tr>
</tbody>
</table>

Separate experiments were performed under normoxic conditions and after a chronic, 22-day anoxic exposure. Values are means ± 1 s.e.m. (N=6 for the normoxic group and N=5 for the anoxic group). Significant differences (P<0.05) from pre-injection values are indicated by an asterisk. All comparable normoxic and anoxic values were significantly different.

¹,²,³ See Table 4.
increased $R_{sys}$ by 235% relative to the pre-nadolol values measured (Table 3). However, the changes in $f_1$ and $P_{sys}$ observed at 22 °C were not present at 5 °C. In addition, the absolute changes in cardiovascular status were much smaller than at 22 °C even though the percentage changes were similar. In 5 °C-acclimated anoxic turtles, nadolol caused a significant (88%) increase in $R_{sys}$ only, so it appears that chronic anoxic exposure in 5 °C-acclimated turtles largely blunted the response to this β-adrenergic antagonist compared with 22 °C-acclimated turtles. However, nadolol injection clearly caused the heart to fail in some individual turtles (Fig. 2) even though there was no statistical significance to the 41% lower $Q_{sys}$.

Effects of adrenaline

The effects of adrenaline injection in 22 °C-acclimated turtles are summarized in Table 4. Under normoxic conditions, adrenaline injection significantly decreased $V_{s,sys}$ by 40% and significantly increased $R_{sys}$ by 135% and $P_{sys}$ by 73%. $Q_{sys}$ and $P_{Osys}$ were unchanged. Similar cardiovascular changes were observed with adrenaline injections after either atropine alone or nadolol 2 h after atropine (Table 4).

Acute anoxic exposure blunted the cardiovascular responses to an adrenaline injection in 22 °C-acclimated turtles (Table 4). The only significant change was an increase in $P_{Osys}$ when adrenaline was injected after nadolol and atropine had both been applied. Anoxic blunting of the adrenergic pressor response possibly reflected the fact that the acute anoxic exposure had itself increased $R_{sys}$ to a similar level as the adrenergic response under normoxic conditions.

The effects of an adrenaline injection in normoxic 5 °C-acclimated turtles (Table 5) were similar to those observed in 22 °C-acclimated turtles (Table 4). Adrenaline significantly increased $P_{sys}$ and $R_{sys}$ and significantly decreased $V_{s,sys}$ and $Q_{sys}$, while $P_{Osys}$ and $f_1$ were unchanged. However, except for a significant increase in $R_{sys}$ when it followed nadolol treatment, adrenaline had no significant effect after atropine and nadolol treatments. There was no significant cardiovascular change in response to any of the adrenaline injections in the 5 °C-acclimated turtles after chronic anoxic exposure (Table 5).

β-adrenoreceptors

$B_{max}$ and $K_D$ were calculated from Scatchard plots of the binding affinity of CGP to the ventricular punches (Table 6). $B_{max}$ and $K_D$ were not significantly different for the dorsal and ventral aspects of the ventricle. Although male turtles had a larger relative ventricular mass, there was no sexual dimorphism for either $B_{max}$ or $K_D$ (data not shown). $B_{max}$ values were unaffected by cold acclimation. However, both acute and chronic anoxic exposure significantly decreased β-adrenoreceptor density. $B_{max}$ decreased by 40% (from 80 to 48 fmol mg$^{-1}$ protein) in 22 °C-acclimated turtles and by 39% (from 62 to 38 fmol mg$^{-1}$ protein) in 5 °C-acclimated turtles. $K_D$ values were not significantly different among the experimental groups (Table 6).

<table>
<thead>
<tr>
<th>Group</th>
<th>$B_{max}$ (fmol mg$^{-1}$ protein)</th>
<th>$K_D$ (nmol l$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22 °C normoxia</td>
<td>79.6±8.8$^{a}$</td>
<td>0.25±0.038</td>
</tr>
<tr>
<td>22 °C anoxia</td>
<td>48.1±10.3$^{b,c}$</td>
<td>0.25±0.060</td>
</tr>
<tr>
<td>5 °C normoxia</td>
<td>61.7±8.2$^{ab}$</td>
<td>0.18±0.047</td>
</tr>
<tr>
<td>5 °C anoxia</td>
<td>38.2±2.4$^{c}$</td>
<td>0.23±0.034</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for $N=6$ in each group. Dissimilar letters indicate significant difference between groups ($P<0.05$).

$B_{max}$, cell surface β-adrenoreceptor density; $K_D$, binding affinity.

Discussion

Haematological variables

As expected, blood pH decreased significantly with anoxia. Anaerobic metabolism is the primary means of energy production during anoxia, and the resultant load of lactic acid decreases blood pH (Driedzic and Gesser, 1994). Although the acidemia after a 6 h anoxic exposure at 22 °C exceeded that of a longer anoxic exposure at 5 °C, caution should be used in interpreting these pH values because of the invasive manner in which blood was sampled. A further complication is that at 22 °C the acidemia probably had a major respiratory component, whereas at 5 °C a decreased metabolic rate (Herbert and Jackson, 1985a) could have slowed the development of acidemia.

Acute and chronic anoxia caused a 25% decrease in haematocrit. Possible explanations include an increase in plasma volume, removal of red blood cells (perhaps sequestration in the spleen) and a reduction in red blood cell volume. A reduction in red cell volume is an unlikely explanation, however, since mean cell haemoglobin content did not change. Ultsch and Jackson (1982) previously reported an 8–10% decrease in haematocrit after a 10-day anoxic exposure that could be reversed within 10 days of return to normoxia. Their observation suggests that red blood cells were sequestered and then later released to the circulating blood. An advantage of sequestering red blood cells during anoxia would be a reduction in blood viscosity and with it cardiac work. This modification would be of some importance since the oxygen-carrying capacity of the blood is eliminated under anoxia, but the transport of glucose and of lactate and buffering of the resultant acid load are still critical roles played by the blood.

Cardiovascular control

Autonomic control is an important modulator of normal cardiovascular status. Cholinergic-mediated bradycardia during diving and apnoea is well established (White, 1976; Signore and Jones, 1995; Akselrod et al., 1985; Furilla and Jones, 1987). Therefore, the present finding that bradycardia associated with acute anoxia in 22 °C-acclimated turtles also involved a vagal inhibitory tone ($f_1$ increased after atropine infusion) is not surprising. Indeed, Hicks and Wang (1998)
reported that atropine increased $f_{\text{H}}$ by 35% and $Q_{\text{sys}}$ by 50% in *T. scripta* after a 2 h anoxic exposure at 25 °C. The percentage changes we observed were somewhat larger, perhaps reflecting the longer anoxic exposure period used in the present study. Even studies of the effects of anoxia on isolated hearts that have no neural input suggest that acute anoxia has only modest effects on intrinsic $f_{\text{H}}$. For example, Bailey and Driedzic (1995) reported a similar $f_{\text{H}}$ for isolated turtle hearts perfused with normoxic and anoxic saline at 15 °C. Similarly, Farrell et al. (1994) reported that short-term anoxia decreased $f_{\text{H}}$ by only a few beats per minute *in situ* in turtle hearts at 5 °C and 15 °C.

The present study, however, is the first to report on the relative importance of these control systems in cold-acclimated turtles. Unlike at 22 °C, cold acclimation clearly reduced the role of vagal inhibition of $f_{\text{H}}$ to a minor one. Atropine infusion into 5 °C-acclimated turtles had no significant effect on $f_{\text{H}}$ under either normoxic or anoxic conditions. This suggests that the intrinsic response to cold acclimation and possibly additional effects of anoxia and acidosis, to account for the difference in $f_{\text{H}}$ between anoxic and normoxic turtles, were the primary effectors of the massive bradycardia rather than an increase in vagal cholinergic inhibition of the heart.

The importance of temperature in dramatically adjusting rate functions in turtles was previously highlighted by the $Q_{10}$ of 7.5 for metabolic rate (Herbert and Jackson, 1985b) and the $Q_{10}$ of 8.8 for cardiac $P_{\text{O}_{2}}$ in the accompanying study of Hicks and Farrell (2000). However, acute temperature effects can only partially explain high $Q_{10}$ values since the $Q_{10}$ for maximum cardiac $P_{\text{O}_{2}}$ was only 4 for *in situ* turtle heart preparations acutely exposed to 15 °C and 5 °C (Farrell et al., 1994). In fact, the higher intrinsic $f_{\text{H}}$ in perfused hearts acutely exposed to 5 °C compared with the *in vivo* $f_{\text{H}}$ in 5 °C-acclimated turtles provides further support for an acclamatory adjustment in $f_{\text{H}}$. An acute anoxic exposure decreased the spontaneous beat frequency of cardiac muscle strips (Bing et al., 1972; Jackson, 1987) and *in situ* turtle hearts irrespective of temperature (Farrell et al., 1994). This anoxic effect on $f_{\text{H}}$ could also be augmented by the accompanying acidosis *in vivo*. In fact, acidosis has been implicated as an important controlling factor of the down-regulation of metabolism, i.e. an $H^{+}$-mediated metabolic depression in hibernating mammals (Malan, 1988) and anoxic turtles (Wasser et al., 1991). Turtles with a more pronounced acidosis were better at defending their glycogen stores, suggesting that acidosis lowered energy demand.

Under normoxic conditions, adrenaline caused a large pressor response by increasing $R_{\text{sys}}$ by 135% at 22 °C and by 185% at 5 °C. This was probably brought about by $\alpha$-adrenergic vasoconstriction, although we did not confirm this possibility using $\alpha$-adrenergic antagonists. We did not observe the tachycardia observed by Hicks and Wang (1998) for normoxic 25 °C *T. scripta*, but this difference could reflect complicating barostatic reflex control of $f_{\text{H}}$. The responses to adrenergic injections after cold acclimation and anoxic exposure are more difficult to interpret for two reasons. First, $R_{\text{sys}}$ was increased by cold acclimation and, to a greater degree, anoxic exposure. Thus, adrenaline was acting on a high background $R_{\text{sys}}$, some of which could have been due to circulating catecholamines, levels of which are known to be elevated in anoxic turtles (Wasser and Jackson, 1991). Second, hypoperfusion of certain critical organs during anoxia may mean that the injected drug did not reach the same target sites as in normoxic turtles. Nonetheless, the adrenaline-mediated pressor response was similar in cold-acclimated turtles and in 22 °C-acclimated turtles. There were subtle indications that this pressor response may have been modified in cold-acclimated turtles because there was no adrenaline-mediated pressor activity after exposure to either atropine or nadolol, unlike in 22 °C-acclimated turtles.

Both acute and chronic anoxia completely blunted the pressor response to adrenaline. While the finding for chronic anoxia in cold-acclimated turtles is novel, it is not unexpected given that Hicks and Wang (1998) had shown that a short (2 h) anoxic exposure in 25 °C *T. scripta* blunted the cardiovascular responses to adrenaline. This anoxia-mediated reduction in cardiovascular responsiveness could come about through a number of mechanisms, all of which would tend to depress cardiac function at a time of energy constraint. One possibility is that the elevated levels of circulating catecholamines in turtles exposed to anoxia (Wasser and Jackson, 1991) prevent further $\alpha$-adrenergic vasoconstriction. This certainly may be true for the systemic circulation, where there are massive increases in systemic resistance with both cold-acclimation and chronic anoxic exposure. In hindsight, injections of $\alpha$-adrenergic antagonists would have been particularly informative in this regard.

In contrast to the systemic circulation, it seems more plausible that adrenergic sensitivity is reduced in cardiac tissues, where $\beta$-adrenoreceptors predominate. Certainly, the observed decreases in $f_{\text{H}}$ and blood pressure (Herbert and Jackson, 1985b; Hicks and Farrell, 2000) and the absence of cholinergic cardiac inhibition with cold acclimation are consistent with a blunting of adrenergic sensitivity. Even so, a $\beta$-adrenergic stimulation of the heart apparently remains important after cold-acclimation and during anoxia despite the blunted response to adrenaline injections. Nadolol significantly decreased systemic $Q_{\text{sys}}$ in normoxic turtles at both temperatures, although this effect was reduced by anoxia in 22 °C-acclimated turtles, and in 5 °C-acclimated turtles the effect of nadolol was cardiac arrhythmia in some turtles. These observations raise interesting questions about the role of cardiac $\beta$-adrenoreceptors in mediating these changes.

**Cardiac $\beta$-adrenoreceptors**

Anoxia reduced ventricular cell-surface $\beta$-adrenoreceptor density to almost half of the normoxic value. This response is consistent with observations on avian and mammalian hearts. Cultured ventricular myocytes from rats and chicks decreased cell-surface $\beta$-adrenoreceptor density by 29% and 62%, respectively, when cells were exposed to hypoxia for 2 h (Rocha-Singh et al., 1991; Marsh and Sweeney, 1989).
Furthermore, exposure of rats to high-altitude hypoxia resulted in a 50% reduction in the density of ventricular β-adrenoreceptors (Voelkel et al., 1981). In mammals, this response may represent an effective mechanism for cardiac down-regulation that would guard against myocardial over-stimulation at a time of energy constraint. Cardiac depression certainly occurs in turtles in the presence of elevated catecholamine levels, but whereas Wasser and Jackson (1991) showed that the anoxia-induced elevation of catecholamine level was more pronounced at higher temperatures (20 °C versus 3 °C), β-adrenoreceptor density did not differ at 22 °C and 5 °C. Therefore, the anoxia-induced reduction in β-adrenoreceptor density in turtles appears to be an acute response to anoxia and independent of temperature acclimation and chronic exposure to anoxia.

The reduction of β-adrenoreceptor density does not exclude the possibility that other portions of the signal transduction pathway are also altered to reduce adrenergic sensitivity. For example, Keen et al. (1993) found that cold acclimation increased the basal activity of adenylate cyclase as well as increasing the β-adrenoreceptor density in the heart of rainbow trout Oncorhynchus mykiss.

An additional finding by Wasser and Jackson (1991) was that the degree of acidosis was correlated with catecholamine release. Turtles that respired N2 had lower catecholamine levels than submerged turtles, which encounter a greater acidosis. This synergism between hypoxaemia and acidemia has also been noted in various mammals (Rose et al., 1983; Lewis and Sadeghi, 1987) and amphibians (Boutilier and Lantz, 1989). Acidemia is a predictable consequence of submersion and, as such, could provide a strong cue to elevate catecholamine levels during diving. The anoxic groups at 5 °C and 22 °C both had reduced blood pH values (Table 1) and also similar reductions in β-adrenoreceptor density. Whether the acidemia, the down-regulation of cardiac β-adrenoreceptors and the elevated circulating catecholamine levels are connected will require further study, but all three are involved in regulating ionotropy.

Ball and Hicks (1996) found that muscle strips prepared from the dorsal half of the turtle ventricle were more sensitive to applied adrenaline than strips from the ventral half. These authors suggested that fibre orientation accounted for this difference since microscopic examination demonstrated that the majority of muscle fibres in the dorsal muscle strips were oriented longitudinally from apex to base while ventral strips were arranged circumferentially, the force of which would be less easily detected by force transducers. We lend support to their suggestion because we found no difference in β-adrenoreceptor density between the dorsal and ventral aspects of the turtle ventricle.

Male turtles had a larger relative ventricular mass but a similar β-adrenoreceptor density to those of females. Various populations of rainbow trout also show sexual dimorphism in relative ventricular mass (19–35 % larger in males) (Graham and Farrell, 1992). The lack of sexual dimorphism for ventricular β-adrenoreceptor density has also been demonstrated in rainbow trout (Gamperl et al., 1994) and spawning chinook salmon Oncorhynchus nerka (A. K. Gamperl, personal communication).

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References


