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Effects of Acute Hypoxia on the Cerebral Blood Flow and Heart Rate in Carp, *Cyprinus carpio*

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Synopsis

Cerebral blood flow with a laser Doppler flowmetry and heart rate were examined in carp, each weighing approximately 500 g, immobilized with a muscle relaxant (d-tubocurarine chloride, 4 mg/kg) during 60-min hypoxia and subsequent 30-min normoxia at a water temperature of 23±1°C. Under mild hypoxia (water Po_2 of 100 and 75 mmHg), cerebral blood flow and heart rate remained constant relative to the normoxic values (water Po_2 of approximately 150 mmHg). At levels of water Po_2 below 25 mmHg, cerebral blood flow was significantly increased, while heart rate was significantly decreased. At water Po_2 of 50 mmHg, some carp individually examined showed a marked increase in cerebral blood flow without bradycardia. In addition, an intramuscular injection of atropine sulfate (1.2 mg/kg) caused the increase in cerebral blood flow without bradycardia in carp subjected to hypoxia (water Po_2 of 25 mmHg). These findings suggest that the mechanisms involved in the cerebral circulatory regulation in response to hypoxia are different from those underlying the bradycardiac response, indicating a vagal reflex mediated through the muscarinic cholinoceptor on the heart, and that cerebral circulatory regulation begins to act before the bradycardiac response in a respiratory chain. In a preliminary study, we found that elevation of cerebral blood flow in response to hypoxia was completely abolished by an intramuscular injection of an α-adrenoceptor antagonist (phentolamine methanesulfonate, 2 mg/kg).

I Introduction

In response to environmental hypoxia, fish show physiological responses, such as hyperventilation by augmented respiratory movement and/or increased respiratory rate[1-7], a higher affinity of hemoglobin to oxygen associated with decreased ATP levels in the erythrocyte or hyperventilation[6,8], erythrocyte supply from the spleen into the circulating blood[9-11], bradycardia associated with an increased efficiency in oxygen transfer in the gills[12-14], and elevation of plasma catecholamine levels associated with oxygen transport and with regulations of blood oxygen content, vascular resistance, and cardiovascular dynamics[15-18], although species difference exists. These responses aim at transport of oxygen from ambient water to the tissues where oxygen is required. According to HUGHES[19], this adaptational process has been termed as a respiratory chain.

The necessity of oxygen may be variable with the tissue ; hypoxia-sensitive tissue and hypoxia-

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resistant tissue. Probably, the brain is the most hypoxia-sensitive organ because of energy production and metabolism of neurotransmitters, as pointed out by NILSSON[20,21]. YOSHIKAWA *et al.*[22] examined EEGs and cerebral blood flow in hypoxic carp and suggested that cerebral activity was compensated to some degree by an elevation of cerebral blood flow for oxygen supply to the brain. The constant cardiac output in carp under hypoxia[23,24] indicates that the elevated cerebral blood flow during hypoxia may be the result of redistribution of blood, suggesting the presence of cerebral circulatory regulation in response to hypoxia.

Under moderate hypoxia above a critical level, oxygen uptake was held constant owing to hyperventilation in many species, including carp[1,25]. RANTIN[26] and RANTIN *et al.*[27] postulated that decrease in heart rate synchronized with decrease in oxygen uptake during graded hypoxia in carp and two species of genus *Hoplias*, although this coincidence is not general for the hypoxic fishes. The above-mentioned cerebral circulatory regulation is also assumed to exist at water Po_2 below a critical level.

In this study, carp were immobilized with a muscle relaxant (d-tubocurarine chloride, 4 mg/kg) because of a technical difficulty in measurement of cerebral blood flow in unrestrained carp. SHELTON and RANDALL[28] reported that d-tubocurarine chloride (5-10 mg/kg) had no effect on ECG in tench, *Tinca tinca*. Also in carp, d-tubocurarine chloride was considered to have a negligible effect on cardiac function[29]. Therefore, to examine the cerebral circulatory regulation in the respiratory chain and the mechanisms involved in the cerebral circulatory regulation in response to hypoxia, cerebral blood flow and heart rate were examined in carp under hypoxia.

II Materials and Methods

Seventy eight carp, *Cyprinus carpio*, each weighing approximately 500 g, were used in the experiment. They were purchased from a local supplier, and acclimated at 23±1°C under a 14 h light and 10 h dark cycle in plastic tanks placed indoors at least for a month. Water in the tank was recirculated using a filtration apparatus. Carp were fed a commercial diet for carp.

Carp were briefly anesthetized with MS222 (tricaine methanesulfonate) for measurement of body weight, and then immobilized by an intraperitoneal injection of a muscle relaxant (d-tubocurarine chloride, 4 mg/kg) in aerated water. After cessation of respiratory movement, they were held with a wet towel to a U-shaped lead plate, and continuously irrigated with fully aerated water through the mouth. In all the experiments, the flow rate and temperature of water for branchial irrigation were held constant at 1.5 L/min and 23°C, respectively. To measure cerebral blood flow, a small hole (7-8 mm in diameter) was made with a dental drill in the skull. A cylindrical probe (0.5 mm in diameter) for a laser Doppler flowmeter (Advance, ALF2100) was placed on the central surface of the left telencephalon using a micromanipulator. The telencephalon was chosen as the brain region for measurement of blood flow because of easy surgery, which was usually completed within 10 min, with negligible bleeding and less concomitant stress to fish. Blood flow, blood mass, and blood velocity calculated by dividing blood flow by blood mass were obtained in 30-s measurements carried out in 2.5-s intervals, since the parameters of blood circulation maintained relatively constant values during normoxia but fluctuated synchronously with heart beats under hypoxia[23,24]. These parameters were shown as relative values. Simultaneously with measurement of blood flow parameters, heart rate was measured throughout the experiment by recording ECG in lead I from the body surface according to UENO *et al.*[30]. Heart rate was determined from the number of R waves in ECG.

Carp were allowed at least an hour to recover from the above-mentioned surgery before the beginning of the experiment, until blood flow parameters and heart rate showed constant values. They were individually supplied with hypoxic water by adjusted N_2 bubbling for 60 min where

Po_2 in water for branchial irrigation reached a plateau, approximately 100, 75, 50, 25 or 15 mmHg, within 15 min and with normoxic water for the subsequent 30 min. Ten carp served as the control. Exposure to hypoxia was carried out on ten carp in each hypoxic condition after the initial measurement of the above-mentioned parameters. Experimental apparatus was the same as that reported by MITSUDA *et al.*[31].

To block the muscarinic and adrenergic responses, preliminary experiments were undertaken as follows: eighteen carp were subjected to 60-min hypoxia (water Po_2 of 25 mmHg) or 60-min normoxia at 30 min after an intramuscular injection of a muscarinic cholinoceptor antagonist (atropine sulfate, 1.2 mg/kg) or together with an α-adrenoceptor antagonist (phentolamine methanesulfonate, 2 mg/kg) or a β-adrenergic antagonist (propranolol hydrochloride, 2.5 mg/kg). The dosages of three antagonists used in this study were the same as those for coho salmon, *Oncorhynchus kisutch*, reported by AXELSSON and FARRELL[32].

Evaluation of statistically significant differences ($P<0.05$) was made using a Mann-Whitney's U test between the control and hypoxic carp.

III Results

Under mild hypoxia at water Po_2 of 75 and 100 mmHg, three parameters of cerebral blood circulation and heart rate showed constant values relative to the normoxic values (Tables 1-4). Statistically significant changes in cerebral blood circulation and heart rate occurred at levels of

Table 1. Cerebral blood flow in carp subjected to 60-min hypoxia (water Po_2 of 50-100 mmHg) and subsequent 30-min normoxia.

		Water PO_2 (mmHg)		
		50	75	100
Time (min) after the onset of bubbling N_2 gas	0	100± 33	94±28	96±15
	5	103± 35	89±22	95±17
	10	132± 83	96±30	97±15
	15	139± 94	94±28	97±16
	20	146± 89	103±31	91±14
	25	153±105	100±28	95±24
	30	161±118	102±28	90±21
	40	172±143	103±26	100±26
	50	190±157	98±26	98±23
	60	206±187	102±31	97±20
Time (min) after the cessation of bubbling N_2 gas	5	138±87	100±31	101±22
	10	122±64	99±31	102±19
	15	118±52	101±33	103±23
	20	120±56	103±35	106±20
	25	118±47	102±36	97±20
	30	116±52	98±30	96±19

In each series, the experiment was carried out on ten carp.
In this and the following tables 2 and 3, the values of cerebral blood flow, mass, and velocity are expressed in relative values, regarding the mean values of the control at the onset of the experiment as 100, respectively.

Table 2. Cerebral blood mass in carp subjected to 60-min hypoxia (water Po_2 of 50-100 mmHg) and subsequent 30-min normoxia.

		Water PO_2 (mmHg)		
		50	75	100
Time (min) after the onset of bubbling N_2 gas	0	102±14	101±14	100± 9
	5	102±12	99± 9	101±11
	10	105±14	105±15	102±10
	15	107±10	105±13	100±11
	20	109±15	105±11	103±15
	25	107±16	102± 9	101±11
	30	107±18	105±12	97± 7
	40	107±18	106±12	103±11
	50	110±21	103±16	100±10
	60	111±21	104±17	103±14
Time (min) after the cessation of bubbling N_2 gas	5	108±19	104±15	104±12
	10	104±23	102±16	102±12
	15	105±16	103±15	100±12
	20	104±18	104±15	99± 8
	25	105±17	105±14	102± 9
	30	102±23	103±17	102± 9

Table 3. Cerebral blood velocity in carp subjected to 60-min hypoxia (water Po_2 of 50-100 mmHg) and subsequent 30-min normoxia.

		Water PO_2 (mmHg)		
		50	75	100
Time (min) after the onset of bubbling N_2 gas	0	96± 23	93±19	97±17
	5	100± 26	89±18	94±18
	10	119± 58	90±21	95±15
	15	125± 67	94±23	94±17
	20	131± 73	96±22	88±16
	25	135± 75	97±22	94±24
	30	141± 83	97±19	92±19
	40	149±102	97±17	97±23
	50	159±108	94±15	99±24
	60	170±134	97±20	96±25
Time (min) after the cessation of bubbling N_2 gas	5	121±56	96±24	98±24
	10	113±42	96±18	100±18
	15	108±36	98±21	102±21
	20	113±39	98±26	104±26
	25	110±33	98±23	95±19
	30	109±32	94±21	94±20

Table 4. Heart rate (beats/min) in carp subjected to 60-min hypoxia (water Po_2 of 50-100 mmHg) and subsequent 30-min normoxia.

		Water PO_2 (mmHg)		
		50	75	100
Time (min) after the onset of bubbling N_2 gas	0	38±12	37± 6	39±14
	5	39±16	37± 8	40±15
	10	42±15	37±10	38±18
	15	43±20	36±11	34±16
	20	42±13	38± 9	35±19
	25	40±18	39±10	35±16
	30	38±17	37± 7	36±18
	40	37±16	35± 8	36±18
	50	37±15	36± 7	36±16
	60	35±10	34± 7	36±19
Time (min) after the cessation of bubbling N_2 gas	5	38±11	36±9	35±17
	10	40±10	36±7	37±15
	15	38±14	39±9	38±17
	20	39±12	39±7	36±16
	25	37±10	37±7	32±13
	30	31±12	39±7	33±15

water Po_2 below 25 mmHg. Figure 1 shows the changes in these parameters in the control carp and in carp subjected to hypoxia (water Po_2 of 15 and 25 mmHg). In the control carp, all the parameters remained constant for 90 min. In hypoxic carp, heart rate (beats/min) was significantly lowered approximately from 40 to 20 at 25 mmHg or from 35 to 15 at 15 mmHg. During the subsequent normoxic period, a long-lasting tachycardia was recognized at 25 mmHg, but at 15 mmHg the heart rate was restored to normal values within 5 min on return to normoxia without any sign of tachycardia.

Cerebral blood flow showed significant increases by approximately 50% at 25 mmHg and by approximately 100% at 15 mmHg, and then returned to normal levels within 5 or 10 min on return to normoxia, irrespective of the long-lasting tachycardia observed at 25 mmHg. Cerebral blood mass showed relatively small but significant increases throughout the hypoxic period, by approximately 15% at 25 mmHg and by approximately 25% at 15 mmHg. However, the blood mass maintained a significant elevation by approximately 10% during normoxia after exposure to hypoxia at 15 mmHg, although blood flow also increased slightly. On the other hand, cerebral blood velocity showed the same pattern as the blood flow and significant increases by approximately 40% at 25 mmHg and by approximately 75% at 15 mmHg, but the increases in the second half of the 60-min hypoxia were not statistically significant.

At water Po_2 of 50 mmHg, statistically significant changes were not recognized in all the parameters, as mentioned above (Tables 1-4). However, cerebral blood flow showed a large increase by 106% at maximum. This was evidently due to a great individual variation. For instance, the mean±standard deviation of the blood flow was 206±187% at the end of hypoxia (Table 1). As shown in Fig. 2, three carp showed a four- or five-fold increase in cerebral blood flow, whereas the other seven carp showed constant values. Even when individually examined, no bradycardia developed in carp subjected to hypoxia at water Po_2 of 50 mmHg.

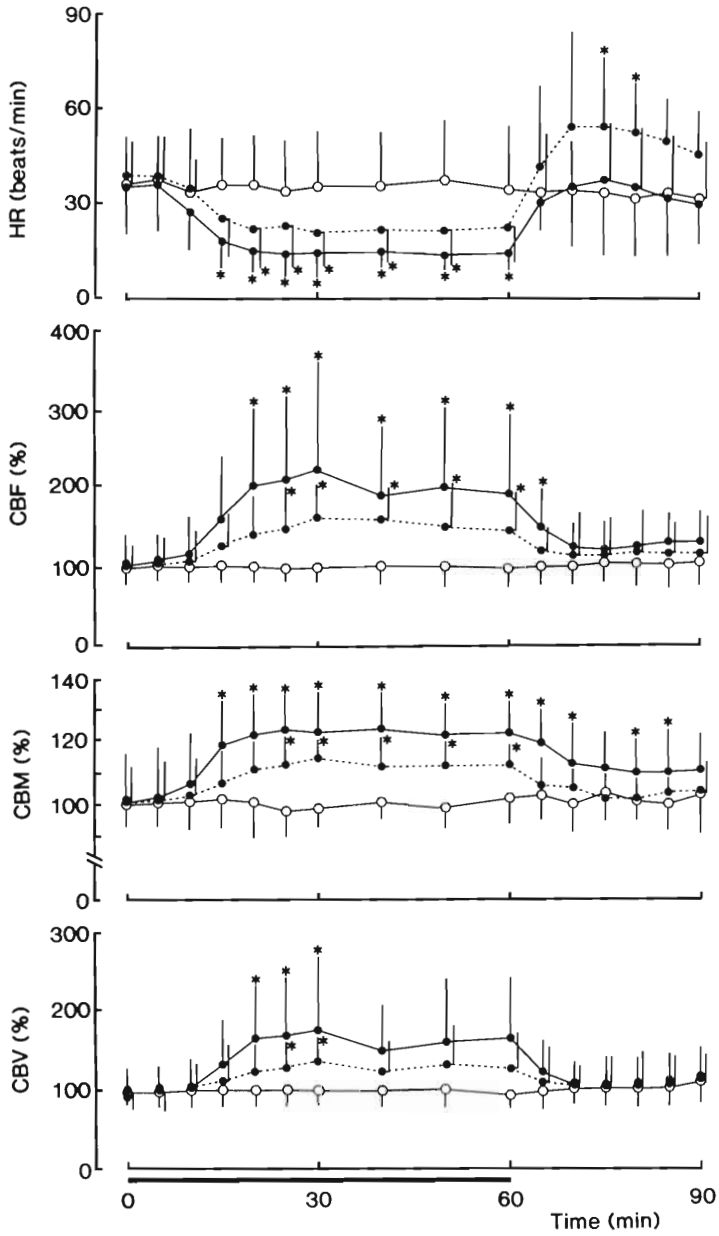

Fig. 1 Heart rate (HR), cerebral blood flow (CBF), cerebral blood mass (CBM), and cerebral blood velocity (CBV) in the control carp (○—○, n=10) and in carp subjected to hypoxia at water Po_2 of 25 mmHg (●···●, n=10) or at water Po_2 of 15 mmHg (●–●, n=10). CBF, CBM, and CBV were expressed in relative values, regarding the mean values of the control at the onset of the experiment as 100, respectively. Each asterisk denotes a statistically significant difference, compared with the control ($P>0.05$). In this and the following figures, the horizontal bold bar indicates the hypoxic period of 60 min.

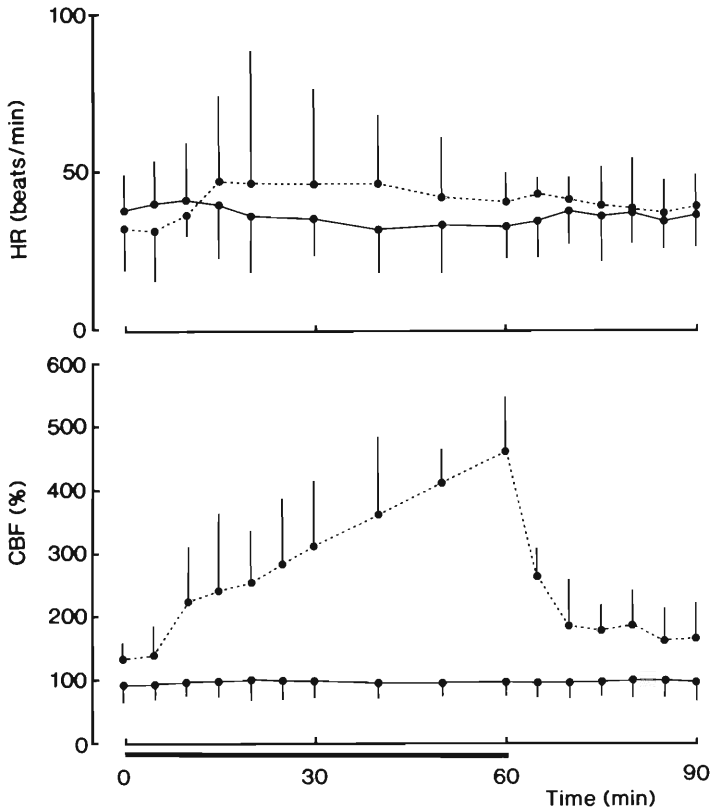

Fig. 2 Heart rate (HR) and cerebral blood flow (CBF) in carp subjected to hypoxia at water Po_2 of 50 mmHg. Three carp (●···●) showed a marked increase in CBF, whereas seven carp (●—●) showed constant values. Note that bradycardiac response was not observed.

Figure 3 shows the cerebral blood flow and heart rate in three carp under a normoxic or hypoxic condition after pretreatment with atropine. Atropinized carp showed a rapid increase in heart rate approximately from 40 to 100 beats/min. During the subsequent hypoxia, cerebral blood flow showed some increase, whereas heart rate showed a constant value. Figure 4 shows the cerebral blood flow in three carp under a normoxic or hypoxic condition after an intramuscular injection of atropine together with phentolamine or propranolol. Phentolamine completely abolished the elevation of cerebral blood flow (Fig. 4a). On the other hand, propranolol did not affect or slightly reduced the elevation of cerebral blood flow in comparison with that by the atropine-treatment (Fig. 3 and 4b). The heart rate in carp treated with phentolamine or propranolol showed a pattern similar to that in carp treated with only atropine.

IV Discussion

Carp used in this study were subjected to various procedures before exposure to hypoxia, netting, handling, anesthesia with MS222 for measurement of body weight, intramuscular injection of a muscle relaxant, attachment with electrodes for recording ECG, and surgical operation

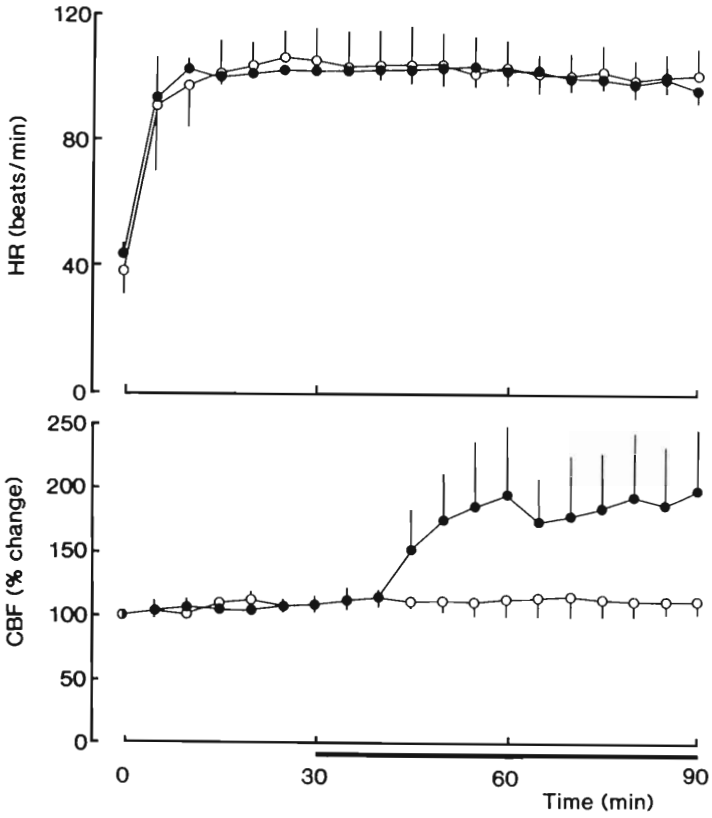

Fig. 3 Effects of atropine on heart rate (HR) and cerebral blood flow (CBF) in the control carp (○—○, n=3) and in carp subjected to hypoxia at water Po_2 of 25 mmHg (●—●, n=3). Hypoxic treatment was commenced at 30 min after an intramuscular injection of atropine sulfate (1.2 mg/kg). In this figure and Fig. 4, CBF was expressed in % change, that is, regarding each CBF value at the onset of the experiment as 100 because of the small number of carp used and a large individual difference.

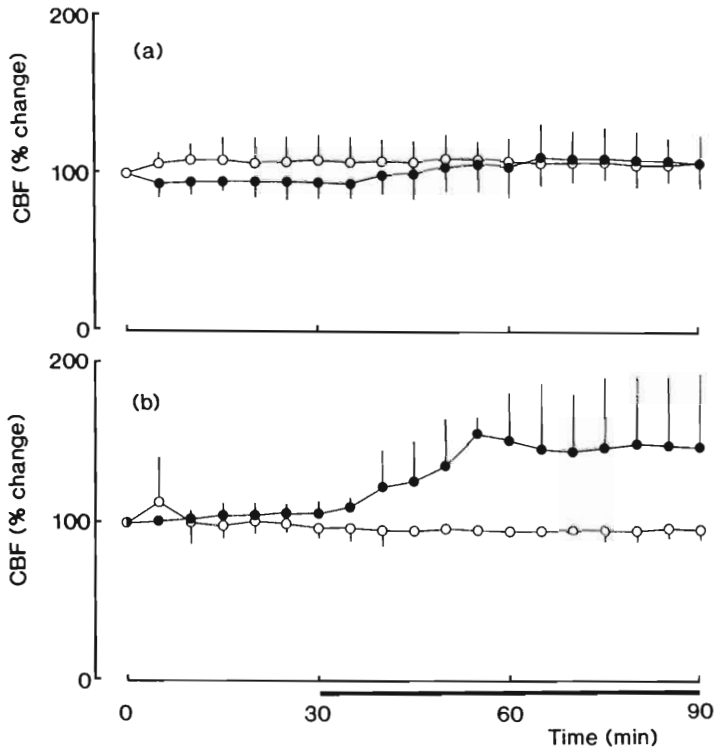

Fig. 4 Effects of phentolamine (a) or propranolol (b) on cerebral blood flow (CBF) in the control carp (○—○, n=3) and in carp subjected to hypoxia at water Po_2 of 25 mmHg (●—●, n=3). Hypoxic treatment was commenced at 30 min after an intramuscular injection of atropine sulfate (1.2 mg/kg) together with phentolamine methanesulfonate (2 mg/kg) or propranolol hydrochloride (2.5 mg/kg).

for implanting a probe for the laser Doppler flowmeter on the brain surface. Therefore, carp might have been in a stressful state before exposure to hypoxia, although they were allowed to recover at least an hour before the beginning of the experiment, since blood flow parameters and heart rate showed constant values. The heart rate in the 78 carp used in this study was 37±13 (mean±SD) beats/min under the initial normoxic period. This value was within the range (27–52 beats/min on average) for unrestrained carp at a similar water temperature of 23–25°C reported by UENO *et al.*[30], MITSUDA *et al.*[31], YAMAMITSU and ITAZAWA[33,34], and GLASS *et al.*[35]. The carp may have been under less stress because of gentle netting and handling, a small dose of MS222, recording of ECG from the body surface instead of a stressful routine method with a needle-type electrode stuck to the muscle, and a short-term operation with negligible bleeding.

During hypoxia, cardiac output has been shown to remain constant due to a compensatory elevation of stroke volume in some fishes[36,37] and has been shown to be reduced in some fishes[38–40]. In hypoxic carp, GAREY[23] reported that cardiac output was independent of water Po_2 at levels above 40 mmHg at 10°C and ITAZAWA and TAKEDA[24] reported that cardiac output remained constant at water Po_2 of 50 and 25 mmHg relative to the normoxic values at 24.5°C. Even under the effect of the muscle relaxant, cardiac output was assummed to remain constant or to be reduced during hypoxia (water Po_2 of 25 and 15 mmHg), where bradycardia and elevated cerebral blood flow were recognized. Because of elevated cerebral blood flow, when considered in conjunction with the constant or reduced cardiac output, blood flow in other tissues must be decreased in hypoxic carp. Indeed, the reduction in blood flow under hypoxia has been reported in the coeliac and mesenteric artery in Atlantic cod, *Gadus morhua*, irrespective of the increase in cardiac output[41], and in the swimbladder in European eel, *Anguilla anguilla*[42]. Also in hypoxic carp, KAKUTA and MURACHI[43,44] and Kakuta *et al.*[45] found that the glomerular filtration rate and urine flow decreased and suggested a decrease in renal blood flow, although SWIFT and LLOYD[46] reported a decreased urine flow in hypoxic rainbow trout, *Oncorhynchus mykiss*. On the other hand, AXELSSON and FARRELL[32] reported a marked increase in coronary blood flow, which perfuses the heart, in coho salmon subjected to hypoxia. These findings suggest that a greater part of blood flow, which would be reduced in the above-mentioned tissues, was distributed to hypoxia-sensitive organs, the brain and heart. Moreover, in this study, cerebral blood flow was restored to normal levels immediately after hypoxia, irrespective of tachycardia associated with a possible oxygen debt during hypoxia. This probably indicated an elevation of blood flow in hypoxia-resistant tissues which had been in an ischemic state during hypoxia. The absence of tachycardia or an overshoot of heart rate on return to normoxia at water Po_2 of 15 mmHg may be due to myocardial damage under severer hypoxia, as pointed out by GLASS *et al.*[35]. The cause of increase in cerebral blood flow was primarily due to increased blood velocity because blood velocity increased to the same degree as blood flow and showed a pattern analogous to blood flow. SCHEICH *et al.*[47] histologically examined cerebral vascularization in a gobiid fish, *Typhlogobius californiensis*, and found an increase in average diameter of cerebral capillaries by 60% under hypoxia. Their findings probably denote an increase in cerebral blood mass. Also in hypoxic carp, a small but significant increase, by 15 to 25%, in cerebral blood mass contributed to the increase in cerebral blood flow. However, this increase does not always indicate the increase in diameter of cerebral capillaries or cerebral vasodilation. The laser Doppler flowmetry is based on the principle that the frequency and amplitude of laser light with a Doppler shift by moving erythrocytes are proportional to the velocity and mass of erythrocytes. The flow of erythrocytes is obtained as the product of velocity and mass. Our instrument measures the flow and mass of blood in approximately 1 mm^3 of tissue. Consequently, the blood mass obtained with the laser Doppler flowmetry is affected by alterations in the hematocrit value of the blood, which is evident from the principle of the laser Doppler flowmetry. At present, it is difficult to conclude that increase in cerebral blood mass results from cerebral vasodilation, because the hematocrit

value is known to increase in hypoxic fishes[11,46,48,49], including carp[45,50].

On assuming that cardiac output was held constant or reduced and blood flow in body tissues was reduced under hypoxia, increased cerebral blood flow in hypoxic carp is considered to be due to a vasoconstriction in dorsal aorta and/or a vasodilation in carotid artery, which perfuses the brain. AXELSSON and FARRELL[32] suggested that both an α-adrenoceptor mediated systemic vasoconstriction and a β-adrenoceptor mediated coronary vasodilation were associated with increased coronary blood flow in coho salmon. We demonstrated that a muscarinic cholinoceptor antagonist, atropine, abolished the bradycardiac response, but not elevated cerebral blood flow. This indicates that regulation of heart rate and development of bradicardia are mediated by muscarinic receptors in carp. Indeed, the regulation of the heart in carp is known to be under inhibitory control by cholinergic vagus nerve fibers[51,52], although in some fish adrenergic innervation of the heart has been reported[52,53]. On the other hand, regulation of cerebral blood flow is not mediated by cholinergic receptors in carp, because increased cerebral blood flow was still recognized in hypoxic carp treated with atropine and a nicotinic cholinoceptor antagonist, d-tubocurarine, used as a muscle relaxant. We also preliminarily found that elevation in cerebral blood flow was abolished by an α-adrenoceptor antagonist, phentolamine, and was not affected or slightly reduced by a β-adrenoceptor antagonist, propranolol. The elevated cerebral blood flow in hypoxic carp may also be due to an α-adrenoceptor mediated systemic vasoconstriction or in combination with carotid vasodilation, although synchronous measurements of cardiac output and blood pressures in the dorsal and ventral aortas and carotid artery are required to test this hypothesis. The mechanisms involved in the regulation of blood flow in the coronary or carotid artery may be different with the fish species. For instance, elevation of dorsal aortic blood pressure supporting a systemic vasoconstriction was reported in some hypoxic fishes, such as sea raven, *Hemitripterus americanus*, Atlantic cod, and coho salmon[32,37,54], while reduction of dorsal aortic blood pressure was reported in some hypoxic fishes, such as lingcod, *Ophiodon elongatus*, and European eel[38,39].

At levels of water Po_2 below 25 mmHg, cerebral blood flow significantly increased and heart rate significantly decreased. Cerebral blood flow and heart rate showed a synchronous change in the course of hypoxia. However, at water Po_2 of 50 mmHg, some carp showed a marked increase in cerebral blood flow without any change in heart rate. Namely, the regulation of cerbral blood flow began before the bradycardiac response in a respiratory chain, although elevation of cerebral and coronary blood flow may occur at the same time in hypoxic carp.

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コイの脳血流と心拍に及ぼす急性低酸素の影響

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摘　要

低酸素時における脳血流量と心拍数を筋弛緩剤（d－塩化ツボクラリン4 mg/kg）で不動化した体重約500 gのコイを用いて水温23±1℃のもとで測定した。脳血流量は終脳表面でレーザードップラー組織血流計により計測した。軽度の低酸素下（呼吸水の酸素分圧が100と75 mmHg）では，脳血流量も心拍数も実験開始時（通常状態）と変わらず一定であった。酸素分圧が25 mmHg以下では脳血流量は通常状態に比べて有意に増大する一方，心拍数は有意に減少（徐拍）した。50 mmHgのときには，徐拍になることなく脳血流量の顕著な増加を示す個体もみられた。さらに，25 mmHgの低酸素状態でも硫酸アトロピンの筋肉注射（1.2 mg/kg）によって徐拍を伴わない脳血流量の増加が生じた。これらの結果から低酸素に対する反応のうち脳循環系における血流調節機構は，徐拍反応の基礎となる機構，すなわちムスカリン作働性のコリン受容体によって仲介される心臓の迷走神経反射とは異なることを示唆し，呼吸鎖の中で脳血液循環系の調節が徐拍を起こす前に働き始めることを示した。また，予備実験として低酸素に対する反応である脳血流量の増加は，α－アドレナリン受容体の阻害剤のメシル酸フェントラミンの筋肉注射（2 mg/kg）で完全に消失することを示した。