Role of Peptides and Proteins in Control of Reproduction

Editors
Samuel M. McCann
Dharam S. Dhindsa

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Editors:

Samuel M. McCann, M.D.

Professor and Chairman
Department of Physiology, The University of Texas
Health Sciences Center at Dallas, Dallas, Texas

Dharam S. Dhindsa, D.V.M., Ph.D.

Executive Secretary
Reproductive Biology Study Section, Division of Research Grants
National Institutes of Health, Bethesda, Maryland

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Foreword

Recently rapid progress has been made in the understanding of the roles of peptides and proteins in the control of reproduction and it has been apparent that several of them have potential for fertility control. A workshop was convened in conjunction with the February 1982 Reproductive Biology Study Section Meeting at Bethesda, Maryland, to explore these advances. The purpose of the workshop was two-fold. First, it would serve as an important educational vehicle for Reproductive Biology Study Section members not working directly in this field. Second, it was to assess potential application in fertility control. Seventeen speakers provided extremely high quality papers which were followed by extensive discussions. Approximately 250 scientists including Reproductive Biology Study Section Members attended this workshop.

The workshop began with a discussion of the role of brain peptides in control of reproduction. The control of LHRH release was thoroughly discussed. It is apparent that peptides, as well as monoamines, are important in this control mechanism. Although LHRH will release FSH as well as LH, considerable evidence suggests the existence of a specific FSH-releasing

factor. Resolution of this problem will await the isolation of the putative releasing hormone. A large number of brain peptides alter release of LHRH and the endogenous opioid peptides appear to have a physiologically important restraining influence on LHRH release. The brain noradrenergic system, which is tonically inhibited by the central opioid peptides, appears to be involved. Attention was focused on LHRH receptors in the pituitary and how they may be linked with membrane events which increase the availability of intracellular calcium to promote release of the gonadotropins.

The regulation of LHRH release in the primate was reviewed. It appears that the system in the monkey may be simpler than that in lower forms and may involve the constant pulsatile release of LHRH from neurons whose cell bodies are located in the arcuate nucleus. Release of FSH and LH and estrogen production by the ovary may be activated by this mechanism. The elevated blood levels of estradiol stimulate the ovulatory surge of gonadotropins via a direct action of estrogen on the pituitary to sensitize the gland to this constant pulsatile LHRH release. This concept has practical implications since several groups have reported the successful induction of ovulation using pulsatile LHRH delivered to patients either by minipumps attached to their arms or by nasal spray of LHRH at frequent intervals.

The current status of the many agonist and inhibitory analogs of LHRH was reviewed. Previous studies have demonstrated that the agonist analogs may be useful as antifertility agents in view of the paradoxical inhibitory action of high dose, continuous LHRH therapy. This may be applied to treat patients with prostatic cancer where it is associated with a reduction of testosterone levels.

Progress in purification of a pineal peptide that inhibits gonadotropin and prolactin release via a CNS action was discussed, and the pituitary hormones involved in reproduction were considered. The report that a receptor for HCG is present in Pseudomonous bacteria, which may be capable of isolation and structural elucidation, should stimulate increased progress in this area. The mechanisms of action of the gonadotropins on the testis were elucidated.

The recent use of dopamine agonist compounds, such as alpha bromoergocryptine, to lower prolactin in hyperprolactinemia states was reviewed. This treatment is in wide use in Europe and appears to be extremely effective. The hyperprolactinemia appears to interfere with LHRH release from the hypothalamus which results in abnormal gonadotropin secretion.

There is a widespread interest in inhibin and the current status of research in this area was thoroughly examined. A review of the evidence was

given for separate control of FSH distinct from the control of LH, which could be explained by interactions involving LHRH, gonadal steroids, and inhibin. Recent studies on the mechanism of action and purification of inhibin were reviewed. Progress on the purification of a large molecular weight form of the protein has been made. Advances were reported in the purification of an inhibin molecule of approximately 67,000 Dalton's and purified material has been used to develop a radioimmunoassay for inhibin.

The isolation of a peptide called "gonadostatin" which inhibits the LHRH-stimulated release of both FSH and LH from monolayer-cultured pituitary cells was described. It was concluded that this is a peptide of 109 amino acids with several disulfide bridges in the molecule which is active in microgram doses per ml to inhibit the response to LHRH in vitro. Because of the questionable relationship of gonadostatin to the previously reported inhibins, purified on the basis of other bioassays, it was chosen to name this peptide "gonadostatin".

The final session of the meeting was devoted to a discussion of placental peptides and proteins. One of the interesting findings reported was that the control of human chorionic somatomammotropin was completely different from that of other pituitary-like hormones in that the release in vitro was enhanced by low calcium media. The releasing mechanism may involve the participation of arachidonic acid-related pathways and phospholipase A. This different behavior of the placental hormone may be related to its presence free in the cytoplasm rather than storage in granules as are pituitary hormones.

The elegant studies on modification of the HCG molecule which would eventually lead to the development of peptides which could have contraceptive applications were described in detail. The extensive evidence for the existence of an LHRH-like peptide in the placenta which may be important in stimulating HCG production and release by the placenta was also reviewed.

In summary, the papers presented a comprehensive review of the current status of peptides and proteins in reproduction and considerable insight into their potential for fertility control.

The program was organized by a Committee consisting of Drs. Dharam S. Dhindsa, Samuel M. McCann, Bryant Benson, Wesley C. Hymer, and John A. Resko, with the assistance and advice of the Reproductive Biology Study Section members. The members of the Committee chaired the various scientific sessions. The Organizing Committee thanks the Reproductive Biology Study Section and the Division of Research Grants of the National Institutes of

Health for sponsoring the workshop. Our special thanks are to Drs. Benson, Hymer, and Resko for their assistance in reviewing the manuscripts. Our sincere thanks are extended to Miss Consuelo B. Chavez and Mrs. Anne L. Holcombe for final typing of most of the manuscripts and for editorial assistance.

Samuel M. McCann, M.D.
Dharam S. Dhindsa, D.V.M., Ph.D.

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PART I
HYPOTHALAMIC AND OTHER BRAIN PEPTIDES

McCann and Dhindsa, editors
Role of Peptides and Proteins in Control of Reproduction

PRESENT STATUS OF LHRH: ITS PHYSIOLOGY AND PHARMACOLOGY

S.M. MCCANN
Department of Physiology, University of Texas Health Science Center at Dallas, 5323 Harry Hines Boulevard, Dallas, Texas 75235 USA

INTRODUCTION

When we became convinced that there was a corticotrophin-releasing factor in addition to vasopressin resident in hypothalamic extracts, we attempted to use the newly described ovarian ascorbic acid depletion (OOAD) assay of Parlow, a very sensitive assay for LH, to search for LH-releasing activity in hypothalamic extracts. Initial experiments revealed that acid extracts of stalk median eminence of rats effectively depleted ovarian ascorbic acid. Cerebral cortical extracts were without effect and there was also no effect of a variety of other biologically active substances, such as norepinephrine, histamine and serotonin.[1] There was a very slight action of vasopressin in this system which was later shown to be due to a direct effect on the ovary. Even at this early time, it was shown that there was small but detectable LH-releasing activity in the overlying basal hypothalamus and in the supra-chiasmatic region.[2]

The principle problem was that the ovarian ascorbic acid levels fell dramatically following hypophysectomy. This was accompanied by loss of sensitivity to the ovarian ascorbic acid-depleting action of LH. Consequently, it was difficult to assess the possible contamination of these extracts with LH. We found an early report by Greep that gonadotropins were heat labile. Heating the hypothalamic extracts to boiling for ten minutes was followed by no loss of activity, whereas LH was completely destroyed. This certainly suggested that the action was due to LH-releasing activity of the extracts rather than to contaminating LH.[2] The problem of decline of sensitivity of the assay following hypophysectomy was settled by carrying out acute hypophysectomy and immediately injecting the extracts. In this circumstance the sensitivity to LH was not impaired and yet there was no demonstrable LH in the extracts.[1] We concluded that we were dealing with an LH-releasing factor.

To establish this more conclusively, we injected the extracts intravenously into rats and assessed the effect on plasma LH again measured by the OAAD method. We were able to show a rapid elevation in LH following the intravenous injection of the extracts in ovariectomized, estrogen-primed rats.[2] No activity was detectable in ovariectomized animals. This was probably due to

the inaccuracies of the assays. Subsequent work has shown that under this circumstance the extracts will also elevate plasma LH.[3]

About the same time and unknown to us, Harris and his collaborators were injecting hypothalamic extracts into the pituitary gland of rats and rabbits. The initial experiments of Nikitovitch-Winer[4] showed that such extracts would indeed evoke ovulation in the rat and that control tissue extracts were inactive. Similar results were obtained by Campbell et al.[5] in the rabbit.

With the realization that there was indeed an LH-releasing factor in hypothalamic extracts, it was very important to attempt to purify the factor, isolate it and determine its structure, so that synthesis could be accomplished. The potential importance of the factor in the control of ovulation was a spur to work in a number of laboratories.

Bioassay of LH-releasing hormone (LH-RH). The first problem in any work of this kind is the assay of the activity. The OAAD method which we had used was not very sensitive and rather imprecise. This led us to look for other methods of assay. Reasoning by analogy with the finding that the corticotrophin-releasing activity of hypothalamic extracts was blocked by treatment with cortical steroids, we felt that LH-releasing activity would be similarly blocked by treatment of ovariectomized animals with gonadal steroids. We found to our surprise that not only did large doses of estrogen fail to block the activity, but estrogen plus progesterone which suppressed LH levels markedly in ovariectomized rats did not block LH-releasing activity.[2] By determining the minimal effective dose of extract in such a preparation it became apparent that we had increased sensitivity instead of a blockade of the response by steroid treatment.[6] We then set out to determine the minimal effective dose of extract in such a preparation. Ramirez found that we could reduce the dose to .01 of a rat stalk median eminence equivalent, whereas in the direct OAAD assay the minimal effective dose was around 0.4 of an equivalent. Obviously we had found increased sensitivity instead of a blockade of the response by steroid treatment.[6] The ovariectomized, estrogen progesterone-blocked rat remains the most sensitive in vivo bioassay for LHRH. It also appears to be relatively precise on the basis of more recent work with radioimmunoassay of LH.

It was shown first by Schally and Bowers[7] that one could also assay the activity utilizing hemipituitaries incubated in vitro and this has been a reasonably good assay. Again the most sensitive preparation is the pituitary from the ovariectomized, estrogen progesterone-treated animal; however, the results with ovariectomized, estrogen-treated animals are similar. Subsequently, it has been shown that dispersed pituitary cells are also a con-

venient assay for LHRH. They are very sensitive to the activity and the precision is excellent.[8]

Chemistry of LHRH. Utilizing these various assays, it was shown in 1962 that LHRH was heat stable (as mentioned above), but was destroyed by proteolytic enzymes.[2] It was completely inactivated by trypsin and partially inactivated by pepsin. This indicated that it was probably a peptide. Gel filtration through Sephadex G-25 columns revealed that the activity eluted just prior to vasopressin suggesting that it was a relatively small peptide of about 1,000 molecular weight.[9] This procedure was sufficient to separate it from most of the other releasing and inhibiting activities of hypothalamic extracts.[10] In our experiments the first activity to be eluted from the column was CRF and this was followed by growth hormone-releasing factor, growth hormone-inhibiting factor, FSH-releasing factor and LH-RF. Further purification was accomplished by chromatography on carboxymethyl cellulose, which again seemed to separate FSH- from LH-releasing activity. All of these assays were bioassays.

With the discovery of the structure of TRH,[11] it was apparent that it was only a matter of time until the structure of LHRH would be revealed. We reasoned that the structure might be similar and might have a pyroglutamic acid at the N-terminal end of the molecule. We also guessed that the structure might be that of a tripeptide, similar to TRH. On the basis of various tests carried out by Fawcett we thought that there was histidine and tryptophane in the molecule. Folkers and his colleagues then synthesized two tripeptides for us, the pyroglutamyl-histidyl-tryptophane-amide and the pyroglutamyl-tryptophanyl-histidine-amide. Our first tests revealed that one of these peptides was active. This caused great excitement; however, the activity could never be confirmed.

At about this time, a brilliant chemist, Matsuo arrived in Schally's laboratory and utilizing very small amounts of highly purified LHRH was able almost single handedly to elucidate the structure and synthesize the decapeptide which was shown to be biologically active.[12] Interestingly, it did contain the pyroglutamyl-histidyl-tryptophane sequence which we had earlier postulated. Guillemin's group confirmed the decapeptide structure using sheep hypothalami.

The determination of structure of LHRH paved the way for many additional studies. It made it possible to prepare antibodies to the hormone which aided in assessing its physiological significance and made possible the development of radioimmunoassays, to develop various analogs which have either augmented

or inhibitory activity and to synthesize sufficient material for use in clinical medicine.

Localization of LHRH in the brain. Radioimmunoassay of sections cut from the brain or of punches removed from it confirmed the early localization of LHRH by bioassay.[13] Immunocytochemical localization of the perikarya and terminals of LHRH neurons was also accomplished.[14] From all of this work it is now apparent that there is a system of LHRH neurons with cell bodies in the preoptic region and long axons which extend along the base of the brain to reach the median eminence. In most species there is probably also another population of LHRH neurons with cell bodies in the arcuate nucleus and short axons extending to the median eminence. It is now apparent that LHRH neurons also have axons extending down into the brain stem in the region of the central gray, a region known to be involved in sexual behavior.[15] This finding is very recent. In addition there is some evidence for projections of LHRH fibers into the amygdala and other brain regions. Very recently it has been shown that there are LHRH neurons in the olfactory system.[16] LHRH cells have even been found in gold fish retina.[17] Lastly, it now appears that LHRH may be located outside the nervous system. An LHRH-like peptide has been found in ovarian follicular fluid[18] and in testicular lymph.[19] Although these peptides do not crossreact in the radioimmunoassay for LHRH, they have similar biological activity on the pituitary and have low molecular weights. One could guess that they are related peptides which do not contain the antigenic determinants for crossreaction in the radioimmunoassay. An LHRH-like peptide has also been reported in the placenta[20] and in milk.[21] Thus, it would appear that LHRH-like peptides are in regions concerned with sexual behavior and sexual function at all levels from olfactory system to peripheral organs, such as gonads and mammary glands.

Actions of LHRH on the pituitary. LHRH acts on the pituitary to promote rapid release of LH and to a lesser extent FSH from the gland. Pulse injection may lead to release of only LH; however, more prolonged exposure of the gland to the peptide leads to significant FSH release.[22] The FSH-releasing potency is often of the order of 20% of the LH releasing potency; however, the FSH-releasing potency varies depending on the hormonal state of the animal and the frequency of LHRH injections. In immature rat and human the peptide tends to produce greater FSH release.[23] Frequent pulses of the peptide provoke primarily LH release, however, with more infrequent pulses a predominance of FSH release appears to occur, perhaps related in part to the longer half-time of disappearance of FSH from the circulation.[24] Responsiveness to LHRH is quite high in the castrate.[3] Following administration of estrogen to the

castrate female, responsiveness declines rapidly in rat, man and other species, and this is followed by augmented responsiveness to the peptide.[25,26] In other words there is a biphasic response, first inhibition and subsequent augmentation in response, produced by estrogen. Progesterone appears to synergize with estrogen in the latter half of the cycle in rat and man to hold gonadotropin secretion in check.[27,28] Injection of progesterone can initially augment and then suppress responsiveness to the decapeptide in both rats and humans in the presence of estrogen.[28,29] Testosterone has an inhibitory-action suppressing responsiveness.[26]

During the menstrual cycle and rat estrous cycle, responsiveness to LHRH increases presumably because of release of estrogen from the developing follicles. Responsiveness becomes maximal just prior to the preovulatory discharge of LH.[26,28,30] This further enhancement in responsiveness is due not only to the action of estrogen but also to the so called "self-priming action" of LHRH which augments response of the pituitary to subsequent LHRH.[31,32] The estrogen secreted by the follicles brings about enhanced LHRH release by an action on the preoptic region and this then produces the self-priming action. Responsiveness to LHRH may increase 50-fold above minimal levels at the time of the preovulatory release in man and rat. In the progestational phase of the cycle, responsiveness is suppressed presumably because of inhibitory actions of both estrogen and progesterone. Responsiveness with regard to FSH release follows a similar pattern.[26,30]

There is clear evidence that there is increased LHRH release from the hypothalamus at the time of the preovulatory LH surge in rat[26] and suggestive evidence for this phenomenon in primates.[33] However, Knobil's group has been able to reinitiate cycles by pulsatile administration of LHRH in Rhesus monkeys with arcuate nuclear lesions which suppress gonadotropin release. He proposes that the preovulatory surge in Rhesus monkey is due to invariant pulsatile release of LHRH from the region of the arcuate nucleus which leads to development of follicles and estrogen secretion.[34] The estrogen then acts to augment responsiveness of the pituitary to this constant pulsatile release of LHRH resulting in the preovulatory discharge. Spies and coworkers on the other hand have been unable to induce cyclic release of gonadotropins with pulsatile LHRH administration in Rhesus monkeys with stalk sections and a permanent barrier placed between the cut ends of the stalk to block regeneration by portal vessels.[35] In these stalk sectioned monkeys the pituitary is presumably completely denervated whereas it is possible that the arcuate nuclear lesions would leave intact the preopticotuberal LHRH pathway which