Role of Endogenous Opiates in the Expression of Negative Feedback Actions of Androgen and Estrogen on Pulsatile Properties of Luteinizing Hormone Secretion in Man

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Abstract. We have tested the participation of endogenous opiate pathways in the negative feedback actions of gonadal steroids on pulsatile properties of luteinizing (LH) hormone release in normal men. To this end, sex steroid hormones were infused intravenously at dosages that under steady state conditions selectively suppressed either the frequency or the amplitude of the pulsatile LH signal. The properties of pulsatile LH secretion were assessed quantitatively by computerized analysis of LH series derived from serial blood sampling over 12 h of observation.

When the pure (nonaromatizable) androgen, 5-α-dihydrotestosterone, was infused continuously for 108 h at the blood production rate of testosterone, we were able to achieve selective inhibition of LH pulse frequency akin to that observed in experimental animals after low-dosage androgen replacement. Under these conditions, serum concentrations of testosterone and estradiol-17β did not change significantly, but serum 5α-dihydrotestosterone concentrations increased approximately two- to threefold, with a corresponding increase in levels of its major metabolite, 5α-androstan-3α, 17β-diol. In separate experiments, the infusion of estradiol-17β at its blood production rate over a 4.5-d interval selectively suppressed LH pulse amplitude without influencing LH pulse frequency. Estrogen infusion increased serum estradiol-17β levels approximately twofold without significantly altering blood androgen concentrations. We then used these schedules of selective androgen or estrogen infusion to investigate the participation of endogenous opiates in the individual inhibitory feedback actions of pure androgen or estrogen on pulsatile LH release by administering a potent and specific opiate-receptor antagonist, naltrexone, during the infusions.

Our observations indicate that, despite the continuous infusion of a dosage of 5α-dihydrotestosterone that significantly suppresses LH pulse frequency, co-administration of an opiate-receptor antagonist effectively reinstates LH pulse frequency to control levels. Moreover, during the infusion of a suppressive dose of estradiol-17β, opiate receptor blockade significantly augments LH pulse frequency and increases LH peak amplitude to control levels.

Thus, the present studies in normal men demonstrate for the first time that the selective inhibitory action of a pure androgen on LH pulse frequency is effectively antagonized by opiate-receptor blockade. This pivotal observation indicates that opiatergic and androgen-dependent mechanisms specifically and coordinately control the hypothalamic pulse generator for gonadotropin-re-

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leasing hormone (GnRH). Moreover, endogenous opiate systems susceptible to blockade by naltrexone also interact significantly with estrogen's negative feedback regulation of LH peak amplitude.

We conclude that the negative feedback actions of gonadal steroids are integrally coupled to endogenous opiate pathways and that such functional coupling is ultimately expressed at least in part at the level of the hypothalamic pulse generator for GnRH. These observations suggest a model for the proximate regulation of gonadotropin secretion in man, in which the regulatory actions of two major inhibitory systems—opiates and gonadal steroids—are effectively integrated by neural mechanisms.

Introduction

Narcotic drugs and endogenous opiate peptides inhibit the elaboration of luteinizing hormone (LH)[1] by the hypothalamic-pituitary axis in the male and female of several mammalian species (1–11). Moreover, the administration of opiate-receptor antagonists alone significantly amplifies the pulsatile mode of LH release, with attendant increases in the frequency and peak amplitude of both immunoactive and biologically active LH pulses (11–15). The ability of opiate-receptor antagonists to enhance episodic LH secretion in vivo (11–15) and to stimulate gonadotropin-releasing hormone (GnRH) secretion from human hypothalamic tissue in vitro (16, 17) has suggested that the inhibitory action of endogenous opiates is exerted at the level of the hypothalamic pulse generator for GnRH.

Gonadal sex steroids also significantly regulate properties of pulsatile gonadotropin release under physiological conditions (18–21). In particular, the negative feedback actions of androgen and estrogen can selectively influence either the frequency or the amplitude of the LH pulse signal (22–27). However, the relationship, if any, between these discrete inhibitory actions of sex steroid hormones and the suppressive effects of endogenous opiates is not known.

Recent investigations in the rat have suggested that endogenous opiates may participate in testosterone and estrogen's suppressive effects on LH secretion (28–30). However, whether functional coupling between these two major inhibitory systems exists in man and is integrated specifically via mechanisms that control one or more distinct properties of pulsatile LH release has not been ascertained. Thus, in the present study, we have investigated functional coupling between the endogenous opiate system and the negative feedback actions of sex steroids on specific properties of pulsatile LH release.

Methods

Studies were approved by the Human Investigation Committee of the University of Virginia School of Medicine. Six healthy male volunteers (age range 21–28 yr) participated. Each had normal basal serum concentrations of free thyroxine, thyroid stimulating hormone, prolactin, immunoactive LH and follicle stimulating hormone, free testosterone, and estradiol-17β. Physical examination and tests of hepatic and renal function were normal.

Serial blood sampling was performed after placebo and naltrexone ingestion in three separate sessions: under basal conditions (control infusions, six men); during infusion of 5α-dihydrotestosterone (the same six men); and during infusion of estradiol-17β (four of the six men). Sessions were 1 mo apart to allow recovery of the gonadal axis. The steroids were administered by continuous intravenous infusions maintained over 4.5 d. 48 μg of estradiol and 7 mg of 5α-dihydrotestosterone were administered per day as described by others (31, 32). Chromatographically pure steroids (assessed by high pressure liquid chromatography) were dissolved in sterile ethanol, which was diluted in 5% dextrose in water immediately before infusion. One liter of 5% dextrose in water was infused continuously every 12 h after the addition of 0.1 ml of stock steroid solution (100% ethanol). A uniform rate of infusion was maintained with an infusion pump (Volumetric 927; Imed Inc., San Diego, CA). Tygon tubing was used to minimize nonspecific steroid adsorption, which was monitored by radioimmunoassay of the effluent (recovery 85–97% at the catheter tip). After 72 h of steroid infusion, placebo diluent was administered orally and blood was sampled for 12 h (0900–2100) at 20-min intervals to characterize pulsatile LH release. After 96 h of steroid infusion, naltrexone elixir (1 mg/kg) was administered orally and blood was sampled again for 12 h (0900–2100) at 20-min intervals. To assess steady state blood levels of sex steroid hormones, blood was also sampled before hormone infusion and every 12 h during the 4.5 d. All blood sampling was performed in the arm contralateral to the infusion.

Blood samples withdrawn from the indwelling intravenous needle were allowed to clot at room temperature, and the serum was stored at −20°C for subsequent immunoassay. Samples from an individual's complete study (all sessions) were analyzed in the same assay to eliminate interassay variability. Serum immunoactive LH concentrations were measured in triplicate by a modification of the method of Odell et al. (33), with the reagents described previously (34). Additional pools of serum were assayed nine times each to define the intraassay variability precisely at multiple points along the displacement curve, since our analysis of pulsatile LH secretion employed intraassay variance that was relevant to the individual subject (see below). In the present studies, the intraassay coefficients of variation were 8.5% for LH concentrations of 2–4 mIU/ml, 7.3% for LH values of 4–8 mIU/ml, and 6.5% for LH levels of 8–12 mIU/ml. Serum concentrations of testosterone, estradiol, 5α-dihydrotestosterone, and 5α-androstan-3α, 17β-diol were determined by radioimmunoassay after celite chromatography exactly as previously described (35, and Samojlik, E., M. A. Kirschner, D. Silber, G. Schneider, and N. H. Ertel, manuscript submitted for publication).

The plasma LH secretion profiles were analyzed for significant fluctuations by a computerized, pulse-detection algorithm modified from that of Santen and Bardin (20). This method estimates the area under the LH concentrations vs. time curve and the fractional amplitude of individually significant pulses (given as percentage above preceding nadir). Our modification requires that a significant pulse exhibit an amplitude at least four times the individual intraassay coefficient of variation (instead of simply 20% as originally described). This somewhat more stringent criterion for an LH pulse minimizes the false-positive error rate for pulse enumeration (15). We used this means of pulse analysis except where noted otherwise, when we compared results with the independent pulse-detection method of Clifton and Steiner (36) as modified by us (15). Interpulse (smoothed base line) LH concentrations were computed

1. *Abbreviations used in this paper:* GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone.

by the program of Merriam and Wachter (37). Although sampling at 20-min intervals can underestimate absolute LH pulse frequency compared with more rapid rates of sampling (38), the present assessment of large amplitude LH pulses at a uniform sampling rate does permit us to evaluate relative changes in LH pulse frequency in relation to specific hormonal effects.

Data are presented as mean±SEM and were analyzed by within-subject comparisons by the use of a paired two-tailed *t* test with correction for repeated measures as appropriate. Significant effects were construed for $P \leq 0.05$.

Results

Characterization of pulse frequency changes. The infusion of 5α-dihydrotestosterone significantly reduced LH pulse frequency in these men from a mean of 3.5±0.3 pulses/12 h (mean±SEM) to 2.0±0.2 pulses/12 h ($P = 0.003$), when pulse frequency was estimated by the modified method of Santen and Bardin. On the other hand, the infusion of estradiol did not significantly alter LH pulse frequency (Fig. 1).

Under conditions in which dihydrotestosterone significantly suppressed LH pulse frequency, the co-administration of naltrexone was able to significantly increase pulse frequency from 2.0±0.2 pulses/12 h to 4.7±0.2 pulses/12 h ($P = 0.005$) (Fig. 1). Moreover, naltrexone restored LH pulse frequency in the presence of continued androgen infusion to a level that was not significantly different from that observed after naltrexone administration during control infusions.

Naltrexone also significantly stimulated LH pulse frequency during estradiol infusion, with an increase from 3.5±0.25 to 5.3±0.22 pulses/12 h ($P = 0.001$) (Fig. 1). The stimulated LH pulse frequencies were not significantly different from those observed when naltrexone was given during control infusions.

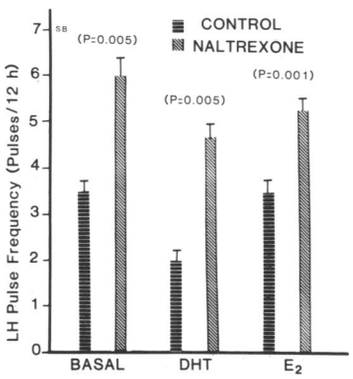

Figure 1. Influence of 5α-dihydrotestosterone (DHT) or estradiol (E_2) infusion on LH pulse frequency in normal men treated with placebo or the opiate-receptor antagonist, naltrexone. Normal male volunteers underwent repetitive venous sampling at 20-min intervals for 12 h to characterize LH pulse frequency under basal conditions (control infusions), and during infusions of 5α-dihydrotestosterone or estradiol (see Methods). Blood sampling was performed after the administration of placebo or naltrexone. Data are mean (±SEM) numbers of LH pulses per 12 h for six men during control and 5α-dihydrotestosterone infusions, and for four men during estradiol infusions. Individual *P* values are given for each session in which the effects of naltrexone and placebo on LH pulse frequency are compared. These data were analyzed by a pulse-detection algorithm modified from the method of Santen and Bardin (SB).

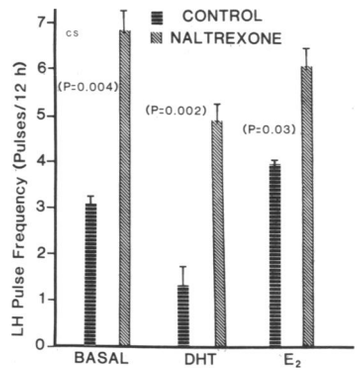

Figure 2. Analysis of LH pulse frequency using an independent pulse-detection algorithm. Blood was sampled as described for Fig. 1, but the LH series were analyzed by the method of Clifton and Steiner (CS) (36), as modified (15). Results are otherwise presented as indicated in Fig. 1.

When all LH data were analyzed by the independent pulse-detection algorithm of Clifton and Steiner (36, 15), the inferred alterations in LH pulse frequency were corroborated (Fig. 2). In particular, naltrexone administration significantly augmented pulse frequency despite continuous infusion of 5α-dihydrotestosterone or estradiol, and these stimulatory actions of naltrexone were not significantly different from those observed during control infusions. The appropriateness of this method of analysis was supported by the high (≥2.2) signal-to-noise ratio in each LH series evaluated, which conforms with the requirement of a signal-to-noise ratio of ≥1.5 (36).

Changes in other parameters of pulsatile LH secretion. The infusion of dihydrotestosterone significantly reduced 12-h integrated LH levels, as estimated by area under the 12-h LH concentration vs. time curve ($P = 0.02$) (Fig. 3). After the co-administration of naltrexone, 12-h integrated LH concentrations increased significantly ($P = 0.006$). A similar pattern of responses was observed during the infusion of estradiol, which significantly reduced the area under the LH concentration versus time curve ($P = 0.05$) for the four subjects who underwent both control and estrogen infusions. Moreover, the co-administration of naltrexone was able to reverse significantly the decrements induced by continuous estradiol infusion (Fig. 3).

The changes in mean serum LH concentrations closely mir-

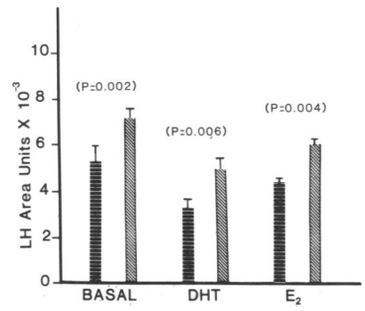

Figure 3. Influence of sex-steroid infusions and opiate-receptor blockade on integrated serum LH concentrations in normal men. Conditions are as described for Fig. 1. Integrated serum LH concentrations were calculated from the LH concentration versus time curves for blood samples withdrawn over 12 h of observation. Individual *P* values are given for each session in which the effects of placebo and naltrexone are compared. Data are given as means±SEM. ▫, naltrexone. □, control.

rored those for 12-h integrated LH levels. In particular, the arithmetic mean of basal serum LH concentrations (milliinternational units per milliliter±SEM) during control infusions was 8.15±0.83 (and 9.69±1.07 after naltrexone, $P = 0.002$), which declined significantly to 6.40±0.15 (10.69±1.33 after naltrexone, $P = 0.003$) during estradiol infusion, and to 4.96±0.57 (7.02±1.41 after naltrexone, $P = 0.006$) during 5α-dihydrotestosterone infusion. Note that during steroid suppression naltrexone significantly increased mean serum LH concentrations in these subjects to levels not significantly different from control (basal).

Androgen and estrogen infusions affected the interpulse basal levels of LH and properties of LH pulse amplitude in distinctive ways (Fig. 4). In particular, dihydrotestosterone administration effectively reduced interpulse basal levels of immunoactive LH ($P = 0.01$ vs. basal) but notably did not significantly influence properties of LH pulse amplitude, whether considered as fractional pulse amplitude (percentage above nadir), incremental pulse amplitude (milliinternational units per millimeter increase above nadir), or absolute LH peak values (milliinternational units) (Fig. 4). Despite continuous infusion of dihydrotestosterone, naltrexone administration was associated with a significant rise in peak LH concentrations ($P = 0.03$) and interpulse basal values ($P = 0.08$).

In contrast to these effects of dihydrotestosterone, estradiol reduced all parameters of LH pulse amplitude: percentage LH pulse amplitude ($P = 0.05$ vs. basal), incremental pulse amplitude ($P = 0.02$ vs. basal), or peak LH pulse amplitude ($P = 0.01$ vs. basal), but did not significantly suppress interpulse basal LH concentrations (Fig. 4). Some of these suppressive effects of estradiol were significantly antagonized by naltrexone, which increased peak LH pulse amplitude ($P = 0.02$) and interpulse basal LH concentrations ($P = 0.004$).

The typical patterns of altered pulsatile LH secretion observed in these studies are illustrated for one subject in Fig. 5.

Steroid hormone concentrations in blood during the infusions. As shown in Fig. 6, serum concentrations of 5α-dihydrotestosterone rose significantly during the infusion of this steroid, reaching stable concentrations within 36 h. For the remaining infusion, 5α-dihydrotestosterone concentrations averaged 3.06 ng/ml, compared with 0.89±0.04 ng/ml basally (normal range, 0.4–1.2 ng/ml). Concentrations of testosterone and estradiol-17β did not change significantly over time but continued to exhibit significant AM-PM diurnal variation throughout the 4.5 d of the infusion, with lower PM values ($P < 0.01$).

When estradiol-17β was infused, its serum concentrations increased approximately twofold above base line within 24 h ($P < 0.01$) and remained at this level thereafter (Fig. 6). During estrogen infusion, serum concentrations of testosterone and 5α-dihydrotestosterone did not change significantly at any time but did exhibit significant AM-PM diurnal variation throughout, with lower PM values ($P < 0.01$).

Circulating concentrations of 5α-androstan-3α, 17β-diol, a major tissue metabolite of 5α-dihydrotestosterone, were also measured in three men by the use of samples collected basally and at 12-h intervals during 5α-dihydrotestosterone infusion. As depicted in Fig. 6, serum concentrations of this 3α-reduced metabolite increased to a transient peak value at 24 h and then declined gradually to stably elevated levels during the remaining 60 h.

Our serial measurements of the principal circulating gonadal steroids of exogenous and endogenous origins thus document the attainment of equilibrium during the infusions. Moreover, these measurements also demonstrate that the doses of steroids infused did not act pharmacologically to suppress the spontaneous diurnal variations characteristic of endogenous steroids.

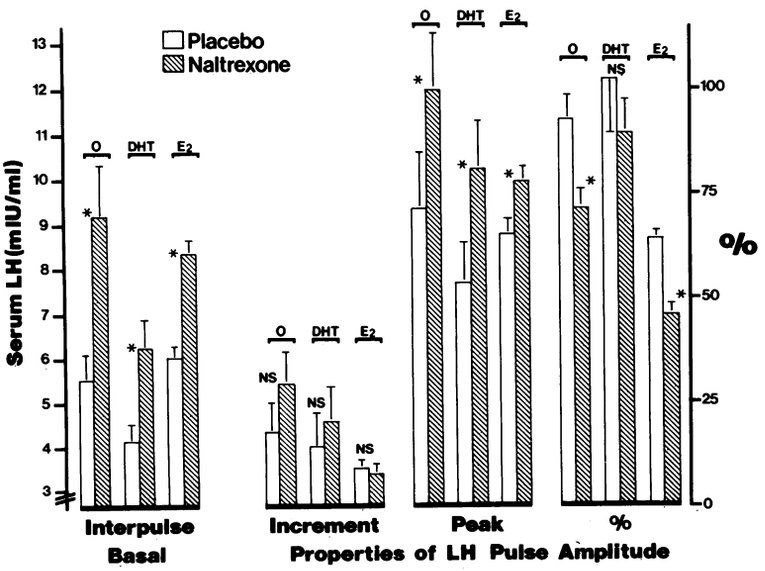

Figure 4. LH pulse amplitude characteristics in relation to gonadal steroid infusions in normal men. Blood was sampled to characterize pulsatile LH release during infusions of control solvent (○), 5α-dihydrotestosterone (DHT), or estradiol (E_2) after the ingestion of placebo elixir or the opiate antagonist, naltrexone (1 mg/kg). The LH pulse profiles were analyzed for mean interpulse basal LH concentrations (milliinternational units per milliliter) (left vertical axis) and LH pulse amplitudes, which were expressed as increments (milliinternational units per milliliter) from preceding nadir to peak, absolute peak LH values (milliinternational units per milliliter), or as fractional (percentage) increases from nadir to peak (right vertical axis). Data are means±SEM ($n = 6$ men for control and DHT, $n = 4$ subjects for E_2 infusion). *$P < 0.05$.

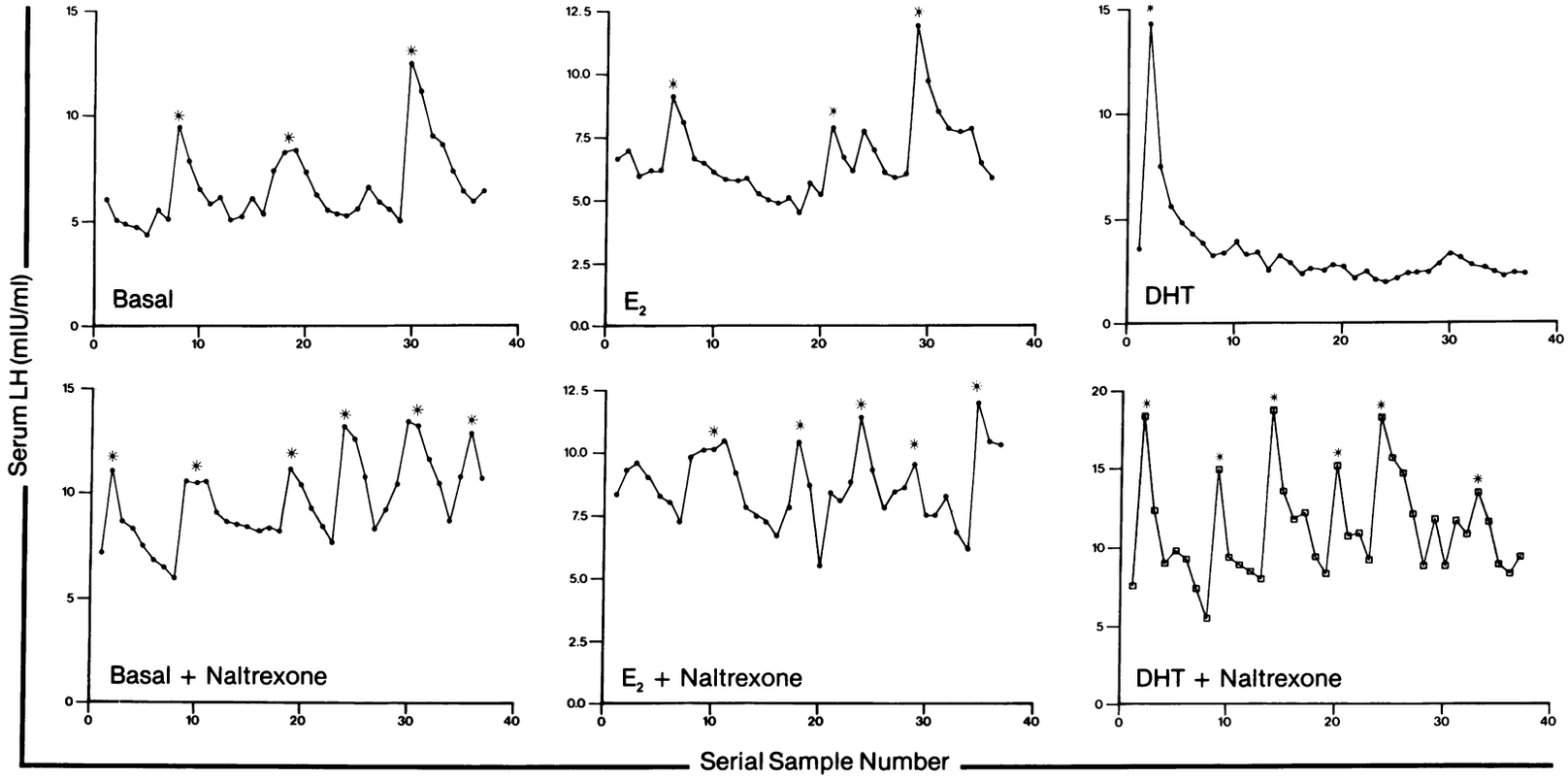

Figure 5. Profiles of pulsatile LH secretion observed in one man during control (basal), 5α-dihydrotestosterone (DHT), or estradiol-17β (E_2) infusions, with or without naltrexone administration. Blood sampled at 20-min intervals for 12 h under the conditions indicated in each panel. Serum LH concentrations (milliinternational units per milliliter) are given on the vertical axis. Individual LH peaks are denoted by asterisks. Complete quantitative data for all the subjects are given in the text and figures.

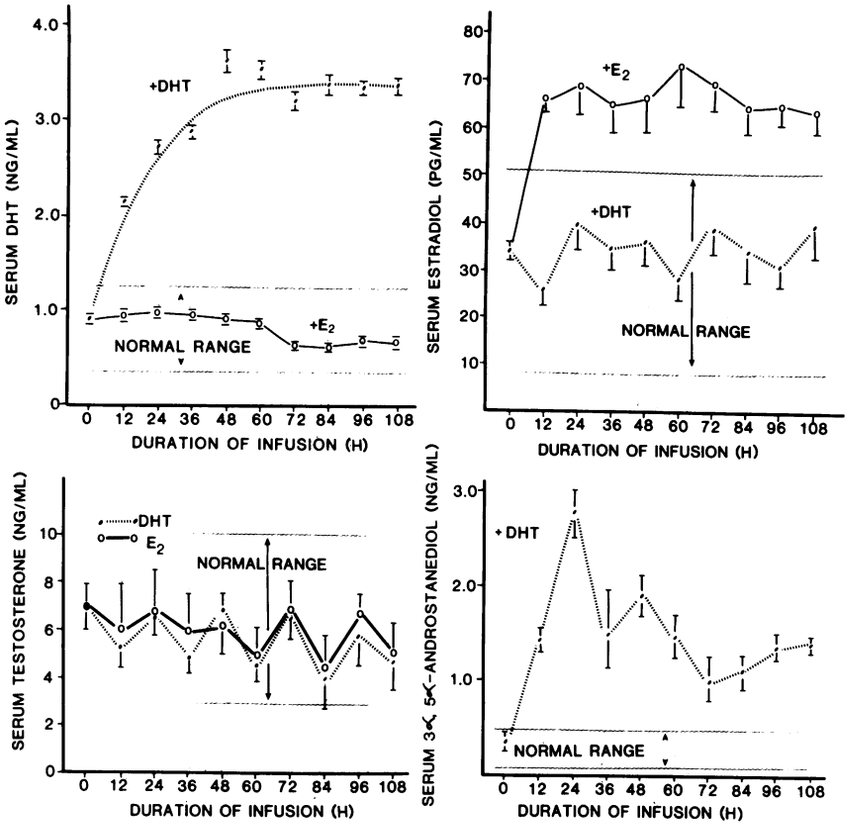

Figure 6. Serum concentrations of principal sex steroid hormones during the infusion of estradiol (E_2) or 5α-dihydrotestosterone (DHT) in normal men. In each panel, serum concentrations of a steroid hormone are given (vertical axes) over time of infusion (horizontal axes). The infusions contained DHT or E_2 as indicated. The normal ranges for basal steroid hormone concentrations in these subjects are given in each panel. Serum 5α-androstan-3α, 17β-diol (*bottom, right*) was measured as a major metabolite of 5α-dihydrotestosterone.

Discussion

We have demonstrated that the specific inhibitory action of a pure (nonaromatizable) androgen on LH pulse frequency is effectively antagonized by opiate-receptor blockade. This pivotal inference was corroborated by two independent algorithms for enumerating LH pulses objectively. Thus, we conclude that endogenous opiate systems are functionally coupled to androgen's negative feedback control of episodic LH secretion in man.

In these studies, we chose a dosage of 5α-dihydrotestosterone that selectively suppresses LH pulse frequency without decreasing LH pulse amplitude (32, and present study), and a dose of naltrexone that antagonizes exogenous opiate challenge for more than 24 h without exerting any discernible agonist effects (15, 39, 40). In this setting of continuous intravenous infusion of an inhibitory dose of 5α-dihydrotestosterone, the co-administration of naltrexone was able to reinstate LH pulse frequency to control levels. Therefore, if changes in LH pulse frequency mirror corresponding alterations in episodic GnRH secretion by hypothalamic neurons (41–43), our data indicate that opiatergic and androgen-dependent mechanisms coordinately regulate the frequency of the hypothalamic GnRH pulse generator.

Certain alternative hypotheses can be considered in relation to the present observations. For example, the possibility that naltrexone simply competes with cytosolic androgen receptors and directly impedes androgen action in brain or pituitary cells can be discounted (44, 45). In addition, opiate-receptor antagonists do not alter the metabolic clearance of androgens or influence the sensitivity of pituitary cells to available GnRH (8, 12, 47, 48). Rather, narcotic antagonists seem to enhance the hypothalamic efflux of GnRH in vitro (47) and in vivo (48). Therefore, our demonstration that a specific opiate-receptor antagonist can reinstate a high frequency of LH pulsations despite the uninterrupted infusion of an inhibitory dose of 5α-dihydrotestosterone implies that endogenous opiates interact with androgen's negative feedback regulation of the GnRH pulse generator.

We infused 5α-dihydrotestosterone, a C_{19}-androgen saturated in the A-ring, because, unlike testosterone, this reduced androgen cannot undergo metabolic conversion to known estrogens (49). In vivo, endogenous 5α-dihydrotestosterone enters hypothalamic or pituitary cells from the circulation or is generated in situ from available testosterone (23, 50, 51). When we infused exogenous 5α-dihydrotestosterone at a rate equal to the daily blood production rate of testosterone in normal men (52, 53), there was a two- to threefold elevation of serum levels of 5α-dihydrotestosterone and its major 3α-reduced metabolite, 5α-androstan-3α,17β-diol. Thus, although brain concentrations of 5α-dihydrotestosterone cannot be determined under these condi-

tions in the human, we presume they increased and consequently influenced gonadotropin secretion. The alternative possibility that infused 5α-dihydrotestosterone altered LH release indirectly by displacing endogenous testosterone from its plasma binding sites is unlikely under these equilibrium conditions, since injected 5α-dihydrotestosterone actually decreases plasma free testosterone concentrations in men within 24–48 h (54). As important, the selectivity of this infusion schedule in suppressing LH pulse frequency without reducing LH pulse amplitude closely mimics the effects of low-dosage (but not pharmacological dosage) androgen replacement in other species, such as the rodent, sheep, and Rhesus monkey (55–57).

In contrast, infusion of estradiol at its blood production rate in normal men (58) significantly attenuated LH pulse amplitude without altering LH pulse frequency. This observation is similar to that reported when estradiol was infused at twice its production rate (32, 59). We documented a suppressive effect of estradiol on LH pulse amplitude whether pulse amplitude was defined as a fractional (percentage) increase above nadir, as an increment (milliinternational units per milliliter) above preceding nadir, or as a peak LH value attained within individual pulses. There was an associated significant decline in mean and integrated serum LH concentrations estimated over 12 h of sampling. These inhibitory actions of estrogen were functionally coupled to the opiatergic system, since the administration of naltrexone during estrogen infusions significantly augmented LH pulse frequency and increased mean and integrated LH concentrations, as well as peak LH pulse amplitude. Because this schedule of estradiol infusion either slightly decreases or does not affect the sensitivity of pituitary LH release to exogenous GnRH in men (25), we infer that the increase in peak LH pulse amplitude observed after opiate antagonism may result from enhanced release of endogenous GnRH rather than increased pituitary sensitivity to endogenous GnRH. In addition, the high LH peaks may reflect the imposition of LH pulses on increased interpulse base line LH concentrations, which accompanied naltrexone's shortening of the interpulse interval.

The present results permit us to suggest a model of functional coupling between androgen and opiate mechanisms (Fig. 7). We have chosen the most conservative interpretation of available data, recognizing that additional considerations are possible. In this model, the negative feedback actions of pure androgen on the hypothalamic pulse generator are mediated at least in part via intervening (or parallel) inhibitory opiate pathway(s). Since naloxone and naltrexone can inhibit several opiate receptor subtypes, the exact nature of the opiate receptor(s) involved in the control of LH secretion in man cannot be ascertained at present. However, recent studies in the rodent suggest that mu opiate receptors in particular mediate LH release (60).

In conclusion, the present studies in normal men have demonstrated that the negative feedback action of pure androgen and estrogen are intimately coupled to endogenous opiate pathways. Moreover, such functional coupling is ultimately expressed at the level of the hypothalamic pulse generator for GnRH with consequent modulation of specific properties of pulsatile LH release.

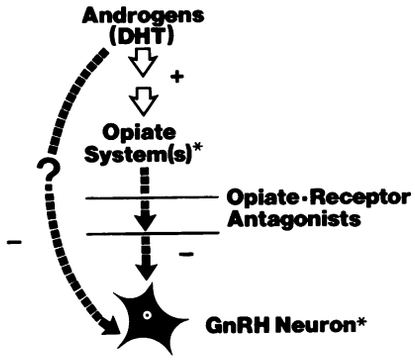

Figure 7. Possible model of the coupling between pure androgen negative feedback mechanisms and the inhibitory endogenous opiate pathway. In this schema, systemically available or locally converted androgen (here designated as 5α-dihydrotestosterone, DHT) acts on brain sites that ultimately stimulate (+) the opiate systems. Activation of the opiatergic pathways in turn leads to suppressed (−) activity of the hypothalamic GnRH pulse generator. The interposition of several arrows indicates that one or more intervening steps may operate within this basic model. In addition, other neuroendocrine systems (e.g., catecholaminergic) could impinge upon these steps, although the exact relationship(s) of such systems to androgen and opiate actions in man cannot be determined at present. The possibility that an opiate-independent pathway of androgen suppression also exists under physiological conditions is denoted by the lateral convex arrow interrupted by a question mark. *Other regulators may also operate at these sites.

Acknowledgments

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