Peripheral O₂ chemoreceptors mediate humoral catecholamine secretion from fish chromaffin cells

Stephen G. Reid and Steve F. Perry

Department of Biology, University of Ottawa, Ottawa, Ontario, Canada K1N 6N5

Submitted 10 July 2002; accepted in final form 18 December 2002

Reid, Stephen G., and Steve F. Perry. Peripheral O₂ chemoreceptors mediate humoral catecholamine secretion from fish chromaffin cells. *Am J Physiol Regul Integr Comp Physiol* 284: R990–R999, 2003. First published January 2, 2003; 10.1152/ajpregu.00412.2002.—This study addressed the hypothesis that the secretion of catecholamines from trout (*Oncorhynchus mykiss*) chromaffin cells, during hypoxia, is triggered by stimulation of O₂ chemoreceptors located within the gills. Sodium cyanide was administered into the inspired water (external cyanide) or injected into the gill circulation (internal cyanide) to pharmacologically stimulate external (water sensing) or internal (blood sensing) O₂ chemoreceptors, respectively. Both of these treatments caused an elevation of circulating catecholamine levels. The response to external, but not internal, cyanide was abolished by removal of the first gill arch. Hypoxia produced an increase in circulating catecholamine levels that was unaffected by removal of the first gill arch or by denervation of the pseudobranch. Cyanide and hypoxia both caused the well-documented cardiorespiratory reflexes normally observed in this species. This study demonstrates, for the first time, that gill O₂ chemoreceptors can initiate the reflex that leads to catecholamine release from the chromaffin cells and that stimulation of internally oriented O₂ receptors on all gill arches appears to be the physiologically important mechanism for initiating release.

epinephrine; cardiorespiratory reflexes; gills; hypoxia; *Oncorhynchus mykiss*

IN VERTEBRATES, STRESSFUL situations (e.g., physical disturbance or environmental perturbation) often result in alterations of cardiovascular and respiratory function as well as the secretion of stress hormones, catecholamines (epinephrine and/or norepinephrine) and cortisol into the blood (15). Together, these physiological responses serve to optimize gas transfer across the lungs/gills, provide adequate oxygen delivery to metabolically active tissues, and mobilize energy supplies to provide for the increased metabolic demands that frequently accompany stress (18, 21).

In mammals exposed to hypoxia, a decrease in arterial blood PO₂ (PaO₂) is sensed by peripheral O₂ chemoreceptors (glomus cells) located within the carotid body at the bifurcation of the internal and external carotid arteries (6). In fish, analogous chemoreceptors are lo-

cated on the gill arches (4, 5, 12, 23). Indeed, the gills of fish are considered to be phylogenetic precursors of the mammalian aortic and carotid bodies. The gill chemoreceptors are either externally oriented so as to monitor the partial pressure of O₂ in the water (PwO₂) or internally oriented so as to monitor changes in PaO₂ (4). Generally, in teleost fish, activation of branchial chemoreceptors during hypoxia results in an increase in breathing (frequency and/or amplitude) as well as a decrease in heart rate (H_r; 5).

Circulating (plasma) catecholamines originate from chromaffin cells. In teleost fish, species with no adrenal gland, the catecholamine-secreting chromaffin cells are located in the walls of the posterior cardinal vein with the greatest concentration of cells found in the rostral region of the vein in the head kidney (13, 22). In all vertebrate species, with the exception of the most primitive fish (cyclostomes), the primary mechanism leading to the secretion of catecholamines from chromaffin cells is stimulation of these cells by the sympathetic nervous system and the actions of the neurotransmitter acetylcholine (1, 21, 22). This cholinergic pathway of release is complemented by an array of noncholinergic mechanisms (1, 9, 22). While the mechanisms of catecholamine release from chromaffin cells have been exhaustively studied, few, if any, studies have focused on sensory aspects of this reflex response. Thus the primary objective of this study was to test the hypothesis that the release of catecholamines into the circulation during hypoxia in fish is triggered initially by O₂ chemoreceptors located within the gills. These experiments were performed using the rainbow trout (*Oncorhynchus mykiss*) because of its robust catecholaminotropic response to hypoxia and the vast physiological database that exists for this species.

METHODS

Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*; 200–250 g) were obtained from a local supplier and transported to the University of Ottawa under hyperoxic conditions. Fish were maintained in fiberglass aquariums supplied with aerated and dechlorinated city of Ottawa tap water. Water temperature was maintained at 12°C, and the light cycle was kept at

Address for reprint requests and other correspondence: S. G. Reid, Division of Life Sciences, Univ. of Toronto at Scarborough, 1265 Military Trail, Toronto, Ontario, Canada, M1C 1A4 (E-mail: sgreid@utsc.utoronto.ca).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

12:12-h light/dark. Fish were fed ad libitum every second day; feeding was stopped 48 h before experimentation.

Animal Preparation

All experimental protocols were approved by the University of Ottawa Animal Care Committee in accordance with guidelines provided by The Canadian Council for Animal Care. The experiments are in accordance with the American Physiological Society guiding principles for research. Fish were anesthetized in a solution of benzocaine (0.1 g/l ethyl-p-aminobenzoate, Sigma) and placed onto an operating table where the gills were ventilated with a solution of the anesthetic.

To sample arterial blood, and/or measure H_f , an indwelling polyethylene cannula (PE-50) was placed into the dorsal aorta (24), secured to the roof of the mouth, and led out of the mouth through a hole in the snout. In those fish in which cyanide was administered into the inspired water (external cyanide), a delivery cannula (PE-160) was placed into the mouth through a second hole in the snout. In those fish in which cyanide was injected into the arterial blood within the gills (internal cyanide), a cannula (PE-50) was placed into either the caudal vein or ventral aorta. Access to the caudal vein was achieved by an incision lateral to the spinal cord slightly caudal to the anal fin. The ventral aorta was exposed, with a ventral incision, immediately rostral to the heart; the pericardium was not damaged in this procedure.

Where appropriate, the first gill arches were removed to abolish the afferent sensory input that originates from chemoreceptors located on these arches. The gill arches were ligated dorsally and ventrally and then removed with scissors. This procedure removed the entire respiratory surface of the gill arch; all that remained was a tiny stump at either end. Despite the ~30% reduction in gill surface area, this ligation procedure was without effect on circulating catecholamine levels and Pa_{O_2} , although a slight respiratory acidosis is associated with this procedure (7). This approach was taken, as opposed to specific gill denervation, because the nerves to the gills are buried under muscle and a venous sinus. Nerve exposure is thus accompanied by significant blood loss. Because the pseudobranch may also be a site of O_2 chemoreception (8), a separate series of experiments examined the effects of pseudobranch denervation with the first gill arches remaining intact. In these experiments, the branch of cranial nerve IX to the pseudobranch was identified as it coursed, superficially, over the inner surface of the operculum. The nerve was isolated and cut with fine iris scissors. After surgery, fish were placed into individual black Plexiglas boxes supplied with flowing, aerated and dechlorinated water and were allowed to recover for 24 h before experimentation.

In Vivo Catecholamine Release

The following experimental series were performed in vivo in which plasma catecholamine levels were measured. Each series was performed on control fish with the gills intact and on a separate group of fish in which the first gill arches were removed. The hypoxic series (see below) was also performed on a group of fish in which the pseudobranch was denervated while the first gill arch was left intact.

Externally administered cyanide. In this series of experiments, a bolus (2 ml/kg) of the O_2 chemoreceptor stimulant sodium cyanide dissolved in water (NaCN; 0.4 mg/ml; Refs. 2, 3, 12) was administered into the inspired water through the cannula in the snout. The NaCN would thus flow across the gills and interact with externally oriented (water sensing)

chemoreceptors on the gill arches (12). The doses of both external and internal (see below) NaCN were determined based on pilot experiments in which dose-response curves for cardiorespiratory responses to NaCN were examined. Preliminary experiments demonstrated that these low doses of cyanide were without effect on arterial blood gases and that they did not cause the fish to become disturbed or agitated.

To determine resting levels of plasma catecholamines before injecting cyanide (pre-cyanide), a blood sample (0.4 ml) was withdrawn and centrifuged and the plasma was immediately frozen in liquid nitrogen. A second blood sample was taken 2 min after administration of the bolus of NaCN into the inspired water (post-cyanide). Control injections of water were also performed and were without effect.

Externally administered cyanide during normoxia, hyperoxia, and hypoxia. This series of experiments was designed to determine whether the level of Pw_{O_2} influenced catecholamine release in response to externally administered cyanide. The procedures for administering NaCN to the inspired water and sampling blood were the same as described above. Three groups of fish were studied: 1) a normoxic group (Pw_{O_2} = 158 mmHg; n = 10), 2) a hypoxic group (Pw_{O_2} = 70 mmHg; n = 9), and 3) a hyperoxic group (Pw_{O_2} = 600 mmHg; n = 10). A Pw_{O_2} of 70 mmHg was chosen for the hypoxic group because this level of hypoxia does not, on its own, elicit catecholamine secretion in this species (16, 17). A dose of 0.2 mg/kg NaCN was used because this dose caused either no, or very little, catecholamine release in normoxic fish; 0.1 mg/kg did not cause catecholamine release under any of the three conditions.

Internally administered cyanide. A third series of experiments examined the effects on catecholamine release of applying NaCN (1.0 ml/kg of 0.10 mg/ml NaCN in saline) into the arterial blood within the gills. In this case, the NaCN would flow through the branchial circulation and interact with internally oriented chemoreceptors within the gills that monitor O_2 levels within the arterial blood rather than the inspired water. Initial experiments focused on administration through the caudal vein because it is easily accessible for cannulation in this species. Blood from the caudal vein flows into the posterior cardinal vein (PCV) and then into the heart and ventral aorta before entering the gill circulation. Given that the caudal vein empties into the PCV, the site of the chromaffin cells, the possibility existed that NaCN injected via this route would directly interact with the chromaffin cells to cause catecholamine release. As such, an additional series of experiments was performed in which NaCN was administered through the ventral aorta. In this case the NaCN would flow directly into the gill circulation without interacting with the chromaffin cells. The results were the same regardless of the injection site. Blood samples were taken before injecting internal cyanide (pre) and 2 min postinjection (post); the plasma was retained as described above.

Hypoxia. In these experiments, fish were exposed to environmental hypoxia (Pw_{O_2} = 40 mmHg). This level of hypoxia is known to cause a significant elevation of plasma catecholamines in rainbow trout (16, 17). To determine resting levels of plasma catecholamines, a blood sample (pre; 0.4 ml) was withdrawn before initiating hypoxia. Hypoxic conditions were achieved by bubbling nitrogen through a water-gas equilibration column prior to the water entering the box housing the fish. A second blood sample was taken at the end of the hypoxic period, ~10 min after the Pw_{O_2} had reached 40 mmHg. After each blood sample, the erythrocytes were suspended in saline and reinjected into the fish. A recovery

(post) sample was taken 1–2 h after the P_{WO_2} returned to normoxic levels.

In Situ Catecholamine Release

In teleost fish, the caudal vein empties into the posterior cardinal vein, which is the location of the chromaffin cells. Although the *in vivo* injections of internal NaCN through the ventral aorta bypassed the chromaffin cells, this series of experiments was performed to confirm that NaCN does not cause catecholamine release via a direct effect on the chromaffin cells. A well described and characterized *in situ* saline-perfused posterior cardinal vein (chromaffin cell) preparation (19) was used to assess any direct catecholamine-releasing effects of NaCN.

Briefly, fish were killed by a blow to the head and the heart was exposed. A cannula (PE-160) was placed into the ventricle; this served as the outflow for the perfusion fluid (Cortland saline). The posterior cardinal vein was exposed and cannulated (PE-160) to serve as an inflow. Perfusion (1 ml/min) was accomplished by siphon. After a 20-min stabilization period, the perfusate was collected for two consecutive 1-min intervals (pre samples). At this point, a bolus dose of NaCN (1.0 ml of 0.10 mg/ml; the same dose used for the internal injections) was administered into the inflow cannula. Perfusate samples were then collected for 1-min periods during the ensuing 5 min. Once collected, the perfusate samples were immediately frozen in liquid nitrogen (and later at -80°C) before analysis for catecholamines.

In a separate series of *in situ* experiments ($n = 6$) cyanide was administered as described above. However, after the 5-min collection period, a bolus dose of homologous ANG II ([Asn¹,Val⁵]ANG II; 5×10^{-7} mol/kg body mass; Sigma) was injected into the posterior cardinal vein, and samples were collected for a further 5 min. ANG II is a potent agonist of catecholamine release in rainbow trout, and these experiments were performed to confirm that the lack of catecholamine release in response to cyanide *in situ* (see below) was not due to the chromaffin cells in this preparation being nonviable.

Cardiovascular and Respiratory Responses

To confirm that hypoxia and the internal/external injections of NaCN evoked the well-described cardiorespiratory responses (see introduction), breathing and H_f were measured in response to the aforementioned treatments. These experiments were performed on separate groups of fish with and without the first gill arches removed. Under anesthesia, a small (1 cm²) brass plate was stitched to the external surface of each operculum to allow the measurement of ventilation amplitude (V_{AMP}) using an impedance converter and amplifier. An impedance converter measures breathing directly by detecting the breath-by-breath displacement of the gill operculae. H_f was measured by connecting the dorsal aorta cannula to a pressure transducer (see below).

Analytic Techniques

P_{WO_2} within the fish box was continuously measured by siphoning water across a P_{O_2} electrode (E101; Cameron Instrument) connected to a Cameron BGM 200 blood gas meter. The O_2 electrode was calibrated by siphoning both a solution of sodium sulfite (20 mg/ml; $P_{\text{O}_2} = 0$ mmHg) and air-equilibrated water ($P_{\text{O}_2} = 158$ mmHg) across the electrode.

V_{AMP} was determined after conversion of the impedance data to linear opercular deflections (in cm) through appropriate calibration (performed by manually displacing the oper-

cular covers known distances on euthanized animals). H_f was determined by connecting the dorsal aorta cannula to a pressure transducer (Bell and Howell) that was precalibrated against a static column of water. Analog blood pressure signals were measured using Harvard Biopac amplifiers (DA 100). All analog signals were converted to digital data by interfacing with a data-acquisition system (Biopac Systems) using Acknowledge data-acquisition software (sampling rate set at 10 Hz) and a Pentium personal computer. Thus continuous data recordings were obtained for blood pressure, f_H (automatic rate calculation from the pulsatile pressure trace), ventilation frequency (automatic rate calculation from the raw impedance), and V_{AMP} (the difference between maximum and minimum impedance values).

Plasma epinephrine and norepinephrine concentrations were determined on alumina-extracted samples (200 μl) using HPLC with electrochemical detection. The HPLC consisted of a Varian Star 9012 solvent delivery system (Varian Chromatography Systems, Walnut Creek, CA) coupled to a Princeton Applied Research 400 electrochemical detector (EG & G Instruments, Princeton, NJ). The extracted samples were passed through an Ultratechsphere ODS-C₁₈ 5- μm column (HPLC Technology, Macclesfield, UK), and the separated amines were integrated with the Star Chromatography software program (version 4.0, Varian). Concentrations were calculated relative to appropriate standards and with 3,4-dihydroxybenzylamine hydrobromide (DHBA) as an internal standard in all determinations. For clarity, total catecholamine levels (i.e., epinephrine plus norepinephrine) are presented in the figures and tables.

Statistical Analysis

The data are reported as the means $\pm$ SE. All data were statistically analyzed by a two-way repeated-measures analysis of variance followed by a post hoc pairwise multiple comparison test (Bonferroni *t*-test). The ED_{50} values reported for Fig. 2B were compared using a two-tailed Student's *t*-test. The limit of statistical significance was 5%.

RESULTS

Externally Administered Cyanide

The effects of externally applied cyanide on cardiorespiratory function and plasma catecholamines are illustrated in Fig. 1. This protocol was designed to stimulate externally oriented (water sensing) O_2 chemoreceptors on the gills. In the control group (gills intact), an injection of cyanide across the gills caused a decrease in H_f (Fig. 1A) and an increase in V_{AMP} (Fig. 1B). These are well-described cardiorespiratory responses to externally applied cyanide (4, 5, 12). The decrease in H_f in response to external cyanide was abolished after removal of the first gill arch, whereas the increase in V_{AMP} was unaffected.

External cyanide caused a significant elevation of plasma catecholamine levels (epinephrine accounted for 80% of the total) that was abolished after removal of the first gill arch (Fig. 1C). Control injections of externally applied water were without effect (data not shown). These results indicate that there are externally oriented receptors on the first gill arch that, on stimulation, produce an elevation of plasma catecholamine levels.

Fig. 1. Heart rate (A; H_f , beats/min; $n = 11$, intact; $n = 9$, ligated), ventilation amplitude (B; V_{AMP} , cm; $n = 9$, intact; $n = 7$, ligated), and plasma catecholamine concentration (C; nmol/l; $n = 19$, intact; $n = 14$, ligated) in response to administration of NaCN into the inspired water (external cyanide). In A and B an arrow indicates the point at which the external NaCN was administered. Open circles or bars represent the control group (gills intact) while the filled symbols or bars represent the group in which the first gill arches were ligated and removed. Data are shown as means $\pm$ SE. In A and B, a single horizontal line represents a difference, from time 0, in either the control or gill-ligated group. In C, *significant difference from the prevalence; †significant difference between the control and gill-ligated group.

Externally Administered Cyanide During Normoxia, Hyperoxia, and Hypoxia

Figure 2 illustrates the effects of water oxygenation on the cardiac response to external cyanide in control

(gills intact) fish. Under normoxic conditions (Fig. 2A), administration of external cyanide (0.4 mg/kg; the same dose used in Fig. 1) caused a decrease in H_f from ~ 75 to 45 beats/min, 20 s postinjection. This was followed by a progressive increase over the next minute. Under hyperoxic conditions, externally applied NaCN did not induce bradycardia.

Figure 2B illustrates dose-response curves for external cyanide-evoked bradycardia under normoxic and hyperoxic conditions. In this figure, the decrease in H_f is expressed as a percentage of the response to the

Fig. 2. A: effect of normoxia [water PO_2 (PwO_2) = 158 mmHg; $\bullet$; $n = 6$] and hyperoxia (PwO_2 = 600 mmHg; $\circ$; $n = 5$) on the cyanide-evoked (0.4 mg/kg) bradycardia. B: dose-response curves for cyanide-evoked bradycardia under normoxic ($\bullet$; $n = 6$ –10) and hyperoxic ($\circ$; $n = 5$) conditions. Dose-response curves were fit by iteration using a sigmoidal 4-parameter equation. ED_{50} values for cyanide-evoked bradycardia were 0.55 ± 0.03 and 0.24 ± 0.05 mg kg^{-1} during hyperoxia and normoxia, respectively. Data are shown as means $\pm$ SE. In A, *significant difference from time 0.

highest dose of externally applied NaCN. Under hyperoxic conditions, the dose-response curve ($R^2=0.99$) is shifted to the right of the normoxic curve ($R^2=0.97$). The ED_{50} values were 0.55 ± 0.03 and 0.24 ± 0.05 mg/kg for the hyperoxic and normoxic curves, respectively. Together, the data in Fig. 2 illustrate that the cardiac response to pharmacological stimulation of O_2 chemoreceptors with NaCN can be modified by altering the P_{O_2} of the water surrounding the chemoreceptor sites.

Figure 3 illustrates the effects of water P_{O_2} on external cyanide-evoked catecholamine secretion. A bolus dose of 0.2 mg/kg NaCN evoked catecholamine secretion under hypoxic ($P_{wO_2} = 70$ mmHg) conditions but not under normoxic ($P_{wO_2} = 158$ mmHg) or hyperoxic ($P_{wO_2} = 600$ mmHg) conditions. A bolus dose of 0.1 mg/kg NaCN did not cause release under any condition (data not shown).

Internally Administered Cyanide

The effects of injecting cyanide into the branchial circulation (internal cyanide), through the caudal vein, are illustrated in Fig. 4. These experiments were performed to stimulate internally oriented (blood sensing) O_2 chemoreceptors. Figure 4A demonstrates that internally injected cyanide caused a decrease in H_f (from 74.4 ± 2.8 to 38.3 ± 6.0 beats/min) that was abolished by ligation of the first gill arches. Similarly, internal cyanide caused an increase in V_{AMP} that also was eliminated after gill arch ligation (Fig. 4B).

An injection of cyanide into the caudal vein caused a substantial elevation of plasma catecholamine levels, with the concentration of epinephrine plus norepinephrine increasing to ~ 150 nmol/l (epinephrine comprised 66% of the total). However, ligation of the first gill arches did not prevent the increase in plasma catecholamines in response to internal cyanide (Fig. 3C). Administration of internal cyanide through the ventral aorta caused plasma catecholamine levels to increase from 7.7 ± 2.7 to 77.2 ± 19.6 nmol/l. Control injections of saline were without effect (data not shown). These

Fig. 3. Effects of water oxygenation on external cyanide-evoked catecholamine release. Closed bars represent catecholamine levels prior to (pre) administration of NaCN; open bars represent catecholamine levels 2 min post-NaCN (0.2 mg/kg). P_{wO_2} for the hyperoxic ($n = 10$), normoxic ($n = 10$), and hypoxic ($n = 9$) groups was 600, 158, and 70 mmHg, respectively. Data are shown as means $\pm$ SE. *Significant difference from the pre value.

Fig. 4. H_f (A; beats/min; $n = 9$ intact; $n = 6$, ligated), V_{AMP} (B; cm; $n = 8$, intact; $n = 6$, ligated), and plasma catecholamine concentration (C; nmol/l; $n = 6$, intact; $n = 6$, ligated) in response to an injection of sodium cyanide into the caudal vein (internal cyanide). In A and B, an arrow indicates the point at which the cyanide was injected. Open circles or bars represent the control group (gills intact), whereas the filled symbols or bars represent the group in which the first gill arches were ligated and removed. Data are shown as means $\pm$ SE. In A and B, a single horizontal line represents a difference, from time 0, in either the control or gill-ligated group. In C, *significant difference from the pre-value. †Significant difference between the control and gill-ligated group.

results indicate that there are internally oriented O_2 receptors that are capable of initiating catecholamine release; furthermore, these internal receptors are not exclusively located on the first gill arch.

In Situ Catecholamine Release

To further examine if the cyanide injections through the caudal vein were causing catecholamine release by directly acting on the chromaffin cells, catecholamine release was measured in situ using a well-described saline-perfused posterior cardinal vein (chromaffin cell) preparation. The data reported in Table 1 indicate that cyanide (the same dose used for the internal injections in vivo) did not cause catecholamine release when applied directly to the chromaffin cells. Indeed, perfusate catecholamine concentration actually decreased 4–5 min post-cyanide.

To confirm that the lack of in situ catecholamine release in response to cyanide was not due to nonviable chromaffin cells, a second series of experiments was performed in which the potent agonist of catecholamine release, ANG II, was administered 5 min after the cyanide injection. In this series of experiments, cyanide did not cause catecholamine release, whereas ANG II caused significant release. Total catecholamine levels pre-cyanide were 86 ± 47 nmol/l, whereas post-cyanide values reached a maximum of 110 ± 82 nmol/l (not significantly different from the pre value). Total catecholamine levels pre-ANG II were 66 ± 25 nmol/l, whereas post-ANG II values reached a maximum of 485 ± 119 nmol/l ($P < 0.05$).

Hypoxia

Figure 5 illustrates the effects of environmental hypoxia on H_f , V_{AMP} , and plasma catecholamine levels. The time course of the hypoxic exposure is shown in Fig. 5A for both the intact group and the group with the first gill arches removed. PwO_2 was reduced to the same extent in both groups of fish, reaching a final value of 40 mmHg.

In the control group, exposure to hypoxia resulted in a substantial decrease in H_f (Fig. 5B) from 69.5 ± 2.6 to 31.9 ± 3.2 beats/min and an increase in V_{AMP} (Fig. 5C) from 0.36 ± 0.06 to 1.13 ± 0.18 cm. The magnitude of the bradycardia during hypoxia was similar to that observed after both external and internal cyanide, whereas the increase in V_{AMP} during hypoxia was

Table 1. Outflowing perfusate [catecholamine] in the in situ saline-perfused posterior cardinal vein preparation in response to NaCN

Time	[Catecholamine], nmol/l ⁻¹
Pre 1st min	17.0 ± 6.1
2nd min	16.1 ± 6.9
Post 1st min	15.7 ± 6.2
2nd min	14.7 ± 6.2
3rd min	12.8 ± 5.6
4th min	$11.4 \pm 5.9^*$
5th min	$11.2 \pm 5.2^*$

Values are means $\pm$ SE. NaCN dose was 1.0 ml of 0.10 mg/ml ($n = 8$). Pre values indicate catecholamine levels before administration of cyanide. Post values are levels from samples collected each minute for 5 min after the cyanide injection. *Significant difference from the second pre value. [Catecholamine], catecholamine concentration.

Fig. 5. A: PwO_2 (mmHg; $n = 9$, intact; $n = 7$, ligated), as a function of time, during environmental hypoxia. Shaded area represents the 20-min period of hypoxia. B: H_f (beats/min; $n = 9$, intact; $n = 7$, ligated) during environmental hypoxia. C: V_{AMP} (cm; $n = 8$, intact; $n = 7$, ligated) during environmental hypoxia. D: plasma catecholamine concentration (epinephrine plus norepinephrine; nmol/l; $n = 18$, intact; $n = 7$, ligated) before, during, and after environmental hypoxia. Open circles or bars represent the control group (gills intact), whereas the filled symbols or bars represent the group in which the first gill arches were ligated and removed. Data are shown as means $\pm$ SE. In A and C, double horizontal line indicates a significant difference in both groups from the normoxic value at time 0 min, whereas, in B, a single horizontal line represents a difference, from time 0, in either the control or gill-ligated group. In D, *significant difference from the pre value. †Significant difference between the control and gill-ligated group.

approximately twice as large compared with the response after cyanide. After removal of the first gill arch, the hypoxic bradycardia was substantially attenuated but not abolished (Fig. 5B). In this group, H_f was decreased by 15 beats/min (compared with 38 beats/min

min in the control group) and the reduction of H_f was not statistically significant until the final 6 min of hypoxia. Removal of the gill arch chemoreceptors also attenuated, but did not abolish, the increase in V_{AMP} (Fig. 5C).

During exposure to severe hypoxia, plasma catecholamines (epinephrine plus norepinephrine) were significantly elevated, reaching a maximum level of ~ 100 nmol/l (the predominant catecholamine was epinephrine; 75% of total). These levels were approximately twice as great as those observed after external cyanide and comparable to the levels measured in response to internal cyanide. Removal of the first gill arch did not abolish the increase in plasma catecholamine levels during hypoxia. Rather, the levels of plasma catecholamines were actually elevated during hypoxia in the gill-ligated group.

In a separate group of fish, the pseudobranch was denervated while the first gill arch remained intact. During these experiments, an additional control group was studied simultaneously with the pseudobranch-denervated group. During exposure to hypoxia in this control group, plasma catecholamine levels rose from 3.9 ± 1.0 to 90.5 ± 31.2 nmol/l, whereas in the pseudobranch-denervated group catecholamine levels rose from 2.2 ± 0.7 to 88.2 nmol/l. These data indicate that there are no chemoreceptors on the pseudobranch that can induce catecholamine secretion during hypoxia.

DISCUSSION

O₂ Chemoreceptor-Induced Catecholamine Secretion

The results of this study demonstrate that, in rainbow trout, the release of catecholamines into the circulation can be triggered by stimulation of cyanide-sensitive O₂ chemoreceptors on the gills. Plasma catecholamine levels were elevated in response to administration of cyanide into both the inspired water and the blood entering the gills. These observations indicate that internally oriented (blood sensing) and externally oriented (water sensing) O₂ chemoreceptors on the gills can, when stimulated, trigger catecholamine release. Despite the extensive body of literature on the physiology of catecholamines in fish (see Refs. 14, 15, 18, 21, 22 for reviews), this study represents the first attempt to determine the sensory loci that trigger the acute adrenergic response in these animals. Given that gill chemoreceptors elicit cardiorespiratory reflexes in fish, it was reasonable to hypothesize that branchial chemoreceptors may also initiate the reflex arc leading to an elevation of catecholamines in the blood during hypoxia.

The cellular mechanisms by which O₂ sensing is achieved are not fully understood (6, 10). Cyanide is a powerful chemostimulant, and, for many years, has been used to activate O₂ chemoreceptors by inhibiting mitochondrial respiration and producing histotoxic hypoxia. In other words, cyanide inhibits oxidative phosphorylation, but arterial P_{O₂} and oxygen content remain normal or are even elevated owing to hyperventilation. Cyanide may, or may not, mimic the exact

physiological mechanism of O₂ sensing, but, because it is easy to work with, cyanide has often been used in chemoreceptor studies, including those on fish (see Refs. 4, 12 for reviews). Regardless of the actual cellular mechanism(s) activated by cyanide, it is apparent that cyanide stimulates O₂ chemoreceptor cells, and in this regard, NaCN is a useful tool to identify the location of O₂ chemoreceptors, induce chemoreceptor discharge, and stimulate cardiorespiratory reflexes.

The vast majority of studies on the cellular mechanisms of O₂ chemoreception have been performed on mammalian species. Although these mechanisms are beyond the scope of this study, we hypothesized that if the chemostimulant actions of cyanide could be modified by altering the P_{O₂} surrounding the chemoreceptor site, this would indicate that NaCN was acting via a specific O₂ chemoreceptor to produce a given reflex response, regardless of the actual cellular mechanism involved. In control (gills intact) fish, increasing the P_{O₂} in the inspired water to 600 mmHg prevented the cyanide-evoked bradycardia that occurred under normoxic conditions. Furthermore, a moderate dose of externally applied NaCN caused catecholamine release under conditions of moderate hypoxia but not under normoxic or hyperoxic conditions. Together these data suggest that the chemostimulant effects of NaCN can be attenuated by raising the P_{W_{O₂}} and enhanced by lowering the P_{W_{O₂}}. Although these experiments do not address the mechanism of O₂ sensing, the data do suggest that NaCN is stimulating specific O₂ chemoreceptors. In this regard the data are consistent with previous work on this species (2, 3) in which NaCN and hypoxia had similar effects on afferent nerve discharge from an isolated gill preparation.

The cardiorespiratory responses to hypoxia and cyanide in fish have been well documented (4, 5, 12, 25, 26). In the current study, cardiorespiratory variables were recorded to ensure that the treatments (hypoxia and cyanide) that evoked catecholamine release also caused the well-documented cardiorespiratory responses. The fact that the more-or-less predicted (based on the literature) receptor-mediated cardiorespiratory responses were observed in response to NaCN supports the conclusion that the elevation of plasma catecholamines, in response to cyanide, was also mediated by specific O₂ chemoreceptors.

Ligation and removal of the first gill arch abolished the increase in plasma catecholamine levels that occurred in response to externally applied, but not internally applied, cyanide. These results demonstrate that the externally oriented O₂ chemoreceptors that trigger catecholamine release are confined to the first gill arch. There are several explanations for the continued response to internally applied cyanide after removal of the first gill arch. It was possible that the internal cyanide, injected through the caudal vein, was having a direct effect on the chromaffin cells located within the posterior cardinal vein. However, the following evidence argues against this explanation.

Internal injections of cyanide into the ventral aorta also evoked an elevation of plasma catecholamine lev-

els, yet this injection site bypassed the chromaffin cells. In this case, it was still possible, however remotely, that some of the cyanide flowed backward through the heart and into the posterior cardinal vein. This possibility was rejected because direct application of cyanide onto the chromaffin cells in an *in situ*, saline-perfused preparation did not evoke the release of catecholamines. The ANG II experiment confirmed the viability of the chromaffin cells in this preparation. The *in situ* result indicates that chromaffin cells in this species are not O₂ sensitive, at least via a mechanism that is activated by cyanide. Additionally, in marked contrast to prior results obtained using Atlantic cod (*Gadus morhua*; 19), it was recently reported that perfusion of trout chromaffin cells, *in situ*, with hypoxic saline or blood, did not elicit the release of catecholamines (20). It would appear, therefore, that unlike in cod and neonatal rats (27), the chromaffin cells in mature rainbow trout are not O₂ sensitive. The simplest explanation for the continued response to internal cyanide after removal of the first gill arch is that there are internally oriented O₂ chemoreceptors located within other gill arches that can trigger catecholamine release on stimulation. It is unclear whether these internally oriented branchial O₂ receptors are located on all gill arches or on more than just the first gill but less than all arches. Given that the chemoreceptors responsible for eliciting cardiorespiratory reflexes in many species of fish are located either on the first arch or all arches, it is likely that the O₂ receptors that elicit catecholamine release are present on all gill arches.

During environmental hypoxia, the concentration of plasma catecholamines was substantially elevated, reaching levels of ~100–200 nmol/l. Removal of the first gill arches did not prevent the release of catecholamines during hypoxia. Similarly, denervation of the pseudobranch, with the first arches intact, had no effect on the release of catecholamines during hypoxia. Given that first arch removal abolished the elevation of plasma catecholamines in response to external, but not internal, cyanide, it is reasonable to conclude that the continued release of catecholamines during hypoxia, after first arch removal, is due to activation of internally oriented receptors on the remaining gill arches.

Previous studies clearly demonstrated that, in teleost fish, catecholamine secretion during hypoxia occurs abruptly when the oxygen content of the arterial blood declines to ~50% of maximal values (16, 17). Thus a model has been developed in which a specific reduction in blood O₂ content acts as the proximate stimulus for catecholamine secretion during environmental hypoxia (14–16). The results of the current study indicate that although stimulation of externally oriented O₂ chemoreceptors can cause catecholamine secretion, activation of these receptors cannot account for the elevation of plasma catecholamines during hypoxia. The implication is that internally oriented receptors are exerting a predominant role in catecholamine release during hypoxia. As such, these results are in agreement with the existing model that the O₂ status

of the arterial blood is the critical factor responsible for catecholamine release during hypoxia. However, current models of O₂ chemoreception involve sensing of P_{O₂} and not O₂ content (6, 10). Given that catecholamine release in fish occurs when O₂ content declines to ~50% of its maximal value, it is unclear whether or not a novel O₂ content receptor exists in fish or whether these O₂ chemoreceptors are detecting changes in P_{aO₂} when it falls to approximately the p50 value.

Chemoreceptor Control of Cardiorespiratory Function

Current models of cardiorespiratory control during hypoxia in teleosts are confounded by a great degree of inter- and intraspecific variation (12). In salmonids, the hypoxic bradycardia is believed to originate largely from stimulation of external receptors localized to the first gill arch. The results of the present study are consistent with this model. However, the marked bradycardia elicited by injection of internal cyanide and the abolition of this response after removal of the first gill arch suggest the additional involvement of internally oriented O₂ chemoreceptors within the first gill arch. A similar situation exists in the traíra, a hypoxia-tolerant neotropical fish (25). Although removal of the first gill arches abolished the bradycardia in response to both internal and external cyanide, a small, delayed bradycardia remained during hypoxia. This observation indicates that extrabranchial O₂ chemoreceptors may exist that can cause a decrease in H_f during hypoxia. Alternatively, hypoxemia may have had a direct effect on the myocardium, slowing the H_f.

Current models for ventilatory control in teleost fish during hypoxia incorporate the participation of both internally and externally oriented chemoreceptors (5, 11, 25, 26). Previous studies, however, have been unable to localize the receptors to one specific region of the gills (5) and thus it is generally believed that the receptors are diffusely distributed within the gills, in other words, located on all of the gill arches. In the present study, ligation of the first gill arch did not abolish the increase in V_{AMP} during hypoxia. However, gill arch ligation did prevent the increase in V_{AMP} to internal but not external cyanide. It would appear, therefore, that the increase in ventilation amplitude during hypoxia is mediated by both internally oriented receptors on the first gill arch and externally oriented O₂ receptors on more than the first gill arch, possibly all gill arches.

Receptor-Mediated Catecholamine Release vs. Cardiorespiratory Responses

Typically during exposure to environmental hypoxia in fish, breathing begins to increase and H_f begins to fall at mild to moderate reductions of P_{wO₂}. In the current study, H_f began to fall at a P_{wO₂} of ~75 mmHg while the increase in breathing began at a P_{wO₂} of ~100 mmHg (Fig. 5). In this study, plasma catecholamines were not measured at these mild to moderate levels of hypoxia. It is clear, however, from pre-

vious studies (14–22) that plasma catecholamines are only elevated during severe acute hypoxia in the rainbow trout. Thus, during hypoxia, cardiorespiratory adjustments begin well before catecholamines are released into the circulation.

In the current study, removal of the first gill arch abolished both the hypoxic bradycardia and the release of catecholamines in response to externally administered cyanide. It is conceivable, therefore, that the same population of O₂ chemoreceptors is triggering both of these responses. In this case, the same receptors may be functioning through different afferent and/or central pathways to produce different effects at different levels of hypoxia. On the other hand, there may be entirely different populations of receptors that trigger the various responses to hypoxia. In this study, gill arch ligation did prevent the increase in V_{AMP} to internal but not external cyanide. This is opposite to the effect of gill arch ligation on cyanide-evoked catecholamine release, suggesting that the O₂ chemoreceptors responsible for triggering an increase in ventilation amplitude are distinct from those that elicit catecholamine release or bradycardia.

Perspectives

The physiology and biochemistry of catecholamines in fish have long been studied. Until recently, the majority of studies focused on the consequences of an elevation of circulating catecholamines in fish (18, 21). The effects of these hormones in fish are not trivial (14). Indeed, given the presence of adrenergically activated Na⁺/H⁺ exchange on the red blood cells of many teleost fish, catecholamines play a significant role in minimizing the decrease in blood oxygen content during exposure to hypoxia, a common occurrence for many fish (5, 28). Over the last decade, much of the work on catecholamines in fish has focused on the afferent limb of the adrenergic stress response, in other words, the mechanisms of catecholamine secretion (22). It is now clear that the mechanisms involved in the release of catecholamines from fish chromaffin cells are likely to be as complex as those functioning in mammals (1, 9, 14, 22). However, to date, no studies have examined the site of chemoreception that initiates the release of catecholamines during respiratory challenges such as environmental hypoxia.

This study demonstrates, for the first time in fish, that peripheral O₂ chemoreceptors, located on the gills, can initiate the reflex that leads to the release of catecholamines from the chromaffin cells into the blood. The gill O₂ chemoreceptors linked to catecholamine secretion are both externally oriented (on the first gill arch) and internally oriented (on all gill arches) and detect changes in O₂ levels within the water and blood, respectively. However, during hypoxia, stimulation of internally oriented O₂ receptors on all gill arches appears to be the physiologically important mechanism for initiating catecholamine release.

Financial support for this study was provided by a Natural Sciences and Engineering Research Council (NSERC) of Canada operating grant to S. F. Perry.

REFERENCES

1. **Burgoyne RD.** Control of exocytosis in adrenal chromaffin cells. *Biochim Biophys Acta* 1071: 174–202, 1991.
2. **Burleson ML and Milsom WK.** Propranolol inhibits O₂-sensitive chemoreceptor activity in trout gills. *Am J Physiol Regul Integr Comp Physiol* 258: R1089–R1091, 1990.
3. **Burleson ML and Milsom WK.** Sensory receptors in the first gill arch of rainbow trout. *Respir Physiol* 93: 97–110, 1993.
4. **Burleson ML, Smatresk NJ, and Milsom WK.** Afferent inputs associated with cardiovascular control in fish. In: *Fish Physiology*, edited by Hoar WS and Randall DJ. New York: Academic, 1992, vol. 12, p. 389–426.
5. **Fritzsche R and Nilsson S.** Cardiovascular and ventilatory control during hypoxia. In: *Fish Ecophysiology*, edited by Rankin JC and Jensen FB. London: Chapman & Hall, 1993, p. 180–206.
6. **Gonzalez C, Almarez L, Obeso A, and Rigual R.** Carotid body chemoreceptors: from natural stimuli to sensory discharge. *Physiol Rev* 74: 829–898, 1994.
7. **Julio AE, Desforges P, and Perry SF.** Apparent diffusion limitations for carbon dioxide excretion in rainbow trout (*Oncorhynchus mykiss*) are relieved by intravascular injections of carbonic anhydrase. *Respir Physiol* 121: 53–64, 2000.
8. **Laurent P and Rouzeau JD.** Afferent neural activity from pseudobranch of the teleosts. Effects of PO₂, pH, osmotic pressure and Na⁺ ions. *Respir Physiol* 14: 307–331, 1972.
9. **Livett BG and Marley PD.** Noncholinergic control of adrenal catecholamine secretion. *J Anat* 183: 277–289, 1993.
10. **López-Barneo J, Pardal R, and Ortega-Sáenz P.** Cellular mechanisms of oxygen sensing. *Annu Rev Physiol* 63: 259–287, 2001.
11. **Milsom WK.** Control of breathing in fish: role of chemoreceptors. In: *Physiology and Biochemistry of the Fishes of the Amazon*, edited by Val AF, Almeida-Val VMF, and Randall DJ. Manaus, Brazil: Instituto Nacional de Pesquisas da Amazônia, 1996, p. 359–377.
12. **Milsom WK, Sundin L, Reid SG, Kalinin A, and Rantin FT.** Chemoreceptor control of cardiovascular reflexes. In: *Physiology and Biochemistry of the Fishes of the Amazon*, edited by Val AF, Almeida-Val VMF, and Randall DJ. Manaus, Brazil: Instituto Nacional de Pesquisas da Amazônia, 1999, p. 363–374.
13. **Nandi J.** New arrangement of interrenal and chromaffin tissues of teleost fishes. *Science* 134: 389–390, 1961.
14. **Perry SF and Bernier NJ.** The acute humoral adrenergic stress response in fish: facts and fiction. *Aquaculture* 177: 285–295, 1999.
15. **Perry SF and Gilmour KM.** Respiratory and cardiovascular systems. In: *Stress Physiology*, edited by Balm PHM. Sheffield, UK: Sheffield Academic, 1999, p. 52–107.
16. **Perry SF and Reid SD.** Relationship between blood O₂ content and catecholamine levels during hypoxia in rainbow trout and American eel. *Am J Physiol Regul Integr Comp Physiol* 263: R240–R249, 1992.
17. **Perry SF and Reid SG.** The effects of acclimation temperature on the dynamics of catecholamine release in the rainbow trout *Oncorhynchus mykiss*. *J Exp Biol* 186: 289–307, 1994.
18. **Perry SF and Wood CM.** Control and co-ordination of gas transfer in fishes. *Can J Zool* 67: 2961–2970, 1989.
19. **Perry SF, Fritzsche R, Kinkead R, and Nilsson S.** Control of catecholamine release in vivo and in situ in the Atlantic cod (*Gadus morhua*) during hypoxia. *J Exp Biol* 155: 549–566, 1991.
20. **Perry SF, Montpetit CJ, and Borowska M.** The effects of acute hypoxia on chemically or neuronally induced catecholamine secretion in rainbow trout (*Oncorhynchus mykiss*) in situ and in vivo. *J Exp Biol* 203: 1487–1495, 2000.

21. **Randall DJ and Perry SF.** Catecholamines. In: *Fish Physiology*, edited by Hoar WS and Randall DJ. New York: Academic, 1992, Vol. 12, p. 255–300.
22. **Reid SG, Bernier NJ, and Perry SF.** The adrenergic stress response in teleost fish: control of catecholamine storage and release. *Comp Biochem Physiol* 120C: 1–27, 1998.
23. **Shelton G, Jones DR, and Milsom WK.** Control of breathing in ectothermic vertebrates. In: *Handbook of Physiology. The Respiratory System. Control of Breathing*. Bethesda, MD: Am Physiol Soc, 1986, sect 3, vol. 2, p. 857–909.
24. **Soivio A, Nyholm K, and Westman K.** A technique for repeated blood sampling of the blood of individual resting fish. *J Exp Biol* 62: 207–217, 1975.
25. **Sundin L, Reid SG, Kalinin A, Rantin FT, and Milsom WK.** Cardiovascular and respiratory reflexes in the tropical fish, *traira (Hoplias malabaricus)*. I. Role of gill O₂ chemoreceptors. *Respir Physiol* 116: 181–199, 1999.
26. **Sundin L, Reid SG, Rantin FT, and Milsom WK.** Cardiovascular and respiratory reflexes in the Tambaqui (*Colossoma macropomum*). Role of gill O₂ and CO₂ chemoreceptors. *J Exp Biol* 203: 1225–1239, 2000.
27. **Thompson RJ, Jackson A, and Nurse CA.** Developmental loss of hypoxic chemosensitivity in rat adrenomedullary chromaffin cells. *J Physiol* 498: 503–510, 1997.
28. **Val A and Almeida-Val VMF.** *Fishes of the Amazon and Their Environment: Physiological and Biochemical Aspects*, edited by Bradshaw SD, Burggren W, Heller HC, Ishii S, Langer H, Neuweiler G and Randall DJ. Berlin: Springer-Verlag, 1995, vol. 32. (Zoophysiology series)