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CHARACTERIZATION OF AN ANCESTRAL GLUCOCORTICOID HORMONE AND ITS COGNATE RECEPTOR IN THE SEA LAMPREY

presented by

David A. Close

has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Fisheries and Wildlife

Major Professor's Signature

10 December 2007

Date

DATE DUE	DATE DUE	DATE DUE

CHARACTERIZATION OF AN ANCESTRAL GLUCOCORTICOID HORMONE AND ITS COGNATE RECEPTOR IN THE SEA LAMPREY

By

David A. Close

A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of the requirements
For the degree of

DOCTOR OF PHILOSOPHY

Department of Fisheries and Wildlife

2007

ABSTRACT

CHARACTERIZATION OF AN ANCESTRAL GLUCOCORTICOID HORMONE AND ITS COGNATE RECEPTOR IN THE SEA LAMPREY

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The goal of this research was to determine if one of the oldest extant vertebrates possesses a stress steroid hormone. Previous studies have been unsuccessful in determining if lampreys have a stress steroid. In order to confirm that a molecule is a hormone, the following criteria must be met: 1) definitive chemical identity, 2) a cognate receptor for that molecule, and 3) the molecule must mediate biological effects. Evidence is provided that 11-deoxycortisol is an ancestral stress steroid hormone in sea lampreys (*Petromyzon marinus*). The current study demonstrated the isolation and identification of two putative glucocorticoids from the blood of the lamprey, 11-deoxycortisol and 11-deoxycorticosterone. Both of these steroids were screened using radioimmunoassays and isolated by chromatography techniques. Positive identification of both steroids was by use of mass spectrometry. The study provided evidence that 11-Deoxycortisol has a receptor in the gill cytosol of the lamprey. The binding moiety for 11-deoxycortisol showed high specificity, affinity, and low capacity in the gill cytosol. Specific binding was highest in the intestine followed by gill, testis, liver, kidney, heart, and muscle. In addition, specific binding of the receptor complex to DNA was demonstrated by DNA-cellulose chromatography. The study showed that circulating levels of 11-deoxycortisol sharply increase after acute stress in spawning phase lampreys.

The stress hormone responds to acute stress in parasitic stage lampreys also. Slow release 11-deoxycortisol implants increased circulating 11-deoxycortisol and decreased classic androgens and estrogens. In addition, the implants increased gill Na^+, K^+-ATPase activity in lamprey. The study demonstrated that mammalian corticotropin releasing hormone increased 11-deoxycortisol. Pituitary extracts also increased circulating 11-deoxycortisol in a dose-response manner. Overall, these results are the first to demonstrate that the ancestral vertebrate, the sea lamprey, has a stress steroid hormone. In conclusion, this study demonstrated evidence for the identification, receptor, and biological effects of 11-deoxycortisol.

ACKNOWLEDGEMENTS

A special thanks to my advisor Dr. Weiming Li and the members of my graduate committee, Dr. Tom Coon, Dr. Don Garling, and Dr. Ken Poff for their guidance and support. A warm thanks to Dr. Sang-Seon Yun for guidance and support. Support from Dr. Alexander Scott was appreciated. A special thanks to Dr. Brad Young for editing and support. I am grateful to the staff of the U.S. Geological Survey, Hammond Bay Biological Station for supplying laboratory space and sea lampreys. Bonneville Power Administration for providing funding and support for this research. I am grateful to Michigan State University and the National Institute of Health for fellowships. A warm thanks to the Confederated Tribes of the Umatilla Indian Reservation for support of this research.

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INTRODUCTION

Concept of stress

During the 19th century, physiologist Claude Bernard established the concept of a constant "milieu interieur" or internal environment in the body (Cannon 1929; Robin 1979). Bernard's concept in 1878 was that an organism regulates a stable internal environment in an ever-changing external environment. Therefore, he is credited with laying the foundation for the concept of homeostasis which was articulated during the 1920s (Cannon 1929). Cannon (1924) demonstrated that adrenal secretions were of importance in maintaining homeostasis. Cannon (1929) actually coined the term homeostasis and related it to maintaining an internal stable state. He also suggested that there were two types of homeostasis involving supplies and processes to maintain a stable state of the internal fluid matrix. After Walter B. Cannon's homeostasis concept came the idea of a General Adaptation Syndrome by Hans Selye (1936).

Some of the most influential studies on stress response in animals were conducted by Hans Selye. Selye (1936) described animals responding to stress in stages of alarm, resistance and exhaustion. He called this phenomenon or theory the General Adaptation Syndrome (GAS). The syndrome included morphological changes such as an enlargement of the adrenal glands and atrophies of the thymus, spleen and lymphoid tissues. If animals were exposed to continued stressors, they would develop resistance. Eventually, the continuous stress would cause the animal to reach exhaustion and die. A few years later, Selye (1946; 1950; 1956; 1973) was able to fill in more details and

provided evidence that non-specific stressors elicited the alarm stage which activated the pituitary-adrenal axis to release corticosteroids. Selye (1950) defined the syndrome as "the sum of all non-specific, systemic reactions of the body which ensue upon long continued exposure to stress." In addition, he divided the idea of stress into stressors and stress response. Selye also reported that the adrenal glands were responsible for "adaptation diseases" although this was later refuted (Sayers 1950). While some of the details of Selye's concept were proven incorrect through time (Munck et al. 1984), his overall principle of the GAS concept was generally accepted.

During the 1980's a different hypothesis was put forth regarding the role of the adrenal stress steroids. Munck et al. (1984) hypothesized that the physiological function of stress-induced increases in glucocorticoid levels is to protect not against the source of the stress itself, but against the possible over-reaction of normal defense mechanisms activated by stress. The glucocorticoids accomplish this function by inhibiting the defense reactions, thus preventing them from overshooting and themselves threatening homeostasis. Sapolsky et al. (2000) provides a comprehensive review of glucocorticoid actions associated with stress.

A relatively new concept of stress has emerged from the biomedical field called allostasis or allostatic load. Sterling and Eyer (1988) define allostasis as the process of adaptation to acute stress including the ability to increase or decrease vital functions to a new steady state on challenge. While this was a seemingly new terminology, others had already explored the concept under different names. Schreck (1981) explored the concept which he termed performance capacity in relation to stress compensation. Several years later the concept was broadened to include energetic load (Schreck and Li

1991). McEwen and Stellar (1993) and McEwen (2000), concerned with diseases in humans, coined the term allostatic load which was defined as, "The strain on the body produced by repeated up and downs of physiologic response, as well as by the elevated activity of physiologic systems under challenge, and the changes in metabolism and the impact of wear and tear on a number of organs and tissues, which can predispose the organ to disease." The primary purpose of the concept was to explore the linkage between stress and disease at a finer scale to uncover the mechanistic causes.

McEwen and Wingfield (2003) expanded the allostasis load concept to include Type 1 and Type 2 overload. The Type 1 overload means that energy demands exceed the energy income, and what can be mobilized from stores. Type 2 occurs if energy demands are not exceeded and the organism continues to take in or store as much or even more energy than it needs. Therefore, Type 1 and Type 2 overloads are in addition to normal allostatic load resulting from unpredictable events.

The role of glucocorticoids in mediating the effects of allostatic loading has been reviewed by Landys et al. (2006). After synthesis of the existing published data, they found 3 levels of allostasis. The first was a "low but critical basal level, the second an elevated seasonal baseline that varies in conjunction with predictable demands, and the third was a high stress-related level in response to unpredictable and life-threatening perturbations."

Fisheries biologists recognized the economic importance of the effects of stress in fish hatcheries (Pickering 1981). Hatchery fish were regularly exposed to acute stressors such as crowding, handling, and transporting. Sometimes the fish were exposed to

chronic stressors also which would lead to increased mortality. Therefore, it became increasingly important for fisheries biologists to quantify stress.

Stress in fish

Most fish biologists developed their ideas about stress from Hans Selye's GAS concept. Brett (1957) thought that Selye's concept was too vague and fisheries professionals needed a better workable definition of stress. His definition is as follows: "stress is a state produced by any environmental or other factor which extends the adaptive responses of an animal beyond the normal range, or which disturbs the normal functioning to such an extent that, in either case, the chances of survival are significantly reduced." He thought that this definition would provide a more quantitative approach by including estimates of chances of survival or by the measure of reduction in capacity of performance. The concept of stress in fish was further expanded to include performance capacity (Schreck 1981) and energetic load (Schreck and Li 1991) similar to the concepts described earlier regarding allostasis. Pickering (1981) reviewed the evolution of the stress concept as it relates to fish and provided accounts of fish biologists beginning to quantify the stress response.

Instead of focusing on the concept of stress, fish physiologists focused efforts on measuring and quantifying the stress response (Pickering 1981; Wedemeyer et al. 1990). The new conceptual framework for stress in fish considered the stress response in terms of primary, secondary, and tertiary changes (Mazeaud et al. 1977; Donaldson 1981; Mazeaud and Mazeaud 1981; Wedemeyer and McLeay 1981). The primary response

was an endocrine system response which included the hypothalamus-pituitary-interrenal axis (HPI). After a perceived stimulus by the central nervous system, catecholamines and stress steroids are released into circulation. Catecholamines (for example, epinephrine), which are the first to increase in the blood, are released from the chromaffin tissues located in various organs of different species (Mazeaud et al. 1977; Mazeaud and Mazeaud 1981). Their principal role is thought to be the stimulation of glucose release for immediate energy used in a fight or flight response. After the catecholamine response is initiated, the stress steroid response is activated by the central nervous system. The central nervous system stimulates Corticotropin Releasing Hormone (CRH) to be released from the hypothalamus which in turn stimulates Corticotrophin Hormone (ACTH) to be released from the pituitary. ACTH circulates and binds to receptors in the interrenal cells of fish (homologue to adrenal gland) to stimulate the release of cortisol or corticosterone (Donaldson 1981; Wendelaar Bonga 1997; Mommsen et al. 1999).

The secondary response includes changes in blood and tissue chemistry in response to stress. Wedemeyer and McLeay (1981) and Wedemeyer et al. (1990) describe these as secondary alterations in blood chemistry such as; hyperglycemia, hyperlacticemia, hypochloremia, and reduced blood clotting time. In addition, they describe measuring changes in liver glycogen or even reductions in vitamin C from interrenal cells.

The tertiary response includes changes to individuals and populations (Wedemeyer et al. 1990). Many of the tertiary responses were observed by hatchery workers in daily operations (Wedemeyer and McLeay 1981). Some examples of tertiary responses are reductions in growth rate, lowered resistance to disease, decreased

reproductive success, oxygen consumption, swimming performance, altered feeding behavior or migratory behavior (Wedemeyer et al. 1990). Chronic stress may eventually lead to reduced performance by individuals and thus eventually impact the population.

Functions of corticosteroids

The corticosteroids are a group of steroids produced and secreted by the adrenal cortex cells in mammals or interrenal cells (in head kidney) in fish. In tetrapods, the adrenal cortex is divided into three zones that produce three different classes of steroids. The zones are the zona glomerulosa (minerlocorticoids), zona fasciculate (glucocorticoids), and zona reticularis (androgens) (Norman and Litwack 1997). Corticosteroids are divided into two subcategories: the glucocorticoids and minerocorticoids. They are both characterized by a hydroxyl group at the 21 carbon position. Further, glucocorticoids are characterized by the presence or absence of hydroxyls at carbon 11 and carbon 17 positions. The principal glucocorticoids in vertebrates are cortisol and corticosterone (Bern 1967; Idler and Truscott 1972; Sandor 1972; Jones et al. 1972) and are released in response to stressors. The only known exception is found in the elasmobranches, which produce 1α hydroxycorticosterone as their major glucocorticoid (Idler and Truscott 1967).

The mineralocorticoids are characterized by a hydroxyl at the carbon 11 position, and the carbon 18 oxidized to an aldehyde (Norman and Litwack 1997). The principal mineralocorticoid is aldosterone in land vertebrates which is important in regulating ion exchange and water metabolism. In fish, there is no evidence that aldosterone exists. It

was thought that cortisol had a dual role as stress steroid and mineralocorticoid in fish. However, a recent study has found that 11-deoxycorticosterone was an agonist for the mineralocorticoid receptor in rainbow trout (Sturm et al. 2005).

Glucocorticoids are considered stress steroid hormones with many functions. Sapolsky et al. (2000) classified the roles of glucocorticoids in the stress response as permissive, suppressive, stimulatory, and preparative. The suppressive functions of glucocorticoids include regulation of reproduction, inflammation, and immune system, while permissive actions are important in adrenal gland insufficiencies or malfunctions (Sapolsky et al. 2000). Stimulatory functions of glucocorticoids include regulating metabolic processes like prolonged elevation of blood glucose by gluconeogenesis and inhibition of glucose utilization (Sapolsky et al. 2000).

The sea lamprey as a model system

The sea lamprey (*Petromyzon marinus*) offers a unique animal model for the studies of stress steroid hormone characterization due to its ancestral lineage. The lampreys are Agnathans, jawless fishes, which are one of the oldest extant vertebrates available for use in the characterization of stress steroid hormones. Characterizing the glucocorticoid in one of the earliest vertebrates helps us understand the evolution of stress hormones in the vertebrate lineage. While we know that most vertebrates utilize cortisol or corticosterone as their glucoccorticoid, it was not known whether these ancient jawless fish possess a stress steroid.

Another benefit in using lamprey as a model is that the stress steroid hormone should be available at different life history stages of the lamprey. During the life cycle of the lamprey, they experience a freshwater and saltwater phase. These different phases include changing environments that may expose the lamprey to different stressors. The larvae go through a transformation called metamorphosis to prepare for the ocean or lake phase of their life cycle. After the parasitic phase, the sea lampreys reenter freshwater to spawn in tributaries where the eggs hatch in nests. The larvae leave the nests and then drift into soft substrate areas and reside as larvae for several years. The process of metamorphosis shares similar characteristics of smolting in salmonids. It is well known that salmonid smoltification is regulated by glucocorticoid cortisol. Therefore, lamprey may have a glucocorticoid hormone that acts similar to cortisol. The parasitic and spawning phases of the adult lampreys should also have a glucocorticoid stress response to help maintain allostasis.

The sea lamprey also offers a good model to understand the glucocorticoid stress response for fisheries management purposes. Sea lampreys are an undesirable pest in the Laurentian Great Lakes. Since the 1950s, substantial amounts of funding and effort to control the sea lamprey population have occurred. However, lamprey populations around the world are in decline due to habitat destruction. In the Pacific Northwest, the Pacific lampreys (*Entosphenus tridentatus*) are important subsistence foods for indigenous peoples. Identifying the glucocorticoid and being able to measure the stress steroid may assist in evaluating environmental stressors and various management activities for better conservation or control.

Introduction to dissertation

There is evidence that lampreys have the primary, secondary, and tertiary stress responses. For example, it has been shown that lamprey exhibit increases in catecholamines after an acute stressor, which is part of the primary stress response (Mazeaud and Mazeaud 1981). Plasma levels of epinephrine and norepinephrine were shown to increase in sea lamprey after various stressors (Mazeaud and Mazeaud 1981). The Hypothalamus-Pituitary-Adrenal (interrenal) Axis may exist in lamprey. While there is no evidence for CRH from the hypothalamus, there is evidence for ACTH in the lamprey pituitary. ACTH from the lamprey pituitary has been cloned and sequenced (Heinig et al. 1995; Takahashi et al. 1995A; Takahashi et al. 1995B). The lamprey ACTH is 60 amino acids long, roughly 20 amino acids longer than other vertebrate ACTHs (Takahashi et al. 2006). However, lamprey ACTH levels have not been shown to increase after acute stress. In addition, there is no direct evidence for a stress steroid hormone or glucocorticoid in the jawless vertebrate, the sea lamprey.

There is evidence that lampreys exhibit a secondary stress response. Different types of stressors such as dewatering and handling can induce hyperglycemia in lampreys (Larsen 1976; Morris and Islam 1969; Leibson and Plisetskaya 1969). Close et al. (2003) provided evidence that handling and tagging can change indicators of secondary and tertiary stress responses such as plasma glucose, ventilation rate, and swimming performance in lamprey.

The goal of this research was to identify and characterize the stress steroid hormone in the ancient vertebrate, the sea lamprey. To accomplish this goal, the first prediction was that lampreys have a stress steroid circulating in the plasma (chapter 1). The second prediction was that any identified steroids have a receptor (chapter 2). The third prediction was that identified steroids that have a receptor will have classic glucocorticoid stress response and show biological effects (chapter 3).

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CHAPTER 1

IDENTIFICATION OF PUTATIVE GLUCOCORTICOIDS IN THE SEA LAMPREY *Petromyzon marinus*

ABSTRACT

This study was undertaken to identify putative glucocorticoids in the blood of the sea lamprey *Petromyzon marinus*. Most glucocorticoid chemical structures are known throughout the vertebrate lineages. However, previous studies have failed to provide definitive evidence that the lamprey, one of the most ancient vertebrates, possess a stress steroid hormone. We hypothesized that lampreys do have a stress steroid hormone in their blood. High Performance Liquid Chromatography (HPLC) was used to fractionate extracted lamprey plasma for screening. Radioimmunoassays and protein binding assays were used to screen fractionated lamprey plasma for cortisol-like and corticosterone-like steroids. Radioimmunoassays were also used to monitor steroids in conjunction with LH-20 partition chromatography followed by HPLC to fractionate 800 ml of extracted lamprey plasma. Corresponding immunoreactive peaks were collected and subjected to mass spectrometry. The blood of the lamprey contained possible stress steroid hormones. Definitive identification was made by mass spectrometry analysis of 11-deoxycortisol and 11-deoxycorticosterone circulating in the blood of lamprey. This study provided evidence that one of these steroids is a putative glucocorticoid in the ancient vertebrate, the sea lamprey.

INTRODUCTION

Corticosteroids are steroid hormones produced by the adrenal cortex in mammals and the interrenal tissue located in the anterior portion of the kidney in teleosts (Bern 1967; Frazer 1992). Corticosteroids are divided into two groups, the glucocorticoids (GC) and mineralocorticoids (MC) based on function. GCs are considered stress steroid hormones with many functions. Sapolsky et al. (2000) classified the roles of GCs in the stress response as permissive, suppressive, stimulatory, and preparative. The suppressive functions of glucocorticoids include regulation of reproduction, inflammation, and immune system, while permissive actions are important in adrenal gland insufficiencies or malfunctions (Sapolsky et al. 2000). Stimulatory functions of glucocorticoids include the regulation of metabolic processes like prolonged elevation of blood glucose by gluconeogenesis and inhibition of glucose utilization (Sapolsky et al. 2000).

The principal stress steroids (GCs) in vertebrates are cortisol or corticosterone (Bern 1967; Idler and Truscott 1972; Sandor 1972; Jones et al. 1972). Mammals secrete cortisol; while rats and mice secrete corticosterone. Reptiles and birds also produce and circulate corticosterone as their primary glucocorticoid (Nelson 2000). In bony fish, the active GC is cortisol (Idler and Truscott 1972) while in elasmobranchs like sharks and rays the active GC is 1α- hydroxycorticosterone (Idler and Truscott 1967). In teleosts, cortisol may act as both a GC and MC which provides homeostasis in salt and water balance. The principal MC in land vertebrates is aldosterone, which functions to regulate

sodium and potassium transport and controls water reabsorption in the kidney (Norman and Litwack 1997). There is no evidence that fish have aldosterone.

The current thinking is that the steroid/receptor complex evolved in the vertebrate lineage (Escriva et al. 1997; Baker 1997). The question of whether stress steroids exist and function in ancient jawless fishes had not been answered. Identifying the possible stress steroids in lamprey would be an important step toward understanding vertebrate steroid/receptor evolution.

Previous studies have provided some evidence that a stress steroid may exist in the jawless vertebrates. The first line of evidence was from *in vitro* experiments using presumptive adrenal tissue incubations (PAT) with tritiated steroid precursors to examine possible corticosteroids in sea lamprey, *Petromyzon marinus*. Weisbart and Youson (1975) incubated PAT from larval and parasitic adult sea lamprey with [4-^{14}C] progesterone and identified the products using thin layer chromatography and recrystallization. The identified steroids were 11-deoxycortisol, 17α-hydroxyprogesterone, and androstenedione. From incubations with testes, the authors also identified 11-deoxycorticosterone. The PAT incubations failed to form cortisol, cortisone, corticosterone, or 11-deoxycorticosterone. Weisbart et al. (1978) conducted another PAT incubation experiment with [1, 2- ^{3}H] cholesterol, which failed to produce any known putative corticosteroids in spawning phase adult sea lamprey. Efforts to find possible corticosteroids in lamprey also included *in vivo* studies.

The second line of evidence was from *in vivo* experiments designed to examine possible corticosteroids in the blood of lamprey. Using double-isotope derivative methods, it was determined that either lampreys had no corticosteroids or levels were too

low to be biologically active (Weisbart and Idler 1970). Weisbart and Idler (1970) measured plasma cortisol levels at < 5 ng/100 ml , while cortisone and corticosterone levels were at 2 ng/100 ml in sea lamprey. Another study using the same methods found serum levels of cortisol, 11-deoxycortisol, corticosterone, at < 3 ng/100 ml and cortisone < 5 ng/100 ml in lamprey (Buus and Larsen 1975). Weisbart and Youson (1977) used chromatography and recrystallization to identify 11-deoxycorticosterone after [^{3}H] progesterone injections into parasitic adult sea lampreys. Weisbart et al. (1980) identified cortisol, corticosterone, 11-deoxycortisol, 11-dehydrocorticosterone by double-isotope derivative assay in sea lamprey after injecting porcine adrenocorticotropin releasing hormone (ACTH). The study failed to identify cortisone or 11-deoxycorticosterone in lamprey.

The third line of evidence was from recent receptor research in lamprey. Thornton (2001) identified DNA sequences which were homologues to estrogen, progesterone, and corticosteroid receptor in the lamprey. The analysis predicted that estrogen receptor evolved first, followed by the progesterone receptor, then the corticosteroid receptor. Recently, Bridgham et al. (2006) synthesized the ligand binding domain of the ancestral corticoid receptor for use in a transactivation assay. The assay showed that aldosterone and 11-deoxycorticosterone had the highest activation levels of the luciferase reporter. In addition, Bridgham et al. (2006) provided phylogenetic evidence that the ancestral corticoid receptor was a mineralocorticoid receptor which then gave rise to the glucocorticoid receptor in teleosts and tetrapods. The three lines of evidence mentioned, give reason to postulate that lampreys possess a stress steroid hormone.

The aim of the study was to establish chemical identification of possible stress steroids present in the plasma of sea lamprey. Our first objective was to use radioimmunoassays to screen lamprey plasma for cortisol-like and corticosterone-like steroids. Our second objective was to extract the steroids from a large pool of plasma, then to isolate them using chromatography methods. Our third objective was to determine the chemical identification of the putative stress steroids by mass spectrometry analysis.

MATERIALS AND METHODS

Maintenance of animals and blood collection

Sea lamprey *Petromyzon marinus* were collected in landlocked streams by the U.S. Fish and Wildlife Service employees, and transported to Michigan State University (East Lansing, MI) or USGS Hammond Bay Biological Station (Millersburg, MI) for experiments and collecting plasma where they were held at 10-13 °C. Blood was obtained by cardiac puncture using vacutainers containing EDTA (Becton Dickinson, Franklin Lakes, NJ), placed on ice for 15 min, and then centrifuged at 1000 x g at 4 ° C for 15 min. The plasma was removed and stored at -80 °C. All experiments were approved by the Michigan State University Institutional Animal Care and Use Committee (AUF # 05/04-077-00).

Plasma Extraction

Pooled plasma (20 ml) from male and female lampreys was diluted 1:1 with 0.9% saline, passed through a 0.45 μM filter (Millipore, Billerica, MA), and loaded onto an activated Sep-Pak (Waters, Milford, MA). The Sep-Pak was washed with 5 ml deionized water and eluted with 5 ml methanol. The methanol elute was evaporated under reduced pressure using a CentriVap Concentrator (Labconco, Kansas City, MO).

High performance liquid chromatography

Samples were dissolved in 1 ml actonitrile/water/trifluoroacetic acid (TFA) (28/72/0.01, v/v/v) and loaded onto a C18 reverse-phase HPLC column (Nova-Pak, 3.9 mm x 300 mm, Waters) fitted with a guard module. Two solvents were used to deliver a gradient to the column. Solvent A was 0.01% TFA in deionized water and solvent B was 70% acetonitrile and 0.01% TFA in deionized water. The pattern of development was as follows: 0 → 10 min, 28% B; 10 → 60 min, 28 → 100% B; 60 → 80 min, 100% B. The eluate was monitored for UV absorption with a photodiode array detector (Waters). Fractions were collected every 1 min between 11 and 70 min into 1.5 ml tubes.

Radioimmunoassay (RIA) and protein binding assay (PBA) procedures

RIAs and PBA were conducted in glass culture tubes (10 mm × 75mm, Fisher Scientific, Pittsburgh, PA). Briefly, the assay buffer consisted of 50 mM sodium phosphate, pH 7.4, 0.2% BSA, 137 mM NaCl, 0.40 mM EDTA, and 0.77 mM sodium azide. Nine standards were made up in duplicate over the range 1.95-500 pg/100 µl/tube. The tubes containing samples also had a volume of 100 µl of RIA buffer. Binding reagent was made by adding radiolabel (American Radiolabled Chemicals, St. Louis, MO) and antiserum (Chemicon, Temecula, CA., 1:100) or rabbit sera (Sigma-Aldrich, St. Louis, MO, 1:40) for PBA to 20 ml of assay buffer in amounts such that, when 100 ul was dispensed to all tubes, each tube contained 5000 dpm and, in the absence of any standard, 50% of the radiolabel was bound to the antiserum or cortisol binding protein.

Blank tubes, to which no antibody was added, and tubes necessary to determine the total and maximum dpm counts were also included in the assay. All tubes were incubated overnight at 4 °C. After overnight incubation, 500 µl of ice-cold charcoal solution at 0 °C (50 mM sodium phosphate, Ph 7.4, 0.1% gelatin, 1.0% dextran-coated charcoal) was added to each tube. The tubes were kept on ice for 20 min, and then centrifuged in an Allegra 6R (Beckman Coulter, Fullerton, CA) at 1000 x g for 12 min. The supernatants were poured into 8 ml scintillation vials, mixed with 6 ml scintillation fluid and DPM were counted with an LS-6500 (Beckman Coulter, Fullerton, CA) scintillation counter.

LH-20 column chromatography

Sample was dissolved in 2 ml 98% dichloromethane and 2% methanol and loaded onto a glass column (450 mm x 15 mm) packed with 20.0 g of Sephadex LH-20 (Amersham, Piscataway, NJ). Two solvents were used to deliver sample through the column. A solution containing 98% dichloromethane and 2% methanol was pumped through the column at 4 ml/min. Fractions were collected every 1 min between 1 and 60 min into 16 x 100 mm glass culture tubes. Elute was dried down and resuspended with 0.5 ml methanol per tube.

Mass spectrometry analysis of plasma

LH-20 fractions corresponding to immunoreactivity of 11-deoxycortisol and 11-deoxycorticosterone were collected and each group combined and dried down under

reduced pressure. Samples were then fractionated by HPLC and assayed to identify immunoreactive peaks in fractions. Immunoreactive fractions were combined, dried down under reduced pressure and then subjected to Atmospheric Pressure Chemical Ionization Mass Spectrometry (APCI-MS) analysis. Mass spectra were obtained using a LCQ-Deca ion trap (Thermo Scientific, Waltham, MA). The vaporizer temperature was 300 °C and the capillary temperature was 250 °C. Samples were compared against authentic 11-Deoxycortisol and 11-Deoxycorticosterone standards (Sigma-Aldrich). Mass spectrometry analysis was performed at the Mass Spectrometry Facility, Research Technology Support Facility at Michigan State University.

RESULTS

To identify possible stress steroids in sea lamprey, plasma was screened for immunoreactive cortisol and corticosterone. In addition, the plasma was also screened for binding activity with cortisol binding protein. Plasma immunoreactive cortisol levels peaked in HPLC fraction 44 at 1561 pg ml^{-1} (Fig. 1A). The peak in fraction 44 corresponds to the retention time of 11-deoxycortisol standard. A small immunoreactive peak occurred at fractions 32/33, where the cortisol standard eluted from the column. However, the peak was only slightly higher than background levels in other fractions. Background immunoreactive cortisol levels appeared to gradually rise throughout the HPLC run.

The cortisol binding protein assay showed results similar to the cortisol radioimmunoassay (Fig. 1B). The cortisol level markedly increased to 2231 pg ml^{-1} in HPLC fraction 44. The 11-deoxycortisol standard eluted from the HPLC column at the same fraction. Cortisol was not detected in HPLC fractions 32/33. Cortisol binding activity, unlike immunoreactivity to cortisol antibody, showed much lower background levels in other fractions.

Immunoreactive corticosterone peaked in fraction 50 at 608 pg ml^{-1} (Fig 2). Plasma levels of corticosterone also peaked in fractions 31, 56/57, and 63. However, no corticosterone was detected in fractions 41/42 where corticosterone standard eluted from the HPLC column. The major peak at fraction 50 corresponded with 11-deoxycorticosterone standard.

To collect a sufficient amount of steroids needed for identification, 800 ml plasma was extracted with Sep-Pak and the sample was subjected to gel filtration on a Sephadex LH-20 column to separate the immunoreactive steroids. Eluted LH-20 fractions were screened with 11-deoxycortisol and 11-deoxycorticosterone RIAs (Fig. 3). Immunoreactive 11-deoxycortisol levels were elevated in fractions 19-28 with a peak of 25 ng ml^{-1}. Immunoreactive 11-deoxycorticosterone levels were elevated in fractions 10-12 with a peak of 23 ng ml^{-1}. Each set of fractions was combined, dried down, and run on HPLC separately. HPLC fractions were screened using 11-deoxycortisol and 11-deoxycorticosterone RIAs. Immunoreactive HPLC fractions corresponding with 11-deoxycortisol (44) and 11-deoxycorticosterone (50) were collected, dried down, and subjected to mass spectrometry analysis.

HPLC fractions corresponding to immunoreactive 11-deoxycortisol and 11-deoxycorticosterone were analyzed by APCI-MS in positive mode to observe parent ions. To confirm the identity of the compounds, the parent ions were subjected to tandem mass spectrometry (MS/MS) and compared to authentic 11-deoxycortisol and 11-deoxycorticosterone fragmentation patterns of daughter ions. The MS/MS spectrum of authentic 11-deoxycortisol $[M+H]^+$ ion at *m/z* 347, obtained by direct infusion of a 10 μg ml^{-1} 11-deoxycortisol standard solution, are shown in figure (4A). The standard fragmented at *m/z* 269, 293, 311, and 329, which matched the fragmented daughter ions of the plasma sample (Fig. 4B).

The MS/MS spectrum of authentic 11-deoxycorticosterone $[M+H]^+$ ion at *m/z* 331, fragmented at m/z 295 and 313 (Fig. 5A). The two most abundant ions obtained by fragmenting the plasma sample $[M+H]^+$ ion, are also at *m/z* 295 and 313 (Fig. 5B).

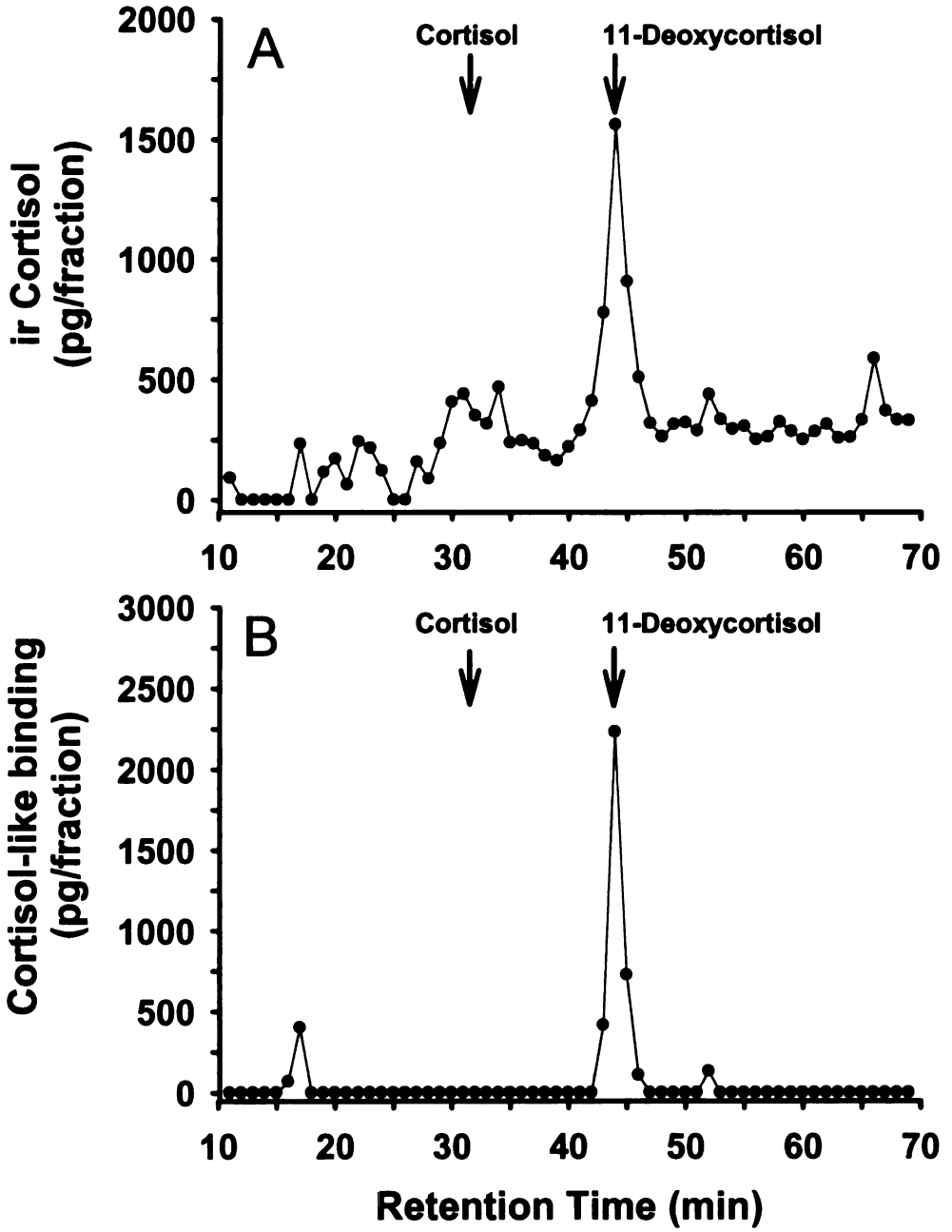

Figure 1-1. Screening of HPLC fractions of lamprey plasma for cortisol-like steroids using RIA and BPA. (**A**) Amount of immunoreactive cortisol found in 0.5 ml HPLC fractions from RIA of 20 µl of each fraction and back calculated fraction volume. (**B**) Amount of cortisol binding activity found in 0.5 ml HPLC fractions from PBA of 20 µl of each fraction and back calculated to fraction volume. Arrows, show where cortisol and 11-deoxycortisol standard elute. ir = immunoreactivity.

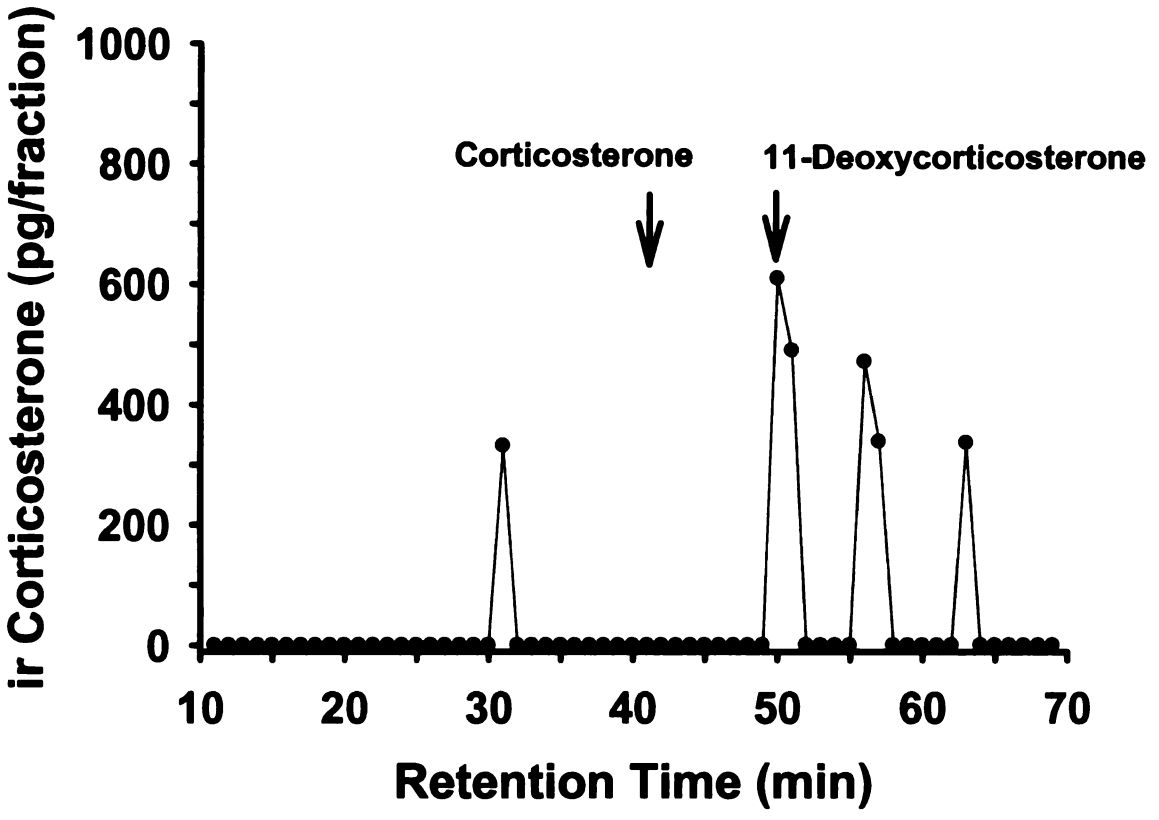

Figure 1-2. Screening of HPLC fractions of lamprey plasma for corticosterone-like steroids using RIA. Amount of immunoreactive corticosterone found in 0.5 ml HPLC fractions from RIA of 20 µl of each fraction and back calculated to fraction volume. Arrows, show where corticosterone and 11-deoxycorticosterone elute. Ir = immunoreactivity.

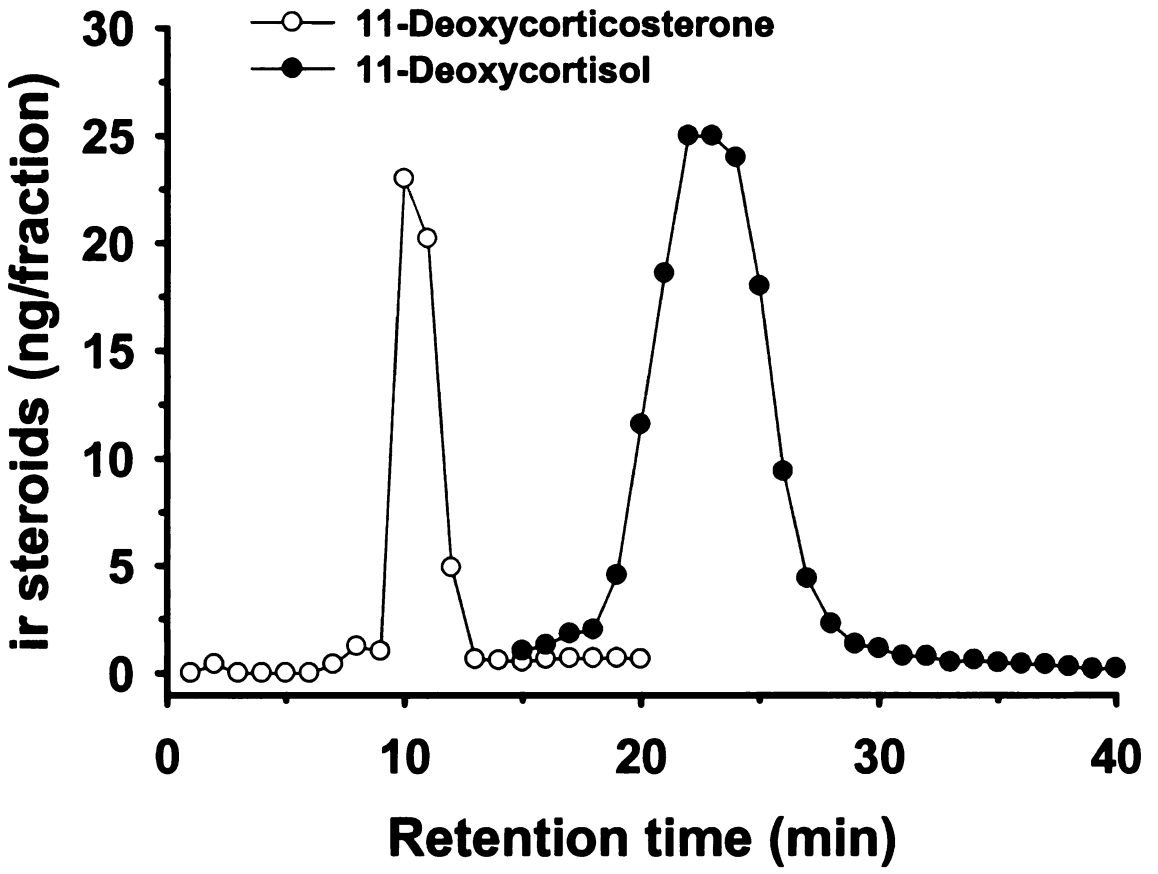

Figure 1-3. Screening of LH-20 fractions of lamprey plasma for immunoreactive 11-deoxycortisol and 11-deoxycorticosterone by RIA. Amount of immunoreactive 11-deoxycortisol and 11-deoxycorticosterone found in LH-20 fractions from a Sep-Pak extract of 800 ml of sea lamprey plasma based on RIA of 20 µl of each fraction and back calculated to fraction volume. Ir = immunoreactivity.

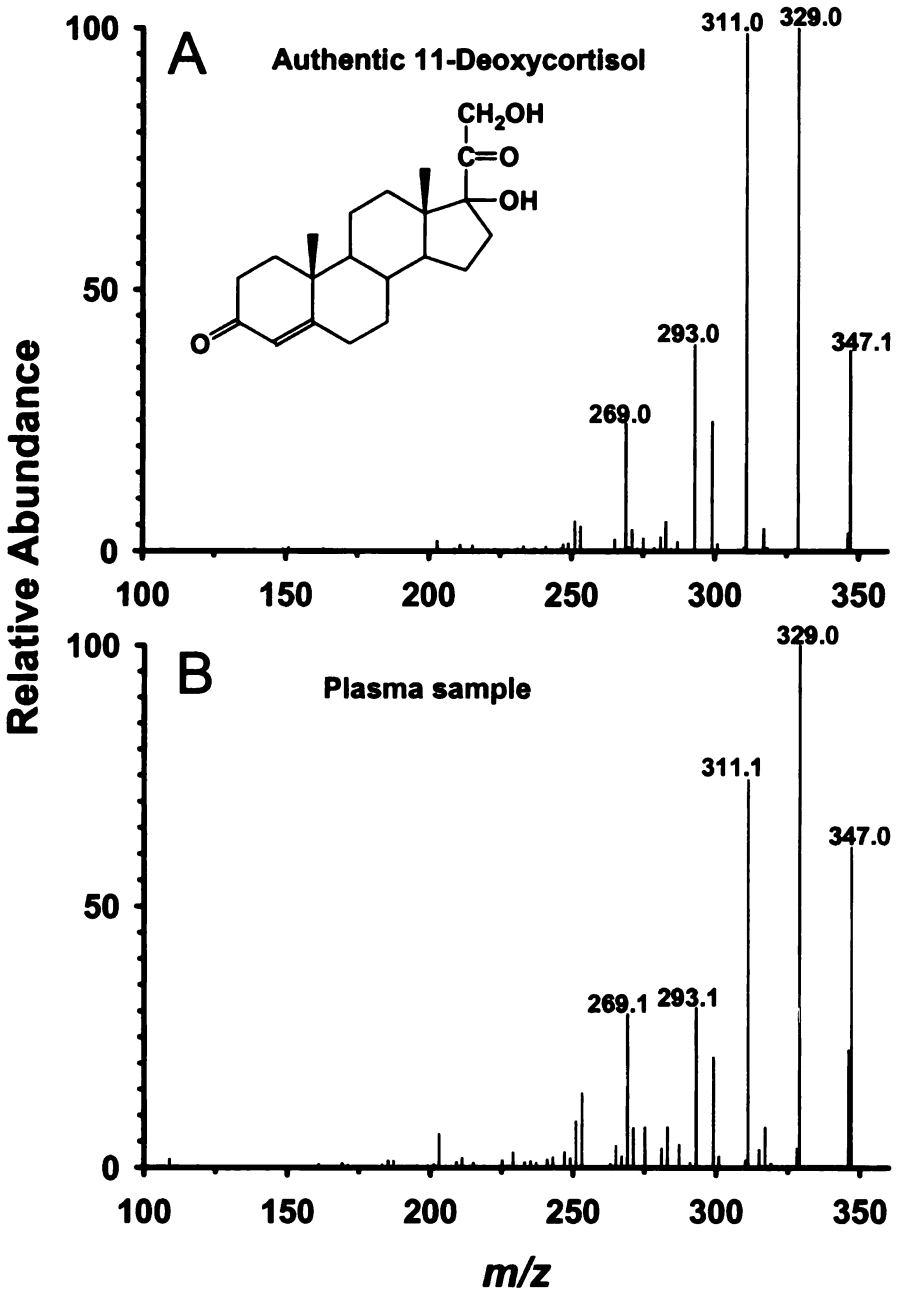

Figure 1-4. Identification of 11-deoxycortisol by APCI MS/MS analysis. Fragmentation patterns generated from both the synthetic 11-deoxycortisol (**A**) and a compound in HPLC fraction 44 (**B**), positively identifying the unknown compound in fraction 44 as 11-deoxycortisol.

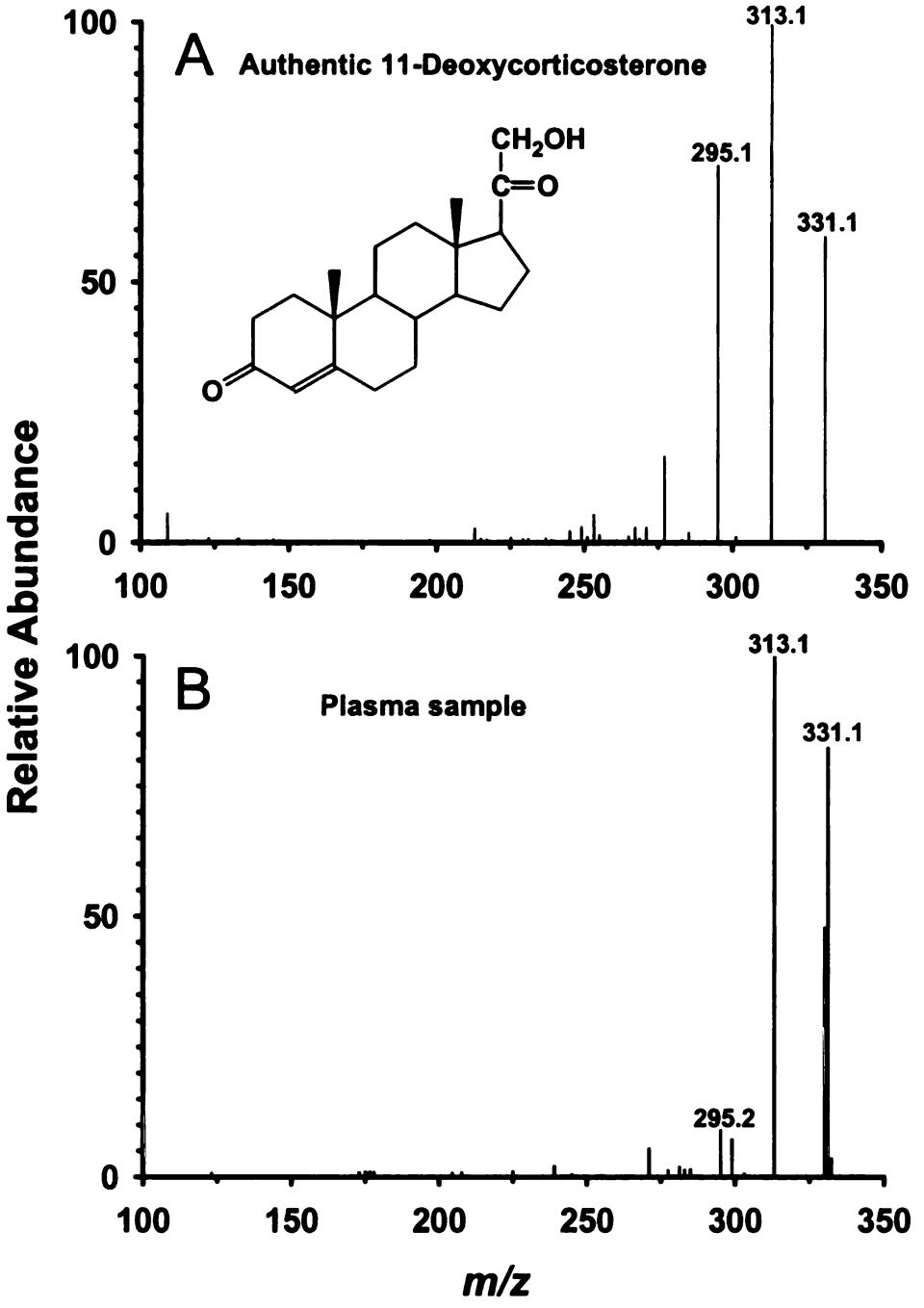

Figure 1-5. Identification of 11-deoxycorticosterone by APCI MS/MS analysis. Fragmentation patterns generated from both the synthetic 11-deoxycorticosterone (**A**) and a compound in HPLC fraction 50 (**B**), positively identifying the unknown compound in fraction 50 as 11-deoxycorticosterone.

DISCUSSION

Biological and chemical analysis provided strong evidence for the identification of 11-deoxycortisol and 11-deoxycorticosterone. There were three lines of evidence to identify these putative stress steroids in the plasma of the sea lamprey. First, the results showed co-elution of immunoreactive cortisol, cortisol binding protein activity, and immunoreactive corticosterone peaks with 11-deoxycortisol and 11-deoxycorticosterone standards in HPLC fractions. Second, the results showed the use of immunoreactive 11-deoxycortisol and 11-deoxycorticosterone to monitor LH-20 and HPLC fractionation with imunoreactive peaks corresponding to elution times of 11-deoxycortisol and 11-deoxycorticoserone standards. Third, the study confirmed the identity of the parent ions of 11-deoxycortisol $[M+H]^+$ ion at *m/z* 347 and 11-deoxycorticosterone $[M+H]^+$ ion at *m/z* 331 from plasma of sea lamprey. To confirm the identity, the parent ions were subjected to MS/MS analysis to observe the fragmentation patterns. The fragmentation patterns of the daughter ions of authentic 11-deoxycortisol and 11-deoxycorticosterone standards matched the fragmentation patterns from the plasma steroid samples.

In earlier studies, steroid identification was problematic due to limitations of the extant techniques and methods. Sandor and Idler (1972) developed the following criteria for identification of steroids; positive, presumptive, tentative, and suggestive based on the level of rigor in methodology. Idler and Truscott (1972) used the criteria to examine earlier reported corticosteroid identifications. They reported that Weisbart and Idler (1970) had used; thin layer chromatography (TLC), derivative methods (Der), recrystallization (Cry), and double-isotope derivative assay (DIDA) to identify cortisol

with plasma levels at 50 pg/ml in sea lamprey. In contrast, they reduced the number techniques to TLC, Der, and DIDA to identify plasma cortisone and corticosterone 20 pg/ml in the sea lamprey (Weisbart and Idler 1970). Thus based on Idler and Truscott's identification criteria, Weisbart and Idler's (1970) identification of cortisol was a presumptive identification, while cortisone and corticosterone were suggestive identifications.

In later studies through the 1970s, *in vivo* and *in vitro* studies continued using the same techniques mentioned above to confirm whether lampreys possessed corticosteroids (Buus and Larsen 1975; Weisbart and Youson 1975; Weisbart and Youson 1977; Weisbart et al. 1978; Weisbart et al. 1980). These studies could not take the identification criteria beyond presumptive. In the most recent study, Weisbart et al. (1980) identified cortisol (130 pg/ml), 11-deoxycortisol (570 pg/ml), and corticosterone (210 pg/ml) in the serum of adrenocorticotropin releasing hormone (ACTH) injected sea lampreys. Although the study reported identifying 11-deoxycortisol, their study also reported cortisol and corticosterone which is in contrast with our study.

In our study, unlike earlier studies, cortisol and corticosterone were absent in the circulation of sea lamprey. After fractionating the lamprey plasma by HPLC, only background levels of immunoreactive cortisol in fractions 32/33 were observed. In addition, the cortisol binding protein activity showed no binding activity in the fractions 32/33 where cortisol standard eluted. Immunoreactive corticosterone was not observed in fractions where corticosterone standard eluted in HPLC fractions. Even though previous studies (Weisbart and Idler 1970; Buus and Larsen 1975; and Weisbart et al. 1980) presumptively identified low levels of cortisol and corticosterone in the circulation of

lamprey, their methods may have been limited by their levels of detection and possibly background noise.

In the current study, *in vitro* PAT incubation experiments were conducted to reproduce the earlier findings by Weisbart and Youson (1975). Incubation experiments with [^{3}H] pregnenolone and [^{3}H] progesterone with parasitic and spawning phase sea lamprey presumptive adrenal tissue (data not shown). A major radioactive peak was observed to co-elute with 11-deoxycortisol standard in the HPLC fractions. The isolated radioactive peak was subjected to TLC. The radioactive peak co-migrated with 11-deoxycortisol standard on TLC. However, changing the solvent system caused the unknown radioactive peak and 11-deoxycortisol to clearly separate. Therefore, the conclusion was that presumptive adrenal tissue was not the source of 11-deoxycortisol. These results are in contrast with the previous report that 11-deoyxcortisol was produced in the sea lamprey PAT (Weisbart and Youson 1975).

In our study, results from RIA, HPLC, LH-20 chromatography, and mass spectrometry analysis demonstrate strong evidence that 11-deoxycortisol and 11-deoxycorticosterone are putative glucocorticoids in sea lamprey. In addition, the results show that cortisol and corticosterone are absent in the plasma of the ancient vertebrate. Weisbart and Youson (1975) suggested that the 11β-hydroxylase may not be present in lamprey. Our study provides evidence that 11β-hydroxylase did not evolve in the steroid biosynthetic pathway in early vertebrates, but evolved later in the elasmobranch lineage, since they produce 1α-hydroxycorticosterone (Idler and Truscott 1967).

In conclusion, this study provided definitive identification of two putative glucocorticoid hormones in sea lamprey. Although the results provide identification of

11-deoxycortisol and 11-deoxycorticosterone in the blood, there is no evidence that these steroids have receptors or biological functions. The positive identification of these two steroids will allow further testing to determine whether or not each has associated receptors. In order to classify these steroids as hormones, they must circulate in the blood, have cognate receptors, and have biological effects. Therefore future research efforts will focus on testing the ability of 11-dexoycortisol and 11-deoxycorticosterone binding affinity to receptor in lamprey.

ACKNOWLEDGEMENTS

A special thanks to the staff of the U.S. Geological Survey, Hammond Bay Biological Station for supplying laboratory space and sea lampreys. A special thanks to Lijun Chen for her assistance with mass spectrometry and the MSU mass spectrometry facility. This research was supported and funded by the Bonneville Power Administration. A warm thanks for the support from the Confederated Tribes of the Umatilla Indian Reservation.

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CHAPTER 2

CHARACTERIZATION OF A GLUCOCORTICOID RECEPTOR IN THE GILL OF THE SEA LAMPREY *Petromyzon marinus*

ABSTRACT

This study was conducted to determine if 11-deoxycortisol or 11-deoxycorticosterone identified in chapter 1 had a receptor in the gill tissue of the sea lamprey *Petromyzon marinus*. Previous research has provided evidence that a corticosteroid receptor may exist in an ancient vertebrate. This prompted the hypothesis that one of the two possible corticosteroids; 11-deoxycortisol and 11-deoxycorticosterone identified must have a cognate receptor in the gill cytosol of sea lamprey. This study identified and characterized high affinity (K_d = 2.66 ± 0.47 nM, mean ± SEM, n = 3), low capacity (B_{max} = 58.10 ± 3.33 fmol/mg protein, n = 3) glucocorticoid receptors in the sea lamprey gill cytosol. The receptor appears to be highly specific for 11-deoxycortisol. Competitive binding assays showed that only 11-deoxycortisol displaced [^{3}H] 11-deoxycortisol compared to nine other steroids. The glucocorticoid receptor had the highest specific 11-deoxycortisol binding activity in the intestine followed by gill, testis, liver, kidney, heart, and muscle. The specific 11-deoxycortisol-receptor complex binds to DNA-cellulose. These results are consistent with characteristics of glucocorticoid receptors in fish.

INTRODUCTION

The nuclear receptor (NR) superfamily arose very early in the evolutionary history of animals (Laudet 1997; Escriva et al. 1997). Recent phylogenetic studies suggest that NRs evolved sometime between the Metazoan and Bilateria divergence (Thornton 2003a; Bertrand et al. 2004). The superfamily receptor groups have the characteristic of binding to small molecules such as steroids, thyroid hormones, fatty acids, and retinoic acids (Robinson-Rechavi et al. 2003). The NR superfamily consists of ligand-dependant transcription factors that provide the bridge between hydrophobic signaling hormones and target gene expression (Bertrand et al. 2004).

The steroid receptor subgroup was thought to be restricted to vertebrates (Escriva et al. 1997; Baker 1997); however, recent research has shown that steroid receptors similar to vertebrate estrogen receptors exist in mollusks (Thornton et al. 2003b; Keay et al. 2006). While chordate estrogen receptors have ligands that are signaling hormones, protostome estrogen receptors were also found, yet it is unclear if they are functional or not (Keay et al. 2006). It is possible that the mollusks have the estrogen-like receptor but do not have the enzymes capable of producing estrogens.

The sea lamprey *Petromyzon marinus* is an agnathan, one of the oldest extant vertebrates which emerged over 500 million years ago (Kumar and Hedges 1998; Shu et al. 1999). Thornton (2001), using PCR techniques, amplified DNA segments from sea lamprey that were homologues to parts of estrogen, progestin, and corticoid receptor genes in higher vertebrates. Phylogenetic analysis using the DNA sequences showed progestin and corticoid receptors evolved from the estrogen receptor. Recently,

Bridgham et al. (2006) using maximum likelihood phylogeny and existing ligand binding domain sequences, inferred the amino acid sequence of the ligand binding domain of the presumed ancestral corticoid receptor. They also synthesized the sequence of the corticosteroid ligand binding domain for use in a transactivation assay. The assay showed that aldosterone and 11-deoxycorticosterone had the highest activation levels of the luciferase reporter. In addition, Bridgham et al. (2006) provided phylogenetic evidence that the ancestral corticoid receptor was a mineralocorticoid receptor which then gave rise to the glucocoricoid receptor in teleosts and tetrapods.

There have been several efforts to characterize steroid receptors from lamprey. Ho et al. (1987) characterized an estrogen binding moiety in the testes of the sea lamprey. The putative receptor shared similar binding characteristics to higher vertebrate estrogen receptor. It was thought that androgen receptor did not exist in lamprey (Thornton 2001). However, Bryan et al. (2007) provided evidence of an androgen receptor in the sea lamprey testes. The study suggested that androstenedione was the ligand; however, the study was unable to convincingly prove that the moiety was not a binding protein typically found in the gonads. There have been no studies characterizing the corticosteroid receptor in lamprey.

The aim of this study was to determine if the two definitively identified steroids 11- deoxycortisol and 11-deoxycorticosterone in chapter 1 are hormones in lamprey. In order to classify identified steroids as hormones, 1) they must be in circulation in the blood; 2) they must have a receptor, and 3) they must mediate relevant biological effects. This chapter will focus on whether or not lampreys possess a receptor for either of the identified steroids from chapter 1, 11-deoxycortisol or 11-deoxycorticosterone.

MATERIALS AND METHODS

Animals and holding facilities

Sea lamprey *Petromyzon marinus* were collected in streams by the U.S. Fish and Wildlife Service employees, and transported to Michigan State University (East Lansing, MI) or USGS Hammond Bay Biological Station (Millersburg, MI) for tissue collection. Lampreys were held at 10-13 °C in flow through tanks. Tissues collected from fish were frozen in liquid nitrogen and held at -80 °C until processed for cytosolic fractions. All experiments were approved by the Michigan State University Institutional Animal Care and Use Committee (AUF # 05/04-077-00).

Materials

Radiolabled steroids were obtained from American Radiolabled Chemicals (St. Louis, MO). Synthetic steroids were obtained from Steraloids (Newport, RI) and Sigma (St. Louis, MO). All other reagents were obtained from Sigma unless otherwise noted.

Preparation of cytosolic fractions of tissues

The decision to use gill cytosol was based on previous research to characterize glucocorticoid receptors in fish. It is well known that gill tissues mainly have glucocorticoid receptors and the receptors are expressed at higher levels than other tissues

in teleosts. Preparation of cytosolic fractions of tissues was adapted from Patino and Thomas (1990). Frozen tissue was ground in liquid nitrogen with a mortar and pestle. Frozen tissue was mixed 1:5 (weight: volume) in HEPES buffer (25 mM HEPES, 10 mM NaCl, 1 mM monothioglycerol, pH 7.4) and kept on ice while being homogenized. The homogenate was centrifuged at 1000 x g for 15 min at 4 °C. The supernatant containing the cytosolic fraction was collected and the pellet discarded. The supernatant was centrifuged at 40,000 x g for 1 h at 4 °C. The supernatant was removed and glycerol (10% v/v) was added. Cytosolic fractions were immediately used in subsequent assays.

Saturation curve and scatchard analysis

Radiolabled 11-deoxycortisol (0.2-20 nM) in ethanol was added to each assay tube with or without 1 µg cold 11-deoxycortisol (to determine non-specific binding). The ethanol was evaporated under nitrogen at 40 °C. Then, 200 µl of gill cytosol were added to the assay tubes and incubated at 0 °C for 2 h. After incubation, 500 µl of ice cold dextran coated charcoal (DCC) solution were added to assay tubes and incubated on ice for 5 min. The samples were then centrifuged at 1000 x g for 5 min at 4 °C, and the supernatants were poured into scintillation vials. Six ml of scintillation cocktail were added to each vial and disintegrations per minute (DPM) were counted by a scintillation counter. The concentration of binding sites (B_{max}) and the dissociation constant (K_D) were determined by hyperbolic regression using Sigmaplot v9.0 (SYSTAT, San Jose, CA, USA). Protein concentrations were determined by *DC* Protein Assay Kit for microplates (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard.

Association and dissociation kinetics

The rate of association was determined by incubating gill cytosol (200 µl) with 3.3 nM of [^{3}H] 11-deoxycortisol with or without 1µg of 11-deoxycortisol at 0 °C for 0-4 hr. To determine the dissociation rate, gill cytosol (200 µl) was incubated with 3.3 nM of [^{3}H] 11-deoxycortisol in the presence or absence of 1µg of 11-deoxycortisol at 0 °C for 2 hr and then the dissociation was initiated by adding 1 µg of 11-deoxycortisol to all assay tubes for another 0-2 hr at 0 °C. Bound and free steroid were separated by addition of DCC. The tubes were kept on ice for 5 min, and then centrifuged at 1000 x g for 5 min. The supernatants were poured into 8 ml scintillation vials, mixed with 6 ml scintillation fluid and DPM were counted using a scintillation counter.

Steroid and tissue binding specificity

To determine steroid specificity of the binding moiety, cold steroids were used for their ability to compete with [^{3}H] 11-deoxycortisol binding. Gill cytosol preparations (200 µl) were incubated at 0 °C for 2 hr with 3.3 nM [^{3}H] 11-deoxycortisol in the presence of different amounts of cold steroid (0.1-1000 nM). Specificity was examined for 11-deoxycortisol (S), 11-deoxycorticosterone (DOC), cortisol (F), corticosterone (B), aldosterone (Aldo), dexamethasone (D), androstenedione (Ad), 17β-estradiol (E_2), 17α-hydroxyprogesterone (17α-P), and progesterone (P).

Relative binding was measured in the gill, intestine, testis, liver, kidney, heart, and muscle by binding assays. Radiolabled S (5 nM) in ethanol was added to each tube in the presence or absence of 1μg of cold S and dried down. Each tube received an aliquot of gill cytosol (200 μl) and was incubated for 2 hr at 0 °C. The reaction was stopped by the addition of DCC. The tubes were kept on ice for 5 min, and then centrifuged at 1000 x g for 5 min. The supernatants were poured into 8 ml scintillation vials, mixed with 6 ml scintillation fluid and DPM were counted using a scintillation counter.

DNA-cellulose chromatography

DNA-cellulose chromatography procedures were modified from Knoebl et al. (1996). Hepes buffer with 0.2 mg/ml BSA consisted of three different concentrations of NaCl, 0.05 M (column buffer), 0.4 M (elution buffer), and 2.0 M (wash buffer). Gill cytosol (1.0 ml) was incubated for 2 hr at 0 °C with 20 nM [^{3}H] S with or without 2 μg cold S. Samples were placed on a lab table for 30 min at 25 °C and then cooled back down with ice for 5 min. The samples were then diluted in 3 ml of column buffer (total volume 4 ml) and added to a 20-ml column (Bio-Rad, Hercules, CA) containing 5 ml DNA-cellulose (Amersham, Piscataway, NJ) in column buffer. The sample was allowed to flow into the DNA-cellulose, and then the flow was stopped to allow absorption for 20 min. The column was then washed with 20 ml of column buffer to remove free radiolabled steroid. To elute the bound receptor complex from the DNA, 7 ml of 0.4 M

NaCl elution buffer followed by 7 ml of wash buffer (2.0 M NaCl) was used. One ml fractions were collected and DPM were counted using a scintillation counter.

RESULTS

Saturation curve and determination of K_d and B_{max}

Saturation binding assays showed specific and saturable binding moiety for S in the cytosolic fraction of the gill homogenate (Fig. 1A). Hyperbolic regression analysis (Fig. 1B) revealed the cytosol binding moiety had a high affinity (K_d = 2.66 ± 0.47 nM, mean ± SEM, n = 3) and low capacity (B_{max} = 58.10 ± 3.33 fmol/mg protein, n = 3) for S.

Association and dissociation kinetics

Kinetic studies of the S binding moiety showed the association rate ($T_{1/2}$) was 2.11 ± 0.32 min (n = 3) and the specific binding remained constant during the experiment (Fig. 2A). The specific binding was reversible with a dissociation rate ($T_{1/2}$) of 26.44 ± 8.41 min (n = 3) during the 2-hr experiment (Fig. 2B).

Steroid and tissue specificity

S had the highest affinity to the cytosolic binding moiety, among the 9 steroids tested (Fig. 3). The other steroids failed to displace 50% of 3.3 nM of [^{3}H] S up to 1000 nM concentration. At 1000 nM concentration, DOC almost displaced 50 % of [3H] S specific binding. The remaining steroids did not compete for the S binding moiety.

Specific binding of S to the binding moiety was found in all tissue tested (Fig. 4). The highest levels of specific binding were found in cytosolic preparations from gill, intestine, and testis. Relatively low specific binding was found in the liver, kidney, heart, and muscle tissues.

DNA-cellulose chromatography

The [^{3}H] 11-deoxycortisol receptor complex showed specific (B_S) DNA binding in the DNA-cellulose chromatography assays (Fig. 5). The specific and non-specific (B_{NS}) binding were both eluted with 0.40 M NaCl buffer. The 2.0 M NaCl wash buffer did not elute any additional specific or non-specific binding.

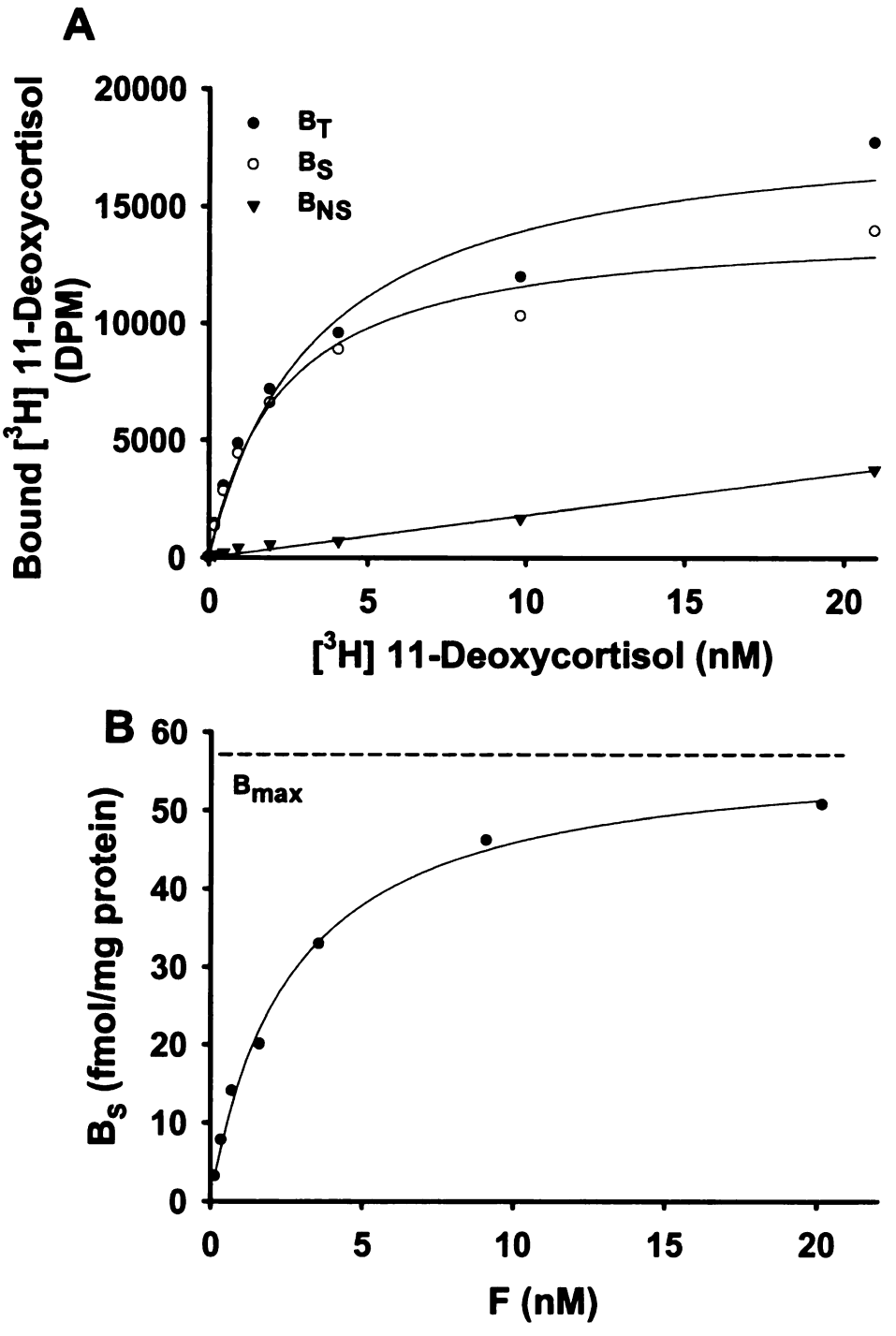

Figure 2-1. Representative saturation curve (**A**) and hyperbolic regression plot (**B**) of 11-dexoycortisol binding to gill cytosol. The abbreviations are; total binding (B_T), specific binding (B_S), non-specific binding (B_{NS}), maximum binding capacity of tissue (B_{max}), free (i.e., unbound) [^{3}H] 11-deoxycortisol (F), and disintegrations per minute (DPM) are shown; n = 3.

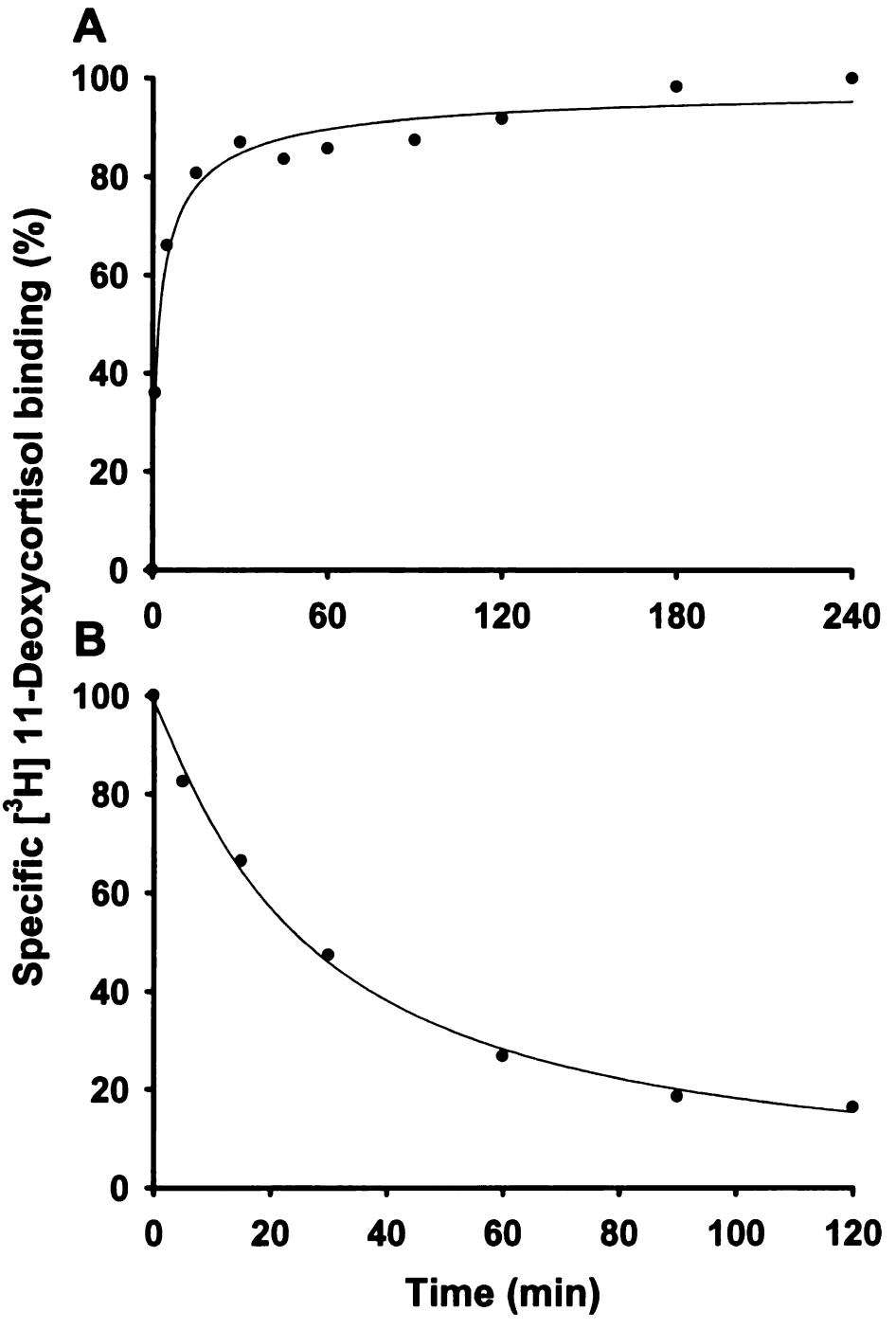

Figure 2-2. Association and dissociation kinetics of a sea lamprey receptor in gill cytosol. For association kinetics (**A**), $T_{1/2}$ was 2.11 ± 0.32 min, n = 3. For dissociation kinetics (**B**), $T_{1/2}$ was 26.44 ± 8.41 min; n = 3.

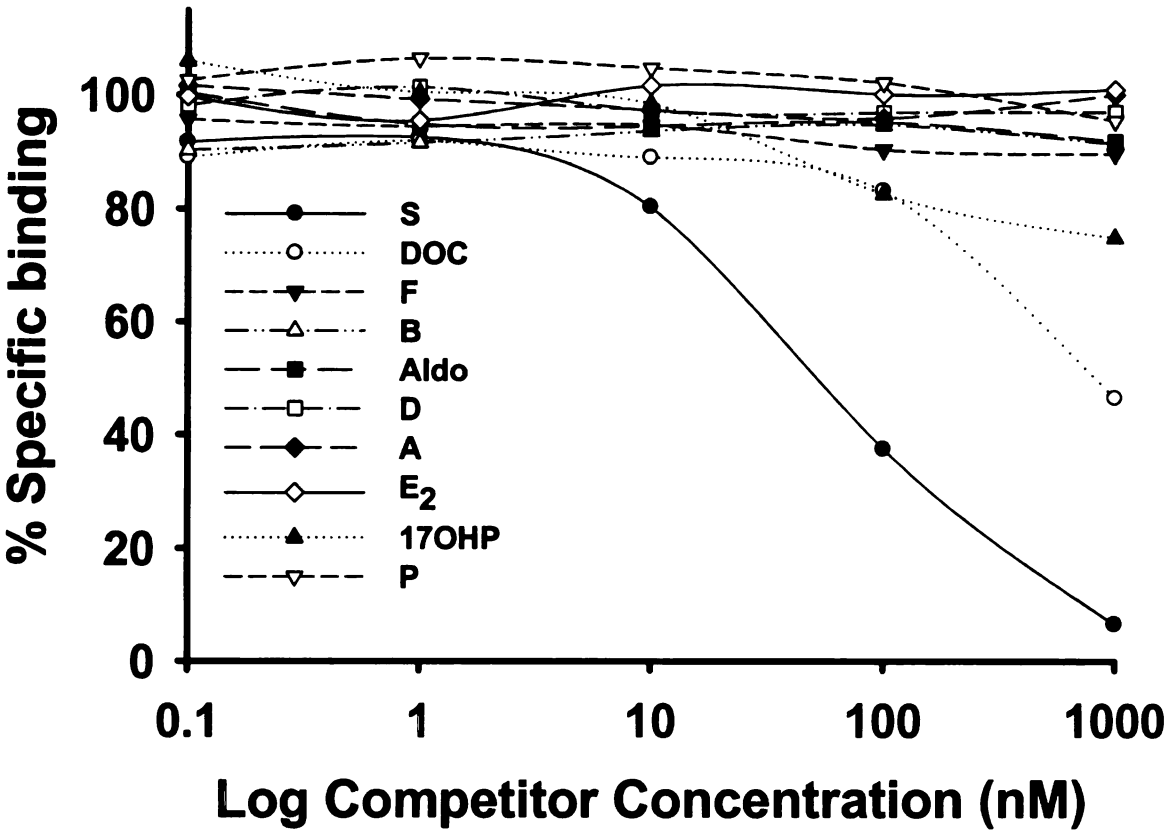

Figure 2-3. Specificity of the lamprey gill cytosolic moiety for various steroids including 11-deoxycortisol (S), 11-deoxycorticosterone (DOC), cortisol (F), corticosterone (B), aldosterone (Aldo), Dexamethasone, (D), androstenedione (A), Estradiol (E2), 17α-hydroxyprogesterone (17OHP), and progesterone (P); n = 3.

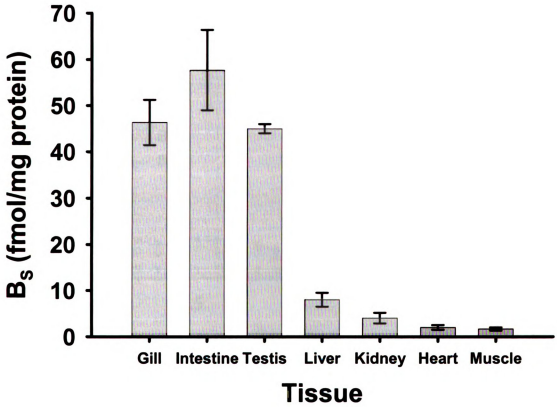

Figure 2-4. Specific binding of [^{3}H] 11-deoxycortisol (5 nM) to cytosolic moiety from different tissues. Abbreviation; specific binding (B_S). Vertical bars represent means ± SEM; n = 3.

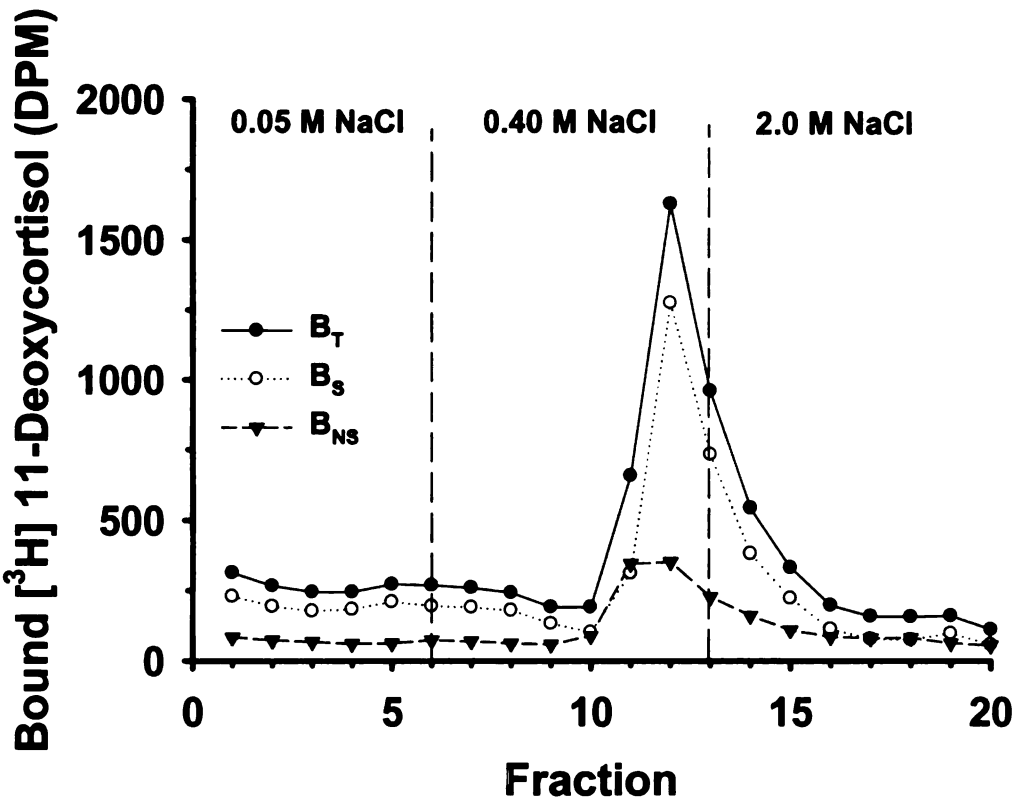

Figure 2-5. Elution of 11-deoxycortisol-receptor complex from DNA-cellulose. Gill cytosol was preincubated with 20 nM [^{3}H] 11-deoxycortisol before binding to the column of DNA-cellulose. Abbreviations are; total binding (B_T), specific binding (B_S), nonspecific binding (B_{NS}), and disintegrations per minute (DPM) are shown; n = 3.

DISCUSSION

This study has provided several lines of evidence to support the hypothesis that 11-deoxycortisol is a stress steroid and has a glucocorticoid receptor in the gill of an ancestral vertebrate, the sea lamprey. Our study demonstrated the presence of a glucocorticoid receptor in the gills of the sea lamprey. The binding moiety was characterized by high affinity, low capacity glucocorticoid binding sites specific to 11-deoxycortisol within gill cytosolic extracts. The dissociation constant (K_d) in our study falls within the range reported for glucocorticoid receptors using [^{3}H] cortisol in the gill, liver, intestine, muscle, leukocytes, erythrocytes and brain of salmonids (Chakraborti et al. 1987; Chakraborti and Weisbart 1987; Pottinger 1990; Maule and Schreck 1991; Knoebl et al. 1996; and Pottinger and Brierley 1997). The concentration of binding sites (B_{max}) in our study was lower than concentrations (224 fmol/mg protein) found in salmonid gill tissue (Chakraborti et al. 1987), but was higher than salmonid brain, intestine, and leukocytes (Chakraborti et al. 1987; Maule and Schreck 1991; Knoebl et al. 1996).

In our study, the glucocorticoid binding moiety shared similar binding characteristics to glucocorticoid receptors in salmonids (Chakraborti and Weisbart 1987; Chakraborti et al. 1987). Although, the association and dissociation rates of the lamprey glucocorticoid receptor were similar to salmonid glucocorticoid receptor, the rates ($T_{1/2}$) were faster. For example, Knoebl et al. (1996) using brain cytosolic extracts estimated

the dissociation rate $T_{1/2}$ = 64.3 min, compared to $T_{1/2}$ = 26.44 min using gill cytosol in our study.

The sea lamprey corticoid binding moiety is highly specific to 11-deoxycortisol. The specificity of the corticosteroid binding moiety in the gill was different than those reported for other glucocorticoid receptors found in fish (Chakraborti et al. 1987; Chakraborti and Weisbart 1987; Pottinger 1990; Maule and Schreck 1991; Knoebl et al. 1996; and Pottinger and Brierley 1997). In this study, the synthetic steroids dexamethasone and triamcinolone acetonide (data not shown), along with natural corticosteroids, were ineffective at displacing 11-deoxycortisol from the binding moiety. Chakraborti et al. (1987) found 11-deoxycortisol had the highest affinity of the natural corticosteroids tested with brook trout (*Salvelinus fontinalis*) gill receptors followed by cortisol and corticosterone.

Recently, Bridgeham et al. (2006) using existing corticoid ligand binding domain sequences, inferred the amino acid sequence of the ligand binding domain of the presumed ancestral corticoid receptor. They synthesized the ligand binding domain of the corticoid receptor in lamprey and developed a transactivation assay. Their assay showed the ligand binding domain to be activated by aldosterone, 11-deoxycorticosterone, 11-deoxycortisol, corticosterone, and cortisol. The current study used native receptor complex and found highly specific binding only for 11-deoxycortisol with limited displacement binding at high concentrations of 11-deoxycorticosterone.

The steroid specificity assay and plasma screening for cortisol and corticosterone (chapter 1) provides strong evidence that the ancestral vertebrate does not produce cortisol or corticosterone and thus lacks the 11 β-hydroxylase enzyme. This could

explain why in our study, the native receptor does not recognize cortisol or corticosterone. The findings using the native receptor contrast the results of Bridgeham et al. (2006). However, they used an inferred amino acid sequence and only included the ligand binding domain of the receptor. This may have changed the protein conformation creating a nonspecific sequence allowing other corticosteroids to bind.

Our study detected an 11-deoxycortisol binding moiety in tissues that have been characterized for glucocorticoid receptor in salmonids (Chakraborti et al. 1987; Chakraborti and Weisbart 1987; Pottinger 1990). In addition, specific 11-deoxycortisol binding moiety was observed in all tissues tested, however, much higher capacity was found in gill, intestine, testis and much lower in liver, kidney, heart and muscle.

Our study has shown that the sea lamprey 11-deoxycortisol receptor complex binds to DNA-cellulose, indicating that it is a nuclear steroid receptor. Similar to earlier glucocorticoid receptor studies, heat activation was important for increasing the levels of specific binding during DNA-cellulose chromatography (Eisen and Glinsmann 1978; Knoebl et al. 1996). This study showed glucocorticoid receptor complex eluting from the DNA-cellulose with 0.4 M NaCl column buffer, which was similar to the findings of Knoebl et al. (1996). No further specific binding or nonspecific binding peaks were found after elution with the 2.0 M NaCl wash buffer, indicating that activated receptor complex binds to DNA-cellulose.

Functional corticosteroid binding globulin (CBG) has not been found in lower vertebrates (Breuner and Orchinik 2002). Breuner and Orchinick (2002) report that a cDNA sequence of a CBG homologue was found in zebrafish, however, the authors doubted that it functioned as a CBG. There is no other evidence supporting CBG in

lower vertebrates. Therefore, it is unlikely that the binding moiety is a CPG in our study. The results demonstrated that the 11-deoxycortisol receptor complex binds to DNA.

Recent studies have focused on steroid receptors and their role in increasing regulatory and developmental complexity in vertebrates (Baker 1997; Baker 2001; Baker 2004; Whitfield et al. 1999). There is evidence that teleostean fish experienced an additional whole-genome duplication event that led to increased speciation and biodiversity (Hoegg et al. 2004; Volff 2005). Thornton (2001) provided evidence that the ancient corticoid receptor gene may have experienced a gene duplication event, which led to the development of glucocorticoid and mineralocorticoid receptors in later vertebrates.

A previous study found 11-deoxycorticosterone circulating in the ancestral vertebrate (chapter 1) and that the steroid had a higher relative affinity to the receptor compared to other corticosteroids tested. Recently, Sturm et al. (2005) using transactivation assays provided evidence that 11-deoxycorticosterone might be the ancestral hormone associated with the mineralocorticoid receptor in rainbow trout *Oncorhynchus mykiss*. Gillmour (2005) suggested that in order to establish that 11-deoxycorticosterone as a mineralocorticoid receptor ligand, it must increase in circulation with a physiological stressor. In addition, it should not only increase, but it must also be used in characterization of the receptor and show biological effects. The recent findings suggest that 11-deoxycorticosterone might be the putative hormone for the ancestral mineralocorticoid receptor which arose after agnathans, but before chondrichthyes diverged (Bury and Sturm 2007).

Our study provided the first characterization of a glucocorticoid receptor in one of the earliest extant vertebrates, the sea lamprey. The results provide evidence that 11-

deoxycortisol is highly specific for the glucocorticoid receptor. The next step in confirming that 11-deoxycortisol is the actual hormone is to provide evidence of biological effects.

ACKNOWLEDGEMENTS

We thank the staff of the U.S. Geological Survey, Hammond Bay Biological Station for supplying sea lampreys. I would like to thank the Bonneville Power Administration for providing funding and support for this research. I would also like to thank the Confederated Tribes of the Umatilla Indian Reservation for support.

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CHAPTER 3

BIOLOGICAL ROLES OF 11-DEOXYCORTISOL IN MEDIATING STRESS RESPONSES OF THE SEA LAMPREY *Petromyzon marinus*

ABSTRACT

This study was conducted to assess whether 11-deoxycortisol serves as a glucocorticoid in the ancient vertebrate, the sea lamprey *Petromyzon marinus*. Earlier research has shown that 11-deoxycortisol circulates in the blood and has a specific receptor in the gills of the sea lamprey. In this study, the hypothesis was that 11-deoxycortisol would exhibit a classical stress response and have biological effects. After acute stress, an increase in 11-deoxycortisol levels occurred by 1 h and remained elevated for at least 8 h, recovering to basal level by 24 h. 11-deoxycortisol implants elevated circulating levels of 11-deoxycortisol and reduced levels of classic androgens and estrogens in the plasma. The implants also increased gill Na^+, K^+-ATPase activity in lamprey. Mammalian corticotropin releasing hormone injections sharply increased plasma levels of 11-deoxycortisol. In addition, lamprey pituitary extract injections increased plasma concentrations of 11-deoxycortisol in a dose response manner. This study provided strong evidence that 11-deoxycortisol is an ancient glucocorticoid in the lamprey.

INTRODUCTION

The stress response is vital to vertebrates and is well conserved among the jawed vertebrates. In tetrapods, an important component of the stress steroid response system is the hypothalamic-pituitary-adrenal (HPA) axis (Chrousos 1998). Teleosts have the same system, except they secrete corticosteroids from interrenal cells located in the anterior portion of the kidneys (Nandi and Bern 1960; Wendelaar Bonga 1997; Barton 2002). The secretion of the corticosteroids is mainly under the control of the neuropeptides corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) (Wendelaar Bonga 1997; Majzoub 2006). The hypothalamus is stimulated to release CRH, which in turn mediates the release of ACTH from the pituitary in jawed vertebrates. ACTH then binds to receptors in the adrenal or interrenal cells to release corticosteroids into circulation. While the physiological stress steroid response is well conserved in vertebrates, the chemical structures of the corticosteroids are different.

There are two types of corticosteroids in vertebrates, the glucorticoids and mineralocorticoids. Glucocorticoids regulate metabolism and are released in response to a stressor (Sapolsky et al. 2000). Cortisol and corticosterone, which differ only by the absence a 17-α hydroxyl, are the two principal glucocorticoids in animals. Mammals secrete cortisol; while rats and mice secrete corticosterone. Reptiles and birds also produce and circulate corticosterone as their primary glucocorticoid (Nelson 2000). Fish,

on the other hand, secrete cortisol as their glucocorticoid (Idler and Truscott 1972; Mommsen et al. 1999; Wendelaar Bonga 1997; Barton 2002), with the exception of elasmobranchs which synthesize 1α-hydroxycorticosterone (Idler and Truscott 1967; Nunez and Trant 1999). It was previously thought that glucocorticoids in fish also functioned as mineralocorticoids (Wendelaar Bonga 1997).

Aldosterone is the principal mineralocorticoid in terrestrial vertebrates (Nelson 2000). Aldosterone is important in regulation of mineral and ion balance in vertebrates (Norman and Litwack 1997). Evidence to support aldosterone circulating in fish seems to be absent (Gilmour 2005; Bury and Sturm 2007). However, Sturm et al. (2005) has shown that 11-deoxycorticosterone is specific for a cloned portion of the mineralocorticoid receptor in rainbow trout. Their study suggests that 11-deoxycorticosterone may be a putative mineralocorticoid in fish (Sturm et al. 2005). Bridgham et al. (2006) found a mineralocorticoid receptor homologue in the skate, *Raja erinacea* but not in lamprey.

It was unclear if one of the earliest vertebrates, the lamprey, contains a stress steroid. The lamprey is an agnathan, one of the oldest extant vertebrates which emerged over 500 million years ago (Kumar and Hedges 1998; Shu et al. 1999). To discover the actual stress hormone would be an important step in understanding the evolution of steroid receptor systems in the vertebrates.

Two putative glucocorticoids were identified, 11-deoxycortisol and 11-deoxycorticosterone, circulating in the lamprey plasma (Chapter 1). Further, the results from chapter 2 have shown that 11-deoxycortisol has a specific glucocorticoid receptor in the gill of lamprey. The last lines of evidence needed to support the claim that 11-

deoxycortisol is an ancestral glucocorticoid was to show classic glucocorticoid response to stress and to show biological effects. The specific questions for this study are: 1) Does 11-deoxycortisol show a classical stress steroid response, sharply rising and returning to basal levels? 2) Will 11-deoxycortisol implants reduce classical androgens and estrogens in circulation and increase gill Na^+, K^+-ATPase activity? 3) Will mammalian CRH increase circulating levels of 11-deoxycortisol? 4) Will lamprey pituitary extract increase circulating levels of 11-deoxycortisol?

MATERIALS AND METHODS

Source of Animals

Sea lamprey, *Petromyzon marinus*, were collected in streams by U.S. Fish and Wildlife Service employees, and transported to Michigan State University (East Lansing, MI) or Hammond Bay Biological Station (Millersburg, MI) for collecting tissues where they were held at 10-13 °C. Tissues collected from fish were frozen in liquid nitrogen and held at -80 °C until processed. All experiments were approved by the Michigan State University Institutional Animal Care and Use Committee (AUF # 05/04-077-00).

Handling and Salinity Stressors

Adult lampreys were acclimated in flow through tanks (254 L) at least 2 weeks before stress tests were conducted. Tanks were isolated to keep people from disturbing the lampreys during acclimation. In the first stress experiment (1-48 h recovery), 140 lampreys were distributed in tanks at a density of 7 lampreys/tank with replicate tanks for each treatment at each time. No lampreys were sampled more than once. In the second stress experiment (1-24 h recovery), 80 parasitic lampreys were distributed in tanks at a density of 5 lampreys/tank with replicate tanks for each treatment at each time. No

lampreys were sampled more than once. Lampreys were netted out of tanks and placed in a dry bucket for 5 min then transferred to 3 % salt water for 10 min. Handling and salinity tests are routine methods to elicit a stress steroid response in teleosts (Wedemeyer et al. 1990). To obtain plasma, lampreys were netted out of tanks and immersed in anesthetic dose of 400 mg/L of tricaine methanesulfonate (MS-222) buffered with sodium bicarbonate. Blood samples were collected at 1, 4, 8, 24, and 48 h after stressors to measure steroid levels. For parasitic lampreys, blood was sampled at 1 and 24 h after stressors. Lampreys were then euthanized with a lethal dose of MS-222. Blood was centrifuged at 1000 x g at 4°C for 15 min and plasma removed. Plasma was stored at -80 °C until analysis.

Radioimmunoassay (RIA) procedure

RIAs were conducted in glass culture tubes (10 mm × 75mm, Fisher Scientific, Pittsburgh, PA). Briefly, the assay buffer consisted of 50 mM sodium phosphate, pH 7.4, 0.2% BSA, 137 mM NaCl, 0.40 mM EDTA, and 0.77 mM sodium azide. Nine standards were made up in duplicate over the range 500-1.95 pg/100 µl/tube. The tubes containing unknowns also had a volume of 100 ul. Binding reagent was made by adding radiolabel and antiserum to 20 ml of assay buffer in amounts such that, when 100 ul was dispensed to all tubes, each tube contained 5000 dpm and in the absence of any standard, 50% of the radiolabel was bound to the antiserum. Blank tubes, to which no antibody was added, and tubes necessary to determine the total and maximum dpm counts, were also included in the assay. All tubes were incubated overnight at 4 °C. After overnight incubation, 500

µl of ice-cold charcoal solution (50 mM sodium phosphate, Ph 7.4, 0.1% gelatin, 1.0% dextran-coated charcoal) was added to each tube. The tubes were kept on ice for 20 min, and then centrifuged at 1000 x g for 12 min. The supernatants were poured into 8 ml scintillation vials, mixed with 6 ml scintillation fluid and DPM were counted using a scintillation counter.

Steroid implants

11-Deoxycortisol (Sigma-Aldrich, St. Louis, MO) time release pellets were made by Innovative Research of America (Sarasota, Fl). The 21-day slow release steroid implants (5 mg/pellet) were injected between the muscle and the skin near the front dorsal fin of the sea lamprey. A total of 48 lampreys were distributed in flow through tanks (254 L) at a density of 6 lampreys/tank with replicate tanks for each treatment. On the 21st day, blood samples and gill tissues were collected. Plasma was analyzed by RIAs for 11-deoxycortisol (S), 11-deoxycorticosterone (DOC), dehydroepiandrosterone-sulfate (DHEA-S), dehydroepiandrosterone (DHEA), androstenedione (AD), testosterone (T), estrone (E1), and estradiol (E2).

Gill Na^+, K^+-ATPase Activity

A gill pouch was removed and placed in ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen immediately at -80 °C. Na^+, K^+-ATPase activity was determined with a kinetic assay run in 96-well microplates at 25 °C

and read at a wavelength of 340 nm for 10 min as described in McCormick (1993). Gill tissue was homogenized in 500 ul SEID (SEI buffer and 0.1% deoxycholic acid) and centrifuged at 5000 x g for 30 s. Ten µl samples were run in two sets of duplicates; one set containing the assay mixture and the other assay mixture and 0.5 mM ouabain. The resulting ouabain-sensitive ATPase activity is expressed as µmoles ADP/mg protein/h. Protein concentrations were determined using BCA (bicinchoninic acid) Protein Assay (Pierce, Rockford, Il). Both assays were run on a THERMOmax microplate reader using SOFTmax software (Molecular Devices, Menlo Park, CA).

Corticotropin Releasing Hormone Injections

Mammalian corticotropin releasing hormone (CRH) (Sigma-Aldrich) was dissolved in 0.9% saline and injected intraperitoneally with a dose of 100 µg/Kg. Saline solution was used as a control. Blood samples were collected 1 hr after injections by cardiac puncture using vacutainers (Becton Dickinson, Franklin Lakes, NJ,). Blood samples were centrifuged at 1000 x g for 15 min; plasma was collected and stored at -80 °C until analyzed by RIA for S.

Pituitary extract injections

To obtain pituitary extract, sea lamprey pituitary glands from 400 adults were collected in June 2000, at Hammond Bay Biological Station (Millersburg, MI, USA). The frozen pituitary glands were homogenized in 20 ml of 20-mM Tris buffer, pH 7,

containing protease inhibitor cocktails (Roche, Nutley, NJ, USA). This mixture was centrifuged at 1000g for 20 min allowing recovery of the supernatant. The protein concentration was determined using DCA protein analysis kit (Pierce, Rockford, IL). The protein concentration for the extract was 6.7 mg/ml. One milliliter of the extract was equivalent to 20 lamprey pituitary glands. Lampreys were given a single intraperitoneal injection of equivalent to 1, 5, or 10 pituitaries or a 0.9% saline as a control (4 treatments total, 10 lampreys per treatment). Blood was sampled at 0, 6, 12, 24, and 48 h after the injection. Blood samples were centrifuged at 1000 x g for 15 min; plasma was collected and stored at -80 °C until analyzed by RIA for S.

Statistical analysis

Statistical analysis of the acute stress experiments was done using Analysis of Variance (ANOVA) in which time was an independent variable. Comparisons among time intervals were compared using Bonferroni's multiple comparison tests. Analysis of the implant experiments was done using an Unpaired t test with Welch's correction. Males and females were analyzed separately. The corticotropin releasing hormone injection experiment was also analyzed by an unpaired t test. Analysis of the pituitary extract experiment was done with repeated measures two-way ANOVA in which pituitary equivalent dosage and time were factors.

RESULTS

Acute Stress Response

Spawning phase adult lampreys subjected to a 5-min dewatering and 3% salt water bath (10 min) exhibited a sharp increase in S levels within 1 hr (Fig 1A). Plasma S levels in control lampreys were 1.09 ± 0.06 ng ml^{-1}, while stressed lampreys elevated levels to 2.38 ± 0.14 pg ml^{-1} ($P < 0.001$) at 1 h. Concentrations of plasma S remained elevated at least for 8 h, returning to control levels by 24 h.

Parasitic male and female lampreys subjected to a 5-min dewatering and 3% salt water bath (10 min) exhibited a sharp increase in S levels within 1 hr (Fig. 1B). Plasma S levels in stressed male lampreys was 1.08 ± 0.09 ng ml^{-1}, compared to control levels 0.45 ± 0.04 ng ml^{-1} ($P < 0.001$) at 1 h. Plasma S levels in stressed female lampreys was 1.46 ± 0.19 ng ml^{-1}, compared to the control levels 0.39 ± 0.01 ng ml^{-1} ($P < 0.001$) at 1 h. Plasma levels of S in male and female parasitic phase lampreys returned to control levels by 24 h. We found basal levels of S nearly doubled from the parasitic stage to the spawning phase in lampreys.

Effects of steroid implants

The S implants caused an increase in plasma levels of S and DOC in male and female lampreys (Fig. 2). S implanted male lampreys had a mean S level of 18.9 ± 2.0 ng ml^{-1}, compared to 1.1 ± .06 ng ml^{-1} ($P < 0.0001$) in control implanted lampreys (Fig. 2A).

Implanted female lampreys had a mean S level of 20.5 ± 3.0 ng ml^{-1}, compared to 1.2 ± 0.09 ng ml^{-1} ($P < 0.0001$) in control lampreys. The S implants also increased circulating DOC in males from 0.84 ± 0.02 ng ml^{-1} to 2.55 ± 0.14 ng ml^{-1} ($P < 0.0001$) (Fig. 2B). The S implants increased circulating DOC levels from 0.54 ± 0.02 ng ml^{-1} to 1.95 ± 0.17 ng ml^{-1} ($P < 0.0001$) in female lampreys.

The S implants caused a decrease in plasma levels of DHEA-S and DHEA in male and female lampreys (Fig. 3). Implanted male and female lampreys decreased mean plasma DHEA-S levels from 2.66 ± 0.15 ng ml^{-1} and 1.61 ± 0.07 ng ml^{-1} to 2.16 ± 0.11 ng ml^{-1} and 1.30 ± 0.07 ng ml^{-1} ($P = 0.01$) respectively (Fig. 3A). Implanted male and female lampreys decreased mean plasma DHEA levels from 0.32 ± 0.01 ng ml^{-1} and 0.28 ± 0.008 ng ml^{-1} to 0.29 ± 0.008 ng ml^{-1} ($P = 0.04$) and 0.21 ± 0.02 ng ml^{-1} ($P = 0.02$) respectively (Fig. 3B).

The mean plasma levels of AD in male and female lampreys implanted with S were not significantly different (Fig. 4A). However, plasma levels of T decreased in male and female lampreys implanted with S (Fig. 4B). Implanted male and female T levels decreased from 0.31 ± 0.005 ng ml^{-1} and 0.23 ± 0.01 ng ml^{-1} to 0.27 ± 0.01 ng ml^{-1} ($P = 0.002$) and 0.17 ± 0.01 ng ml^{-1} ($P = 0.005$) respectively.

Implants caused a decrease in mean plasma level of E_1 in male lampreys, but not in female lampreys (Fig. 5A). Mean plasma E_1 levels in males implanted with S was 1.76 ± 0.15 ng ml^{-1}, while plasma levels in control lampreys were 2.46 ± 0.18 ng ml^{-1} ($P = 0.009$). Implants decreased plasma levels of E_2 in male and female lampreys (Fig. 5B). The mean plasma level of E_2 in control males and females was 3.50 ± 0.28 ng ml^{-1} and

2.03 ± 0.13 ng ml^{-1}, with levels decreasing to 2.44 ± 0.21 ng ml^{-1} (P = 0.008) and 1.12 ± 0.12 ng ml^{-1} (P = 0.0001) respectively.

Na^+, K^+-ATPase Activity

The S implants nearly doubled gill Na^+, K^+-ATPase activity in both male and female lamprey after 21 days (Fig. 6). The gill Na^+, K^+-ATPase activity was 2.9 ± 0.23 μmol ADP/mg protein/h and 2.8 ± 0.22 μmol ADP/mg protein/h, in control male and female lamprey, with levels increasing to 5.7 ± 0.53 μmol ADP/mg protein/h (P < 0.0001) and 4.1 ± 0.30 μmol ADP/mg protein/h (P = 0.002).

Corticotropin Releasing Hormone Injections

Mammalian CRH injections markedly increased plasma S levels in male and female lampreys 1 hr after injection (Fig. 7). Mean plasma S level in saline injected male lampreys was 0.99 ± 0.13 ng ml^{-1} and increased to 2.08 ± 0.39 ng ml^{-1} (P = 0.02). Mean plasma S level in saline injected female lampreys was 0.72 ± 0.12 ng ml^{-1} and increased to 2.84 ± 0.28 ng ml^{-1} (P = 0.001).

Pituitary Extract Injections

Pituitary extract injections increased plasma S levels in a dose dependent manner (Fig. 8). The 1, 5, and 10 pituitary equivalent treatments significantly increased plasma S

levels to 2.11 ± 0.51 ($P = 0.002$), 3.16 ± 0.55 ng ml^{-1} ($P < 0.0001$), and 3.31 ± 0.52 ng ml^{-1} ($P < 0.0001$) from a control level of 1.35 ± 0.40 ng ml^{-1} at 6 h. By 12 h, all treatment groups except for the 10 pituitary equivalent group returned to control levels. At 48 h, the 1 pituitary equivalent group was slightly reduced compared to control levels.

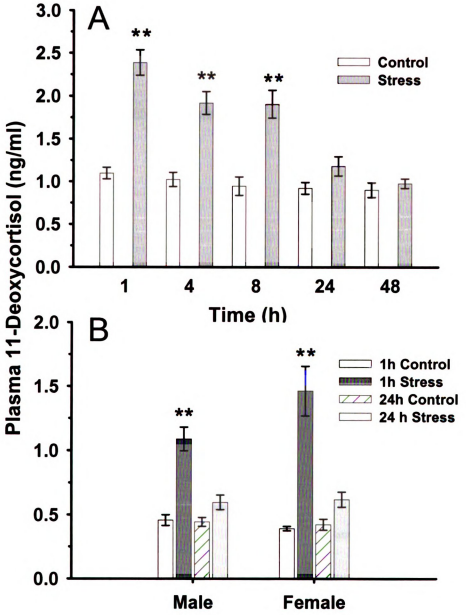

Figure 3-1. Effect of handling and salt challenge stressors on plasma levels of 11-deoxycortisol for spawning (**A**) and parasitic (**B**) phase lamprey during recovery. Vertical bars represent means ± SEM of 6-14 observations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

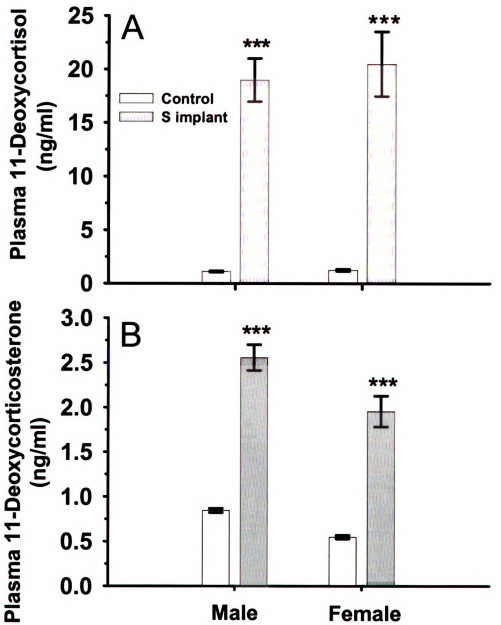

Figure 3-2. Effect of 21 d 11-deoxycortisol implant treatment on plasma levels of 11-deoxycortisol (**A**) and 11-deoxycorticosterone (**B**). Vertical bars represent means ± SEM of 11-12 observations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

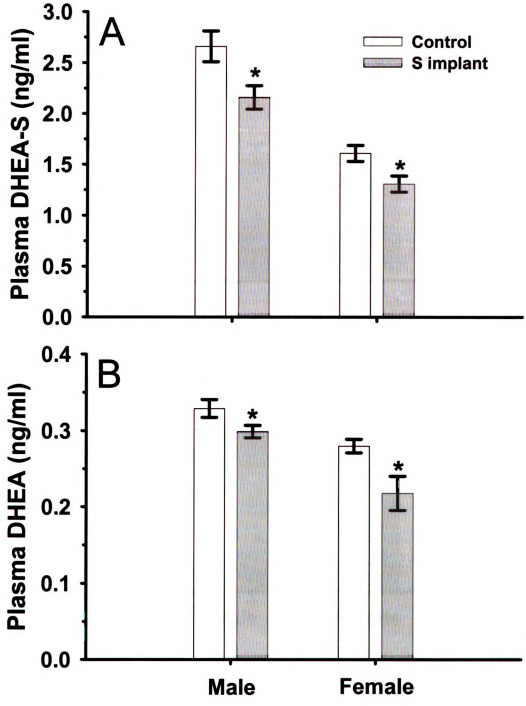

Figure 3-3. Effect of 21 d 11-deoxycortisol implant treatment on plasma levels of Dehydroepiandrosterone-sulfate, DHEA-S (**A**) and Dehydroepiandrosterone, DHEA (**B**). Vertical bars represent means ± SEM of 11-12 observations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

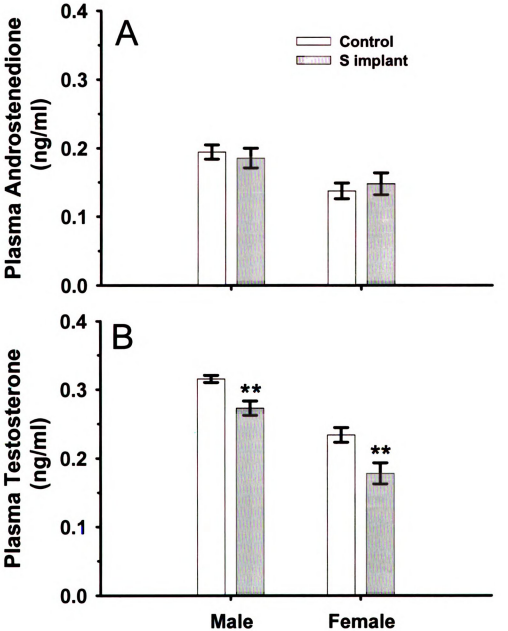

Figure 3-4. Effect of 21 d 11-deoxycortisol implant treatment on plasma levels of androstenedione (**A**) and testosterone (**B**). Vertical bars represent means ± SEM of 11-12 observations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

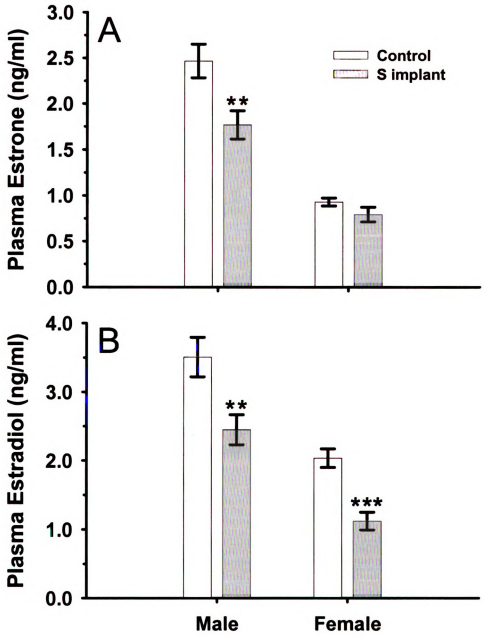

Figure 3-5. Effect of 21 d 11-deoxycortisol implant treatment on plasma levels of estrone (**A**) and estradiol (**B**). Vertical bars represent means ± SEM of 11-12 observations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

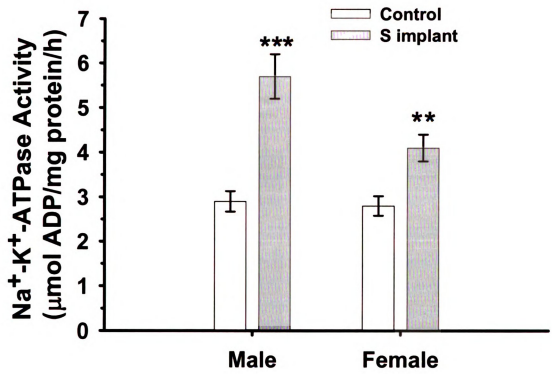

Figure 3-6. Effect of 21 d 11-deoxycortisol implant treatment on gill Na^+, K^+-ATPase activity. Vertical bars represent means ± SEM of 11-12 observations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

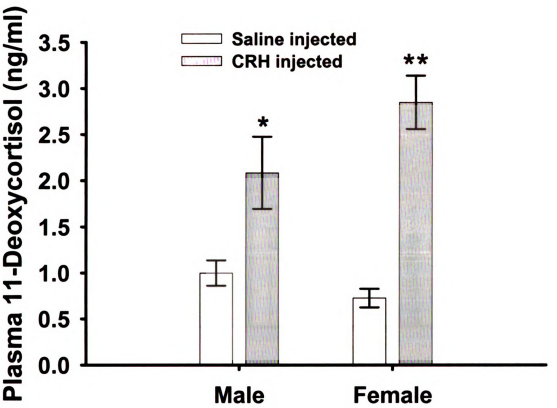

Figure 3-7. Changes in concentration of plasma 11-deoxycortisol following injections of mammalian CRH. Vertical bars represent means ± SEM of 3-5 observations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

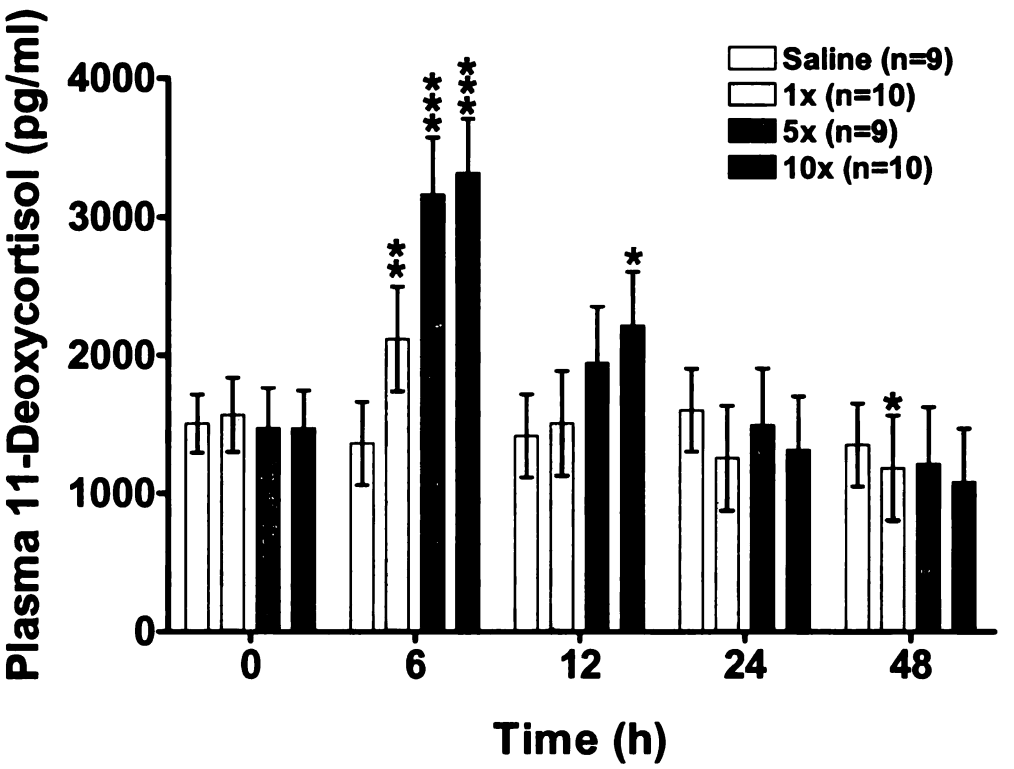

Figure 3-8. Changes in concentration of plasma 11-deoxycortisol following injections of pituitary extract. Vertical bars represent means ± SEM of 9-10 observations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

DISCUSSION

Our study has provided several lines of evidence to support the hypothesis that S is an ancestral glucocorticoid hormone. The results demonstrated that circulating S responds to acute stress in the ancient vertebrate, the sea lamprey. In lamprey, S exhibits similar characteristics to the classical cortisol responses in teleosts. In this study, S levels increased significantly within 1 hour of an acute stressor, remained elevated for at least 8 h, and returned to basal levels by 24 h. In most teleosts, cortisol levels generally increase rapidly, peaking within 1-2 h after exposure to an acute stressor, remain elevated, followed by recovery (Pickering et al. 1987; Barton 2002; Milston et al. 2006). Plasma S levels doubled after acute stress in lamprey, however, the levels were found to be lower than cortisol levels measured in most fishes (Barton and Iwama 1991; Barton 2002). This study showed that S response to acute stress in lamprey was most similar to cortisol levels in stressed Pallid sturgeon *Scaphirhynchus albus* (Barton 2002). Parasitic phase lamprey had lower basal plasma S levels compared to spawning phase lamprey. The pattern of increasing cortisol levels during spawning phase salmonids is well documented (Carruth et al. 2000).

Our study demonstrated that S has classic glucocorticoid effects such as decreasing androgens, estrogens, and increasing gill Na^+, K^+-ATPase activity in the ancient vertebrate. This study found that S implants decreased classical androgens in the plasma of lamprey. The S implants also significantly reduced plasma levels of androgens DHEA-S, DHEA and T. The synthetic glucocorticoid dexamethasone has been shown to decrease DHEA-S, DHEA, AD, and T in humans (Abraham 1974; Kalimi et al. 1994).

Acute and chronic stressors have been shown to elevate plasma glucocorticoid, while decreasing T in fish (Pickering et al. 1987; Pankhurst and Dedual 1994), amphibians (Moore and Zoeller 1985), and reptiles (Lance and Elsey 1986). Cortisol implants were also shown to be effective at reducing plasma levels of T in fish (Carragher et al. 1989; Foo and Lam 1993A,B). Interestingly, S implants did not reduce circulating AD in lamprey, even though Bryan et al. (2007) provided evidence that AD was an androgen in the sea lamprey. One possibility is that AD is not the principal androgen and that the ancestral vertebrate utilizes the 5-ene pathway to T. It is well known that the biosynthetic pathway can go from DHEA to androstenediol then to T (Grower 1995).

Our study demonstrated that plasma levels of E_2 in both sexes and E_1 in males significantly decrease after S implants in lamprey. Similar to the current findings, acute stress and cortisol implants were shown to decrease plasma levels of E_2 in teleosts (Pankhurst and Dedual 1994; Foo and Lam 1993A). E_2 has been well studied in lamprey and established as a sex hormone in lamprey. Ho et al. (1987) provided evidence for an E_2 receptor in the testis of the sea lamprey. Plasma levels of E_2 were shown to increase during the reproductive stage in lamprey (Bolduc and Sower 1992) and increased with injections of lamprey GnRH I, and III (Deragon and Sower 1994; Gazourian et al. 1997). In addition, E_2 was shown to stimulate vitellogenesis in female and male lampreys (Mewes et al. 2002).

The S implants increased gill Na^+, K^+-ATPase activity in adult lamprey. Our results show that S implants significantly increased ion regulating proteins in the gills of male and female lampreys. In juvenile salmonids, cortisol and growth hormone have been shown to increase gill Na^+, K^+-ATPase activity (McCormick 1996). Cortisol

administration by itself has also been shown to increase gill Na^+, K^+-ATPase activity (Madsen 1990; McCormick et al. 1991; McCormick 1996; Seidelin et al. 1999; Quinn et al. 2003) and intestinal Na^+, K^+-ATPase activity (Veillette and Young 2005) in juvenile salmonids.

Our results have demonstrated that S is regulated by the hypothalamus-pituitary axis in the ancient vertebrate. The mammalian CRH injections increased plasma levels of S in male and female sea lampreys. CRH elicits a pronounced increase in plasma S compared to the saline injected lampreys. In teleosts, the regulation of cortisol release is under the hypothalamus-pituitary-interrenal axis (Mommsen et al. 1999). CRH neuropeptide controls the release of ACTH from the anterior pituitary which circulates in the blood and binds to receptors in the interrenal cells, which in turn, stimulates release of cortisol (Wendelaar Bonga 1997). In goldfish, injections of CRH, urotensin I, and sauvagine elicited significant increases in plasma cortisol and stimulated release of ACTH from interrenals (Fryer et al. 1983). Pepels et al. (2004) showed significant increases of plasma levels of CRH and cortisol by 5 min in tilapia. The CRH peptide sequences in the vertebrates are highly conserved (Lovejoy and Balment 1999). Lovejoy and Jahan (2006) suggest the CRH system based on CRH homologues found in insects and chordates may have evolved in a metazoan ancestor.

Pituitary extract injections increased plasma levels of S in a dose-response manner. The increases in plasma S from control levels were substantially elevated at 6 h for all treatment groups. All treatment groups recovered to control levels by 24 h. ACTH secreted from the pituitary is the principal hormone that controls cortisol secretion in teleosts (Wendelaar Bonga 1997). Pickering et al. (1987) have shown increased levels

of plasma ACTH in brown trout after acute stress. In lamprey, ACTH has been cloned and sequenced from the pituitary (Heinig et al. 1995; Takahashi et al. 1995A; Takahashi et al. 1995B). The lamprey ACTH is 60 amino acids long, roughly 20 amino acids longer than other vertebrate ACTHs (Takahashi et al. 2006). However, plasma ACTH levels have not been shown to increase in lamprey and direct stimulation of 11-deoxycortisol by lamprey ACTH has not been shown.

In teleosts, there is evidence of other pituitary hormones producing corticotropic activity (Wendelaar Bonga 1997; Mommen et al. 1999). Schreck et al. (1989) demonstrated that gonadotropin hormone stimulated higher levels of cortisol release than ACTH in coho salmon *Oncorhynchus kisutch* interrenals. Recently, Sower et al. (2006) cloned and sequenced a gonadotropin like protein from the pituitary of sea lamprey. Therefore the stimulatory effect from the pituitary injection might not be lamprey ACTH, but instead another factor or peptide hormone causing the increase in plasma S levels.

In conclusion, this study has demonstrated that S functions as a glucocorticoid and has biological effects in the ancestral vertebrate, the lamprey. The results have shown that S, like cortisol in higher vertebrates, exhibits a classical stress steroid response in lamprey. In addition, the glucocorticoid hormone S reduced circulating levels of classic androgens and estrogens in the plasma of lamprey. The glucocorticoid hormone S, like cortisol in teleosts, up regulates ion regulating proteins in the gills of sea lamprey. Lastly, we provide evidence that the lamprey glucocorticoid hormone S is controlled by the hypothalamus-pituitary axis in these ancient vertebrates.

ACKNOWLEDGEMENTS

We thank the staff of the U.S. Geological Survey, Hammond Bay Biological Station for supplying sea lampreys. I would like to thank the Bonneville Power Administration for providing funding and support for this research. I would also like to thank the Confederated Tribes of the Umatilla Indian Reservation for support.

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SUMMARY OF DISSERTATION

At the beginning of this research it was unknown if the ancient vertebrate, the sea lamprey, had a stress steroid hormone. However, there were some indications that it might exist. Research in the 1990s showed that lamprey ACTH might be an active hormone in circulation by cloning and sequencing an ACTH homologue. Recent evidence confirmed that lamprey ACTH 1-60 is produced in the pituitary. However, evidence is lacking that it increases in circulation or that is stimulates the release of a stress steroid. Another piece of evidence supporting the notion that lamprey might have a stress steroid was cloning of a corticoid receptor homologue.

Recent research has shown that lamprey possess a corticoid receptor homologue. Researcher's using PCR techniques amplified DNA segments from sea lamprey, which were homologues to parts of the corticoid receptor genes in higher vertebrates. However, the receptor had not been proven to be functional or characterized in the lamprey.

In chapter 1, the first prediction was that lampreys have a stress steroid circulating in the plasma. In the laboratory, various chromatography and mass spectrometry methods were used to address this hypothesis. A large a quantity of blood was extracted to increase the abundance of steroids for identifications. Radioimmunoassays were used to screen the plasma for cortisol or corticosterone like steroids. These two steroids were chosen due to their prevalence as stress steroids (glucocorticoids) in almost all vertebrates. Once immunoreactive peaks were identified, a large quantity of the steroid was purified for definitive identification. LH-20 chromatography and HPLC were used in conjunction with RIA to clean up the extract matrix before mass spectrometry. These

steps enabled the definitive identification of two possible glucocorticoids in the sea lamprey.

In chapter 2, the second prediction was that one of the two definitively identified steroids from chapter 1 have a receptor. An important criterion to classify a hormone is that it must have a cognate receptor. Both radioligands were used for testing of tissue cytosol. 11-deoxycortisol, but not 11-deoxycorticosterone, bound to gill cytosol containing glucocorticoid receptor. Our study showed that the receptor in lamprey gill tissue had similar characteristics to glucocorticoid receptors in fish. The receptor was found by competitive assay to be highly specific for 11-deoxycortisol. However, 11-deoxycorticosterone was the only steroid that nearly displaced 50 percent of 11-deoxycortisol but only at 1000 nM. Finally, this study showed that 11-deoxycortisol-receptor complex bound to DNA-cellulose, an important step in characterization of a receptor.

In chapter 3, the last prediction was that 11-deoxycortisol will have classic glucocorticoid stress response and show biological effects. This study demonstrated that adult lampreys exposed to acute stress responded with a classic glucocorticoid response. Steroid levels were elevated and remained elevated for hours, but returned to basal levels by 24 h. The 11-deoxycortisol implants decreased circulating androgens and estrogens in the sea lamprey, a typical glucocorticoid effect. In addition, the implants stimulated gill Na^+, K^+-ATPase activity which is mainly controlled by cortisol (glucocorticoid) in teleosts. Evidence was shown that the hypothalamus-pituitary axis controls 11-deoxycortisol just like cortisol in higher vertebrates. Mammalian CRH injections sharply

increase plasma 11-deoxycortisol. In addition, pituitary extract injections also increased plasma 11-deoxycortisol in a dose-response manner.

The results from our research confirm that one of the earliest vertebrates, the sea lamprey, contains a stress steroid hormone (glucocorticoid). These findings are an important step in understanding the evolution of steroid biosynthesis in the vertebrate lineges. It is clear that 11-β hydroxylase is absent in the early vertebrates, which is an important enzyme to in the biosynthetic pathway to make cortisol and corticosterone which are glucocorticoids in higher vertebrates. The 11 position hydroxyl is converted to a ketone which inactivates the steroid. Thus cortisol is converted to cortisone and corticosterone is converted to 11-dehydrocorticosterone. An important question is; how do the ancient vertebrates control excess stress steroid? Now research can proceed to better understand the roles of the glucorticoid in the early vertebrates. In addition, these results may have an impact on the management activity to control or conserve lamprey.

In the Great Lakes, management agencies have been trying to control sea lamprey for many years. On the Pacific coast, fisheries management agencies are concerned about negative impacts to Pacific lampreys. Both groups now have a tool to measure or monitor the stress steroid in lampreys, an important indicator of stress.