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RAPID COMMUNICATION

Remarkable Blood Catecholamine Levels in Forced Dived Ducks

DENNIS M. HUDSON AND DAVID R. JONES
Department of Zoology, The University of British Columbia, 6270 University Boulevard, Vancouver, British Columbia, Canada V6T 2A9

ABSTRACT Levels of circulating epinephrine (E) and nor-epinephhrine (NE) were measured in duck blood before, during, and after forced dives which were extended close to the limit of underwater endurance. Both E and NE increased by, on occasion, three orders of magnitude. After surfacing, plasma catecholamine levels fell rapidly. The reason for this enormous amine release in forced dived ducks remains a matter of speculation.

Like that to the brain and heart, and in contrast to the majority of tissues, blood supply to the adrenal glands continues during forced dives. In ducks, adrenal blood flow increases (Johansen, '64; Jones et al., '79), whereas in Weddell seals adrenal flow is reduced, but only by 40% (Zapol et al., '79). In nutria (*Myocastor coypus*), adrenal flow is unchanged from the predive rate (McKean, '82). Continued perfusion, allied to the large size of the adrenal glands in habitual divers (Slijper, '58; Katomski and Ferrante, '74) suggests that the adrenals play an important role in forced diving. Despite this, only two observations have been made to date on plasma catecholamine levels in surmerged homeothermic divers and both were done during short dives. Plasma catecholamines double in ducks after 1 minute of forced submergence (Huang et al., '74), whereas after 4–6 minutes diving in seals, catecholamines may increase from five to 20 times (Hance et al., '82). This is nothing like the exceptional increases which have been observed in human fetuses during parturition, particularly when birth is traumatic (Lagercrantz and Bistoletti, '73). Since parallels are often drawn between the forced dive response and cardiovascular events associated with birth (Scholander, '61–'62), we felt that measurements of plasma catecholamines in ducks forced dived to the limit of their underwater endurance would be of great interest.

METHODS

Materials and protocol

Measurements were made on five ducks varying in mass from 2.5 to 3.0 kg. All experiments were done at room temperature (22°C), and ducks were acclimated to this temperature for 2 weeks before the start of measurements. A cannula was inserted in the brachial artery, which was exposed under local anaesthesia, and advanced until its tip lay in the brachiocephalic artery. The cannula was filled with heparinised saline (40 IU ml^{-1}) and connected to a Bio-Tec BT 70 blood pressure transducer. A three-way stopcock was used for this connection and blood samples were taken from the side arm. Arterial blood pressure was displayed on a Harvard pen recorder writing on curvilinear coordinates and heart rate was derived from the blood pressure trace. Ducks were gently restrained, in a supine position, on an operating table with the head held pointing into a large filter funnel. Ducks were left in this position for several hours during which a couple of brief dives (1–2 minutes) were performed. The last of these introductory dives was performed 1 to 2 hours before the dive in which blood samples were taken. Only one dive was done in which blood samples were taken and the animal was held underwater until diving bradycardia "broke", when the duck was allowed to surface. Blood samples were taken before and around 2, 5, and 10 minutes during the dive. A final sample was taken just before emergence.

Dennis M. Hudson's present address is Department of Physiology, School of Medicine, University of California, Los Angeles, CA 90024. Address reprint requests to him there.

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Blood sampling procedures

Arterial blood was withdrawn from a cannulated brachial artery in 1 ml plastic syringes containing a sample vehicle consisting of EGTA ($90 mg \cdot ml^{-1}$) and reduced glutathione ($60\ mg \cdot ml^{-1}$). Samples were gently mixed with the sample vehicle in the syringe and immediately transferred to evaluated glass tubes (Vacutainer, Becton and Dickinson Co.) and placed in an ice water bath. Within 15 minutes of sampling, the iced vacutainers were centrifuged in prechilled centrifuge cups at about 300 × g for about 6 minutes. Care was taken to avoid haemolysis. After centrifugation, the supernatant was transfered to a second evaculated tube and frozen (−20°C) until later analysis. All plasma samples were analyzed for catecholamine content within 4 weeks of an experiment.

Enzymatic O-methylation procedures

The analysis is based on the enzymatic O-methylation of catecholamine by COMT (catecholamine O-methyl transferase) using tritiated ^3H-SAM (CH_3-S-adenosyl-L-methionine) as a methyl donor in order to create a radioactive catecholamine derivative proportional to the original catecholamine concentration of the blood sample. After methylation, the labeled catecholamines are then phase-separated from unreacted ^3H-SAM and proteins. Nonlabeled O-methylated catecholamines (metanephrine, normetanephrine, and methoxytyramine) are then added as carriers for subsequent isolation by thin layer chromatography.

All catecholamine standards (E, NE), their O-methylated derivatives, COMT, and ^3H-SAM were purchased from Upjohn Diagnostics (The Upjohn Co., Kalamazoo, MI) and kept frozen (−40°C) until immediately before use. All reagents were used within 2 months of receipt.

For enzymatic O-methylation of the catecholamines, quadruplicate 50 μl aliquots of freshly thawed plasma were added to each of four disposable polypropylene microfuge tubes (500 μl, Eppenndorf) which were at 0°C in an ice bath. Plasma samples suspected of high catecholamine content were diluted 10–50 times with sample vehicle before the above step. To each of two of the aliquots was added 10 μl of an acidic glutathione solution, while the other two aliquots received 10 μl of the glutathione solution containing 20–50 $ng \cdot ml^{-1}$ of E and NE as internal standards. Blanks consisted of 50 μl of sample vehicle, 10 μl of glutathione solution, and 40 μl of reagent mixture. The reagent mixture contained, 2M tromethamine buffer (pH 9.6), 15 mM EGTA, 40 mM $MgCl_2$, COMT, and dithiotreitol. 5 μCi ^3H-SAM was added to each tube. The tubes were lightly vortexed and the reaction initiated by incubating the tubes for 37°C for 1 hour.

After completion of enzymatic labeling, the reaction was stopped by placing the tubes in an ice bath and adding 50 μl of a borate buffer (1 M, pH 11) containing EDTA and 4 mM metanephrine, normetanephrine, and methoxytyramine as carriers for the labeled derivatives. In order to separate the labeled catecholamine derivatives and carriers from unreacted ^3H-SAM and protein, 50 μl of a 20% solution of phosphotungstic acid in 1.2N HCl was added to each tube. The tubes were vortexed vigorously and centrifuged at 5000 × g for 10 minutes. After centrifugation, supernatant containing the labeled reaction products and carriers was decanted into new tubes and replaced in the ice bath.

Separation, extraction, and scintillation counting procedures

The procedures consist of isolating the labeled products of O-methylation by thin-layer chromatography and the subsequent conversion of the catecholamine derivatives to ^3H-vanillan by oxidation with periodate. The products are extracted and the radioactivity attributable to each catecholamine is determined by liquid scintillation counting.

For the isolation of methylated catecholamines, 100 μl of the solution containing the reaction products was spotted by hand onto prescribed silica gel thin-layer chromatographic plates (Silica GEl GF, 250 μm; Analtech Uniplate). Each plate was then dried with a blow dryer and placed in the dark under a dry N_2 atmosphere for 1 hour. The plates were then removed from the drying chamber and placed in a developing tank containing a freshly prepared mixture of tert-amyl alcohol, toluene, and 40% methylamine (6:2:3, v/v/v). Plates were developed in the dark for 2–3 hours. After separation, plates were removed from the developing tank and dried thoroughly. Zones of silica gel containing the methylated catecholamines were visualized under 254 nm ultraviolet light and marked carefully with a scribe. The gel from each zone was separated with care and scraped into a numbered glass scintillation

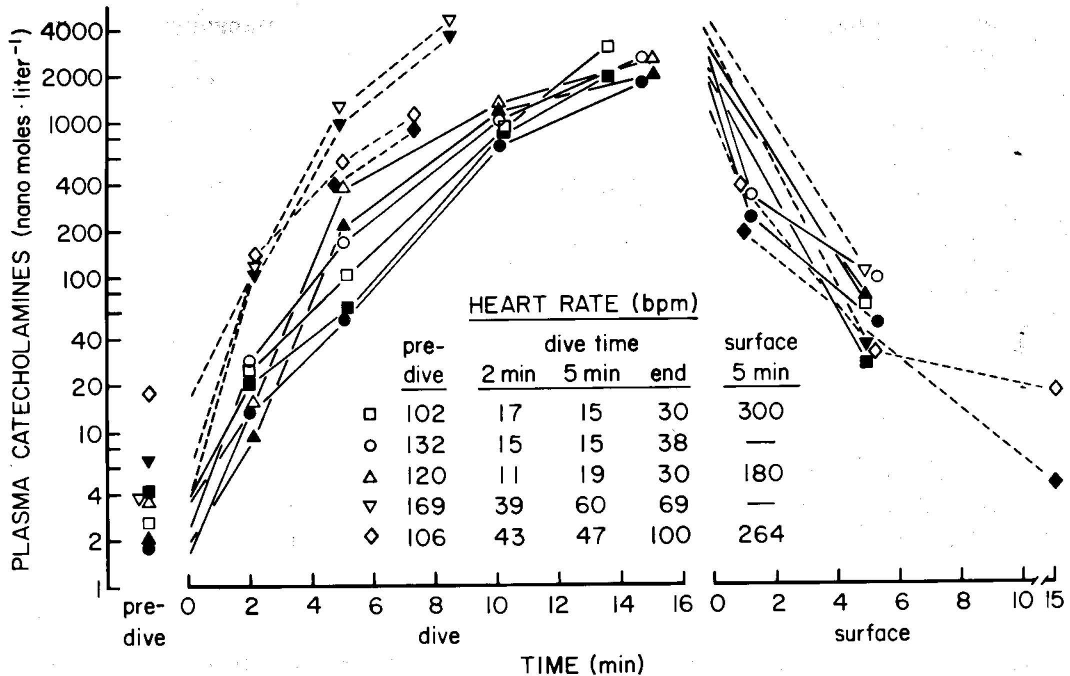

Fig. 1. Increase in NE (open symbols) and E (closed symbols) during forced dives in ducks. All dives started at dive time 0 and ended when the last sample was taken. Recovery time 0 represents when each animal was allowed to surface. The broken lines link values from two ducks which had about half the maximum underwater tolerance of the other three ducks (solid lines). Heart rates before, during, and after diving are given in the insert. Each duck is identified by the same shaped symbol as is used in the graph.

vial. One ml of 50 mM NH_4OH was added to each vial, which was then vigorously vortexed for 30 seconds. In addition, each vial containing metanephrine or normetanephrine received 50 μl of a 40% solution of Na metaperiodate. The periodate oxidation was allowed to continue for 5 minutes while each vial was gently agitated. At the end of this period, 50 μl of 10% glycerol was added to each vial. Finally, 1 ml of 0.1M acetic acid was added and the vials vortexed vigorously for 30 seconds.

Ten ml of a scintillation cocktail containing toluene and Liquiflour (1000:50, v/v) was added to each vial containing the metanephrine or normetanephrine oxidation products. All vials were then capped, vortexed vigorously, and dark controlled overnight. The vials containing the tritiated catecholamine derivatives were assayed at ambient temperature in a Beckman LS-3000 liquid scientillation spectrophotometer with counting efficiency of 50%. Radioactivity determined in samples and internal standards (counted to less than 2% counting error) was corrected for the CPM found in the corresponding blank. The concentration of the amines in each sample was then calculated based upon the radioactivity of the internal standard and the sample dilution factor.

RESULTS

Two of the five ducks only dived for about half the time period of the other three, despite all being of similar mass. These two ducks dived for 7.4 and 8.6 minutes, whereas the other three animals dived between 14 to 15 minutes. The cardiac chronotropic response was also different between ducks diving for the shorter and longer periods. In all ducks, the lowest diving heart rate occurred between the second and fifth minute of the dive, but in the two ducks with the shorter underwater endurance time this rate was 23% and 40% of the predive rate, whereas in the others heart rate fell to 9 to 15% of the predive rate (Fig. 1). Heart rate then increased and was usually two times the mini-

mum diving rate towards the end of the dive (Fig. 1). Blood pressure fell slightly in the early part of the dive, but returned close to predive levels when heart rate increased as the dive was prolonged.

Both NE and E in arterial blood increased spectacularly (Fig. 1). In ducks diving for 14 to 15 minutes, circulating catecholamines went up from predive levels between 2 and 4 nmole$\cdot l^{-1}$ to reach values between 2 and 3 $\times 10^3$ nmole$\cdot l^{-1}$ at the end of the dive. Both E and NE increased together in dives. Higher levels of circulating catecholamine occurred in the two animals which could only tolerate the shorter dives. In one of these ducks, values of 4.28×10^3 and 3.44×10^3 nmole$\cdot l^{-1}$, for NE and E, respectively, were obtained after 8.6 minutes diving (Fig. 1). On surfacing, catecholamine levels fell rapidly, and in the ducks which dived the longest were down by an order of magnitude 1-minute after surfacing. After 5 minutes recovery, all values for E and NE, in all ducks, were between 25 and 100 nmoles$\cdot l^{-1}$ (Fig. 1).

DISCUSSION

Much higher levels of circulating catecholamines have been reported from experimentally stressed birds (Jurani et al., '80) than have been recorded in mammals, but even so, the values obtained from forced dived ducks are quite exceptional. This raises the question as to whether the present data suffers from a systematic error. However, some circumstantial evidence indicates it does not. 1) Increases of at least two orders of magnitude have been recorded in human newborns undergoing breech deliveries which is similar to the increases we obtained in forced dived ducks (Langercrants and Bistoletti, '73). 2) The predive values for the majority of our ducks were lower than those usually reported for resting birds, being only two to three times the resting values for mammals. 3) In a recent study in seals (Hance et al., '82), plasma catecholamines increased after 4–6 minutes of submergence to values for NE and E between 10 and 25 nmol$\cdot l^{-1}$. Similar values were obtained in some of our ducks after 2 minutes of diving. Certainly, we feel that a 2 minute dive in a duck is equivalent to a 4 minute dive in a seal (Butler and Jones, '82). 4) We have run equivalent (although not the same) samples on an HPLC using electrochemical detection and find that increases in plasma catecholamines of two or three orders of magnitude in long forced dives are not uncommon (Mangalam and Jones, personal communication).

The fact that NE and E increase in tandem suggests that they are derived from the adrenal medulla. However, with the present information we can only speculate on the rate of catecholamine production and degradation before, during and after dives. For instance, even if the rate of synthesis and liberation is unchanged from predive, then catecholamines in the central arteries will increase by the same proportionate amount as cardiac output falls. Furthermore, the amount by which the circulating blood volume is effectively reduced in a dive will also influence the build-up of catecholamines, as will contributions from peripheral adrenergic nervous activity. Offsetting this will be uptake by peripheral tissues. On the other hand, the very rapid decline in catecholamines during recovery suggests dilution of the central blood pool with peripheral blood low in catecholamines rather than metabolic degradation.

What is the functional role of these enormous amine levels in dives? The effects of high levels of catecholamines of the cardiovascular adjustments to diving are equivocal (Butler and Jones, '82; Hance et al., '82). Vasoconstriction will be fostered, but both cardiac inotropic and especially chronotropic effects must be overruled. Effects on metabolism of the heart and other perfused tissues, due to catecholaminergic promotion of increased glycolytic flux, could contribute to underwater survival (Clark and Patten, '81), as would the possibility that high levels of nor-epinephrine may prolong the electrocortical response in asphyxia (Boëthius et al., '69). Finally, circulating catecholamines may play a role in stimulating breathing in recovery (Lillo and Jones, '82).

The opinion has been stated recently that a psychogenic reaction (fear) is an important component of the forced diving response (Kanwisher et al., '81). Certainly, the high levels of catecholamines we observed would appear to support this idea. Yet, it has been claimed for both seals and ducks that more intense diving responses are obtained from relaxed or "calm" animals (Irving et al., '41; Folkow et al., '67). Furthermore, ducks which dived the longest all had low predive catecholamine levels and had a more pronounced bradycardia than the two animals which tolerated only the shorter dives. Also, ducks are so quiescent in long dives, despite the enor-

mous amine release, that it seems likely that the psychogenic effects of catecholamines are being overruled. Obviously, high catecholamine levels are an important adjunct to forced diving although their role must remain a matter of speculation at present.

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