19-Hydroxy steroids in the aromatase reaction: Review on expression and potential functions

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Disclosure Statement: The authors have nothing to disclose.
ABSTRACT

Scientific evidence related to the aromatase reaction in various biological processes spanning from mid-1960 is abundant, however, as our analytical sensitivity increases, a new look at the old chemical reaction is necessary. Here, we review an irreversible aromatase reaction from the substrate androstenedione. It proceeds in 3 consecutive steps. In the first two steps, 19-hydroxy steroids are produced. They can dissociate from the enzyme complex and either accumulate in tissues or enter the blood.

In this review, we want to highlight the potential importance of these 19-hydroxy steroids in various physiological and pathological conditions. We focus primarily on 19-hydroxy steroids, and in particular on the 19-hydroxyandrostenedione produced by the incomplete aromatase reaction. Using a PubMed database and search terms aromatase reaction, 19-hydroxylation of androgens and steroid measurements, we detail the chemistry of the aromatase reaction and list previous and current methods used to measure 19-hydroxy steroids.

We present the evidence of the existence of 19-hydroxy steroids in the brain tissue, ovaries, testes, adrenal glands, prostate cancer and also during pregnancy and parturition and in Cushing’s disease. Based on the available literature, a potential involvement of 19-hydroxy steroids in the brain differentiation process, sperm motility, ovarian function, and hypertension is suggested and warrant future research.

We hope that with the advancement of highly specific and sensitive analytical methods, future research into 19-hydroxy steroids will be encouraged, as much remains to be learned and discovered.

Keywords: 19-hydroxyandrostenedione, 19-oxoandrostenedione, ACTH, POR, HPA and HPG axes
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Abbreviations

19-OH AD - 19-hydroxyandrostenedione

19-Oxo AD - 19-oxoandrostenedione

3β HSD - 3β hydroxysteroid dehydrogenase

ACTH - adencorticotropic hormone

ANP - atrial natriuretic peptide

ArKO - aromatase-deficient

BNST - bed nucleus of the stria terminalis

CRH - corticotropin releasing factor
DHEA - dehydroepiandrosterone

DHT - dihydrotestosterone

E1 - estrone

E2 - 17β-estradiol

E3 - 17β,16α-estriol

FSH - follicle stimulating hormone

GnRH - gonadotropin releasing hormone

HPA - hypothalamic pituitary adrenal axis

HPG - hypothalamic pituitary gonadal axis

HSD17B - 17β-hydroxysteroid dehydrogenase

LH - luteinizing hormone

PE - pre-eclampsia

POA - preoptic area

POR - cytochrome P450 oxidoreductase, NADPH cytochrome reductase

RAAS - renin-angiotensin-aldosterone system

SDN - sexually dimorphic nucleus of the POA

T - testosterone

VIP - vasoactive intestinal peptide
Introduction

The functional aromatase enzyme is a product of CYP19A1 gene and consists of 503 amino-acid residues and a heme group (protoporphyrin IX). Aromatase is a monooxygenase that transfers one oxygen atom from molecular dioxygen to the substrate and one to the water. There are several endogenous substrates for aromatase: androstenedione, testosterone (T), 16-α hydroxytestosterone and dihydrotestosterone (DHT), although DHT is only oxidized, but not aromatized \(^1\)\(^2\)\(^3\).

Aromatase complex consists of two highly conserved components: P450 aromatase and NADPH P450 reductase, a product of the POR gene. The expression of POR starts at the two-cell stage of embryonic development, and a germ-line deletion of this gene in mice results in embryonic lethality indicating its importance for embryogenesis \(^4\). POR contains FAD - and FMN - flavins which bind FAD and FMN, and act as a port of entry and exit, respectively, for the electrons transferred from the NADPH to POR \(^5\). A binding of NADPH induces a conformational change in POR to a “closed form” ready for inter-flavin electron transfer. When the connecting domain loop extends, the whole structure changes to an “open form”, which is also a preferred form when interacting with aromatase \(^6\)\(^7\).

Aromatase catalyzes an irreversible and complex transformation of androgens to estrogens and it is the only enzyme in vertebrates known to catalyze the aromatization of a six-membered ring \(^8\)\(^9\)\(^10\)\(^11\) (Fig 1). This reaction, first reported in 1959, involves three consecutive hydroxylations. When androstenedione is the substrate, the first two steps produce two 19-hydroxy steroids, 19-hydroxyandrostendione (19-OH AD) and 19-oxoandrostenedione (19-oxo AD). The third step of aromatase reaction is aromatization of the steroid A ring which results in the formation of estrone (E1) and formic acid \(^12\)\(^13\)\(^14\)\(^15\)\(^16\)\(^17\). The interaction between aromatase and POR is critical for this reaction and the extent of hydroxylations are highly dependent on this reductase \(^18\). Limitation of
POR results in reduced electron supply and increases the production of 19-OH AD and 19-Oxo AD relative to E1 19. Aromatase exists as a homodimer and oligomer, and forms heterodimers only with POR 20,21. A proposed ratio of 2:2 (1:1 aromatase homodimer x 2 POR) was suggested as this ratio leads to the greater activity and reduced release of 19-hydroxy steroids 20.

Aromatase binding pocket is about 400 Å³ big and consists of heme porphyrin rings and hydrophobic side chains, which form van der Waals contacts and tightly bind androstenedione, with C19 of the methyl group of the substrate androstenedione only 4 Å away from the Fe atom 2,22. Binding pocket hosts the proton relay network, and a doorway/access channel through which oxygen, water, and steroids can pass (Fig. 1 inset, dash-circled blue, the “gatekeepers” residues Arg192 and Glu483 are dash-circled red 22). Aromatase/membrane interface is critical for these access/egress channels and studies have shown that this access channel “breathes”, thus allowing steroids, oxygen, and water to enter and exit the binding pocket 23,24.

Detailed analysis of aromatase reaction steps demonstrated that aromatase allows a free dissociation of 19-hydroxy steroids, which has been attributed to its distributive-dissociative nature 10,25. Similar results were obtained from kinetic studies 14, and also in reconstitution assays 26. Thus, aromatase is a distributive enzyme, and 19-OH AD and 19-Oxo AD as aromatase reaction products can dissociate from the complex and may accumulate in the blood and tissues.
Documented presence and measurements of 19-hydroxy steroids in different cell lines, tissue, and blood

First report of the existence of 19-hydroxy steroids dates back to 1955 when Meyer incubated dihydroepiandrosterone (DHEA) and androstenedione with bovine adrenal homogenate preparations and discovered a 19-OH AD among a “wide galaxy of conversion products” 27. Later, it was demonstrated that 19-OH AD is also produced by placental and brain aromatase 28-30. These early measurements employed a radiometric method, in which a tritiated substrate androstenedione was used, and both a) a transfer of tritium to the water as an indicator of hydroxylation, as well as b) expulsion of the tritiated C19-fragment with the generation of $^3$H-formic acid, as an indicator of aromatization were measured 30-34. These experiments demonstrated much higher concentrations of tritiated 19-OH AD and 19-oxo AD than tritiated estrogens, indicating that these substantial hydroxylations were not followed by the aromatization step. However, it seems that results in these early studies were not corrected for the kinetic isotope effect known to be present in radiometric studies 14, thus they were later largely abandoned. In parallel to the radiometric methods, a radioimmune assay (RIA) was developed and used for measurements of 19-hydroxy steroids in human plasma 35-38. The RIA method was also used to observe an age-related decrease in endogenous plasma 19-OH AD and androstenedione levels 39.

Because different steroids with similar structures and differing only in their hydroxyl or carbonyl groups can cross-react with specific antibodies, their reliability have been recently questioned 40,41. Furthermore, radioimmunoassay can measure only one analyte at a time.

Currently, GC-MS and LC-MS are considered as accurate, efficient, and reliable methods for the measurement of steroids and their various metabolites 42. These methods have sufficient analytical selectivity and specificity and can overcome immunoassay deficiencies. Here, we
review literature related to measurements of 19-hydroxy steroids in the plasma, tissues, and cell lines.

The GC-MS method was used to measure 19-OH AD in serum from pregnant women and in placenta samples\(^\text{43}\). HPLC method was used to measure 19-OH AD production from COS cells with stable expression of porcine ovarian aromatase\(^\text{44}\). HPLC method was also used to evaluate the production of 19-hydroxy steroids from HEK293 cells expressing either porcine placental or gonadal aromatase\(^\text{45,46}\). We have also recently applied the LC-MS method to measure the release of 19-OH AD from the prostate cancer cells\(^\text{47}\). LC-MS method was recently applied to quantify the release of many steroids (using both targeted and untargeted approaches), and among them also 19-OH AD and 16-hydroxytestosterone, from the human adrenal H295R cells\(^\text{48}\), which also express aromatase transcripts\(^\text{49}\) and are positive for aromatase activity\(^\text{50}\).

Production of 19-hydroxy steroids and estrone was also measured using a bioelectrochemical method\(^\text{51}\). In addition, UPLC/MS-MS method was used to assess changes in steroid profiles from boar testis tissue, including 19-OH AD, in the presence and absence of aromatase inhibitors\(^\text{52}\).

Detectable presence of 19-hydroxy steroids in these studies demonstrate they are not just short-lived intermediates of the aromatase reaction but are likely active metabolites which functions have yet to be revealed.

The aromatase enzyme is expressed in brain, ovary, testis, placenta, adrenal gland, adipose tissue, bone, olfactory system, and also in some malignancies (such as breast cancer\(^\text{53}\), prostate cancer\(^\text{54}\), malignant human liver cell line HepG2 and HuH7\(^\text{55}\), malignant human lung carcinoma cell line A549 and LK87\(^\text{56}\) and human endometrial carcinoma\(^\text{57}\)), and its
expression is regulated by tissue-specific promoters. Several excellent reviews and manuscripts on aromatase and steroidogenesis have been already published, and here we will only review literature related to the measurements of 19-hydroxy steroids and their potential effects in various physiological and pathophysiological processes.

**Neurosteroidogenesis and potential roles for 19-hydroxy steroids**

All steroidogenesis enzymes are detected in fetal and adult brain, and aromatase in particular is present in the hypothalamus, preoptic area (POA), limbic lobe, the olfactory bulb, hippocampus, lateral septum, amygdala, bed nucleus of the stria terminalis and nucleus accumbens. Aromatase substrate androstenedione was also detected in the human adult brain. Furthermore, 3β hydroxysteroid dehydrogenase (3β HSD), an enzyme that converts DHEA to androstenedione (Fig 2), has been also detected, indicating that oxidation of DHEA to androstenedione is indeed possible in the adult brain. Recent mass spectrometry analysis of human proteome also detected 3β HSD in fetal brain, indicating that androstenedione can be also synthesized in the fetal brain. These results also suggest the potential presence of 19-hydroxy steroids in both fetal and adult brain, and that was indeed demonstrated.

Aromatization of androstenedione by human fetal hypothalamus and amygdala was first reported in 1971. Studies revealed a 2-fold greater aromatization in the male vs. female hypothalamus. These results provided a basis for the aromatization hypothesis, according to which, T synthesized by fetal testis diffuses into the male brain where it is locally aromatized and thus initiates the process of masculinization. In male mice, this process starts at E17 and extends until postnatal day P10. It is believed that differences that emerge during this initial phase result in sexually dimorphic circuits, and aromatase plays an essential role in brain development and sexual differentiation. Aromatase expression in sexually dimorphic
regions of the brain is at its highest level during this perinatal period, demonstrating its critical role in the development of sexually dimorphic patterning. In female embryos steroid secreting ovaries develop after the first postnatal week, and the process of feminization has been classically viewed as “default”. Estradiol presence was however detected in the female newborn brain, suggesting that female rat fetal brain can also synthesize estrogens de novo. This intriguing finding is hard to explain by the aromatization hypothesis, which implies that only circulating T is a precursor for estrogen because in the female fetal brain T is non-detectable. These results suggest potential roles for aromatase reaction products derived from androstenedione, warrant future research, and call for a re-evaluation of the initial aromatization hypothesis. Recent study by Nugent et al. demonstrated active repression of male-typical genes mediated by DNA methylation during the brain feminization process. Thus, the brain differentiation process is far from being clearly defined, and novel mechanisms, such as epigenetic control is currently being actively investigated.

**Do 19-hydroxy steroids accumulate during a critical perinatal period to contribute to the brain differentiation?**

Brain aromatase activity is developmentally regulated and expressed in different regions of the male rat brain. The number of aromatase positive neurons in rodent studies of both sexes increases during gestation and peaks around birth, with higher expression in males, but estrogen content although increased at birth in the male hypothalamus decreases significantly already at 2 hours after birth, at the time when aromatase activity is still high. Thus, there is a lack of a clear correlation between aromatase activity and estrogen content during this critical period, which may be indicative of potentially incomplete aromatase reaction. The evidence that the first 19-hydroxylation step can exceed the final third aromatization step in
male rat neonatal hypothalamus and amygdala was first published in 1985. These results demonstrated increased accumulation of 19-OH AD and 19-Oxo AD when compared to E1. In addition, it was demonstrated that the ratio of 19-hydroxylation/aromatization was similar in the neonate and adult rat hypothalamus in both sexes. However, since these results were obtained using a radiometric method, a more accurate and specific analysis using a more specific LC-MS method is now necessary to confirm these early results.

Estrogen injection in male rats 4 days after birth produced adults unable to achieve intromission, although they mounted as frequently as control animals. On the contrary, androstenedione injection during this period resulted in normal patterns of sexual behavior in adulthood. Blocking aromatase blocked the brain differentiation of the male rodent brain. These results indicate that aromatase reaction products derived from androstenedione may be implicated in brain differentiation during the critical perinatal period.

The sexually dimorphic nucleus of the POA (SDN-POA) in the hypothalamus is important for male copulatory behavior, and it is several-fold larger in males than in the female rats. T treatment of females during a perinatal period produced larger SDN-POA, similar to the one seen in males and application of aromatase inhibitor during this critical period reduced the size of SDN-POA and changed male copulatory behavior. As no sex differences in estrogen receptor expression (ERs) in SDN-POA has been detected, the current view is that ER expression has not been proven informative as the basis of sex differences during brain development. We suggest that conversion of T to androstenedione by 17β-hydroxysteroid dehydrogenase (HSD17B2) may occur. HSD17B2 is present in the fetal brain, where it can convert T to androstenedione, thus making it available to act as a substrate for aromatase during this critical period (Fig. 2). Recent elegant experiments using brain-
specific ArKO model demonstrated the importance of brain aromatase for testosterone-dependent male sexual activity and feedback regulation of T of testicular origin in the adult mice.

Ovaries

Human ovarian follicles synthesize estrogen in a compartmentalized fashion; androgens are produced in the outer theca interna cells layer, while estrogens are produced in the inner granulosa layer. This “two-cell” organization of follicular estrogen synthesis may have its basis in avoiding the competition between CYP17A1 and CYP19A1 for reducing equivalents provided by POR if both enzymes are expressed in the same cell. Follicle stimulating hormone (FSH) increases both aromatase and POR activity and induces differentiation of rat granulosa cells into steroidogenic cells.

Do ovaries produce 19-hydroxy steroids?

Ovarian synthesis of estrogens in ovarian granulosa cells is associated with the parallel synthesis of 19-OH AD and 19-Oxo AD and tritiated water assay indicated that these metabolites accumulate in higher quantities than estrogens. Production of 19-OH AD was also reported in the human ovarian HOSE 17 cells using RP-HPLC method. How exactly 19-hydroxy steroids affect ovarian function has not been investigated, but an intriguing hypothesis of their potential role in sperm chemotaxis described below is proposed.
Testes

Adult testicular germ cells express aromatase and estrogens play an important role in sperm maturation\textsuperscript{1,116}. Association between aromatase and sperm count and motility has been clearly established, and both 19-OH AD and 19-OH T were also detected in the testicular vein blood, suggesting their role in sperm motility\textsuperscript{117-122}. Recently, it has been demonstrated that prolonged treatment with letrozole decreased 19-OH AD levels in testis when analyzed by highly specific UPLC-MS/MS method, however the effects on sperm motility were not analyzed\textsuperscript{52}.

Is 19-OH AD involved in sperm motility and chemotaxis?

It is interesting to mention that about 90 transcripts of olfactory receptors (ORs) have been found in human spermatozoa\textsuperscript{123}. Previously, another olfactory receptor, hOR17-4, have been implicated in sperm chemotaxis\textsuperscript{124}, and more recently several highly expressed ORs have been detected in seminal plasma, sperm, testes and epididymis, using a high-resolution mass-spectrometer approach\textsuperscript{125}. Olfactory receptor OR51E2 is highly expressed on the acrosome cap, the midpiece, and the entire flagella in spermatozoa\textsuperscript{123}. Recently, 19-OH AD has been identified as a potent agonist for olfactory receptor OR51E2\textsuperscript{47}. Thus, activation of this receptor by 19-OH AD may contribute to sperm motility. In addition, secretion of 19-OH AD from the ovarian cells has also been reported\textsuperscript{115}. Taken together, these studies suggest that: a) 19-OH AD and 19-OH T originating from testes may contribute to sperm motility and b) 19-OH AD secreted from the ovarian cells may activate OR51E2 in the sperm, and thus contribute to sperm chemotaxis. Future studies are necessary to test these assumptions.
**Pregnancy and parturition**

An increase of 19-OH AD measured by GC-MS method during pregnancy was reported and 6-fold higher concentrations were detected at the end of the third trimester. This increase of 19-OH AD in the maternal blood is also combined with its dramatic decrease in the umbilical artery at delivery indicating that all 19-OH AD is completely transferred and taken up by the baby and/or placenta at delivery, while no such effect was observed with either T or E1. High amounts of 19-OH AD were also detected in the end-term placenta tissue, indicating that the placenta may be the major source of 19-hydroxy steroids production. Unfortunately, there was no follow-up on this study.

Taken together, these results suggest that 19-OH AD is likely to originate from the placenta. What is the function of this newly produced steroid and is it important for parturition? These remain open questions, as we still lack a full understanding of the role of different steroids in the parturition process.

A recent transcriptomic study of the fetal-maternal interface from Vento-Tormo R. demonstrate an overlapping aromatase and OR51E2 receptor presence in the syncytiotrophoblasts. As 19-OH AD is a ligand for OR51E2 receptor, it would be interesting to study their potential molecular interactions with respect to aromatase during early placentation period (https://maternal-fetal-interface.cellgeni.sanger.ac.uk/).

No correlation between androstenedione levels and gestation in normotensive pregnant women was found, however, in hypertensive women a highly significant correlation was demonstrated. Furthermore, increased levels of circulating T were found in women with preeclampsia and although the early studies reported an increase in circulating 19-OH AD in the hypertensive pregnant women, a subsequent study on the small number of participants did not support this
Thus, future studies on a larger sample-set are warranted to clarify if 19-hydroxy steroids play any role in pregnancy and parturition.

Adrenal glands

Adrenal steroidogenesis is regulated by sympathetic innervation, ACTH, and by complex paracrine interactions of inter-dispersed medulla and cortex cells. As aromatase is expressed in the adrenal gland it is highly likely that this gland also produces 19-OH AD. This has been indeed demonstrated in several early studies using the RIA method.

The secretion of 19-OH AD increased during ACTH and angiotensin II stimulation. These results support the view that 19-OH has an adrenal origin. In addition, these studies further strengthen the notion that the first C\19 hydroxylation step (in which 19-OH AD is produced under ACTH control) is to a certain extent unaccompanied by subsequent aromatization and thus may represent potentially significant physiologically relevant transformation. Regulation of 19-OH AD secretion from cultured human adrenal cells by ACTH was also demonstrated. Of note, when ACTH is suppressed, angiotensin II acts to stimulate secretion of 19-OH AD. Since the highest expression of aromatase in the adrenal gland was detected in zona reticularis, the synthesis of 19-OH AD is most likely to be in that area, however direct evidence for this is still lacking. Positive correlations between basal plasma 19-OH AD and androstenedione, as well as cortisol were reported, and suppression of 19-OH AD secretion by dexamethasone indicates that 19-OH AD secretion is regulated by the HPA axis (Fig. 3).

Various neuroendocrine modulators, such as epinephrine and vasoactive intestinal peptide (VIP) are released from the adrenal nerves, while androstenedione and other C\19 androgens are released from the adrenocortical cells, suggesting that these cells may also potentially release 19-OH AD. In contrast, it was demonstrated that a atrial natriuretic peptide (ANP)
decreases secretion of 19-OH AD. Cytokines produced by either immune cells within the gland or by adrenal cells can also affect adrenal steroidogenesis. For example, IL-6 activates the HPA axis and stimulates a release of ACTH, and also stimulates a release of aldosterone, cortisol, and DHEA. Thus, cytokines are likely to also increase 19-OH AD secretion. Further research is needed to prove these assumptions.

**What are the functional consequences of increased 19-OH AD secretion in Cushing’s disease?**

High levels of 19-OH AD were detected in Cushing’s disease, a benign pituitary adenoma, characterized by increased secretion of ACTH which stimulates adrenal glands to secrete cortisol and 19-OH AD, while decreased levels of 19-OH AD are seen in Cushing’s syndrome. Whether this secretion exerts a negative feedback loop in the hypothalamus is currently unknown (Fig. 3).

*Obesity* is one of the symptoms of Cushing’s disease, and since aromatase is abundantly expressed in adipose tissue, increased production of 19-OH AD from the adipose tissue is also likely. Future research is needed to unravel the functional consequences of high 19-OH AD found in the blood of patients with Cushing’s disease which also develop a hypertension.

**Hypertension and 19-OH AD**

19-OH AD-treated rats developed hypertension and elevated 19-OH AD was reported in patients with high renin essential hypertension. 19-OH AD and 19-Oxo AD also amplify the sodium-retaining action of aldosterone. These results suggested a potential role of the renin-angiotensin system (RAS) in 19-OH AD secretion. Aldosterone-producing adenoma patients have lower levels of circulating 19-OH AD, likely due to a suppressed RAS; however, it
seems that 19-OH AD does not play a causative role in hypertension seen in these patients.\cite{145} Future experiments are warranted to dissect the role of this steroid in hypertension. It is interesting to note that Olfr78, a mouse ortholog of human olfactory receptor OR51E2 is activated by 19-OH AD and is also expressed in renal afferent arterioles where it can affect renin release when stimulated with short chain fatty acids (SCFA) \cite{47,146}. Olfr78 KO mice also have lower circulating plasma renin \cite{146,147}. Olfactory receptors, like most GPCRs, are quite promiscuous, and it is possible that their activation by various agonists including 19-OH AD contributes to blood pressure regulation.

19-OH AD, POR and cancer

Aromatase overexpression in tumor tissue results from a shift in promoter use, which allows for activation of cAMP-dependent signaling pathway and results in increased estrogen synthesis.\cite{58} Both aromatase and CYP17A1 require POR for their electron transport and catalysis, and if expressed in the same cell, which is the case in the cancer cell, these two cytochrome enzymes compete with one another for POR, reducing equivalents NADPH, and O2. A 30-fold increase in CYP19A1 and a 17-fold increase in CYP17A1 was measured in the metastatic prostate cancer tissue, while the enzyme SRD5A2 (5α reductase) which converts T to DHT is decreased 9 fold (Fig. 3)\cite{148}. A disbalance in T:E ratio has been associated with prostate cancer progression and increased secretion of 19-OH AD was detected in the prostate cancer cells following activation of olfactory receptor OR51E2, indicating a potential role for aromatase reaction products in prostate carcinogenesis.\cite{47,149} Administration of abiraterone acetate (a CYP17A1 inhibitor), may actually save NADPH and POR for the aromatase reaction, thus driving androgen metabolism via aromatase with a consequent release of 19-OH AD, 19-Oxo AD, and estrone. This may potentially contribute to chemotherapy resistance. Steroid hormones stimulate prostate cancer
progression and ArKO mice have reduced prostatic hyperplasia and incidence of prostate cancer following exposure to T and estrogens, indicating that 19-hydroxy steroids are likely involved in prostate carcinogenesis.\textsuperscript{150,151}

Aromatase inhibitors have major roles in the treatment of hormone-sensitive breast cancer and recently POR was identified as an independent prognostic biomarker of short recurrence-free survival of triple-negative breast cancer patients.\textsuperscript{152} It has been demonstrated that patients with triple-negative breast cancer and with high POR expression in the primary tumors have a twofold higher risk of tumor recurrence.\textsuperscript{153}

Conclusion

A list of potential roles of 19-hydroxy steroids, and of 19-OH AD in particular, in various physiological and pathophysiological processes is presented in Fig. 3.

Research in the brain differentiation process started over 5 decades ago, and although much has been learned, it is still not completely understood. We believe that future studies of steroid metabolites, and in particular 19-hydroxy steroids, using state of the art analytical tools will help to better understand this extremely complex process.

Striking data from the pregnancy studies indicate an underappreciated role of 19-OH AD and should be followed by future studies.

Both ovarian and testicular synthesis of 19-OH AD has been documented, but its role has not been examined so far.

Increased secretion of 19-OH AD in Cushing disease may warrant future research to determine its role in disease pathology. Could 19-OH AD serve as a diagnostic biomarker in the Cushing's
As 19-OH AD secretion from the adrenal gland is under the HPA axis, we assume it will be also involved in stress-related behaviors. Does it contribute to hypertension?

Many questions are still remaining unanswered.

Is 19-OH AD an androgen? Does 19-OH AD produced by adrenals send negative feedback to the hypothalamus? Do 19-hydroxy steroids act as ligands for other GPCRs (except OR51E2), transporters, or channels?

Which signaling pathways are regulated by 19-hydroxy steroids?

This review raises interesting questions that merit further investigation. We hope that it will stimulate future studies related to the roles of 19-OH AD and 19-Oxo AD in the brain, pregnancy, blood pressure regulation, Cushing’s disease and cancer.

Numerous studies related to aromatase reaction have been listed here, and many more are certainly missing, but we hope that the information provided will stir discussion and stimulate future research endeavors. As our analytical sensitivity and methodology are nowadays significantly improved, it is time to re-examine 19-hydroxy steroids, products of aromatase reaction.
Acknowledgments

We are grateful to Dr. Maira Harume Nagai and Professors Jennifer L. Pluznick and F. Peter Guengerich for suggestions and critical reading of the manuscript.

Funding

NIH/NIDCD R01DC016224

Data Availability Statement: Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.
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Figure Legends

Figure 1. Aromatase reaction. The oxidation of androstenedione to estrone by aromatase complex involves NADPH, NADPH P450 reductase and aromatase. It does not follow a clear linear trajectory of sequential reactions, but has a distributive character, where 19-OH AD and 19-oxo AD freely dissociate from the aromatase binding site and enter blood and/or tissue or re-enter the aromatase reaction again for further oxidation and estrone production. 19-OH AD=19-hydroxyandrostenedione, 19-oxo AD=19-oxo-androstenedione (adapted from 154 and 25).

Inset: A closeup look at the aromatase binding pocket with a substrate androstenedione. The residues lining binding pocket are labelled as: hydrophobic-green, acidic-red, basic blue, polar-purple and S-containing yellow. Residues associated with a doorway/access channel are dash-circled (adapted from 22) and in dash-circled red Arg 192 and Glu 483 gatekeepers are indicated.

Figure 2. Aromatase acts on substrates androstenedione and testosterone- producing a variety of 19-hydroxy steroids. 17β HSD enzymes catalyzing the conversion of T to AD and AD to T, as well as E1 to E2 and E2 to E1 are indicated. 3β HSD mediates oxidation of DHEA to AD. The third substrate for aromatase reaction is 16-α hydroxytestosterone. It has been omitted here, as very little is known about it, and it has not been discussed. T is converted to DHT by the enzyme SRD5A2 (5α-reductase)
Figure 3. Regulation, detection and the potential involvement of 19-OH AD in human physiology and pathophysiology. Secretion of 19-OH AD is under control of the hypothalamic pituitary adrenal axis (HPA) and is directly stimulated by ACTH. LH and FSH acting on testis and ovary also increase 19-OH AD secretion via the hypothalamic pituitary gonadal (HPG) axis. Cytokines likely indirectly increase 19-OH AD via ACTH. Positive and negative regulation of 19-OH AD secretion by VIP, epinephrine, and ANP are indicated. 19-OH AD increases blood pressure and renin secretion and is increased during pregnancy and Cushing’s disease. Tissues, where 19-OH AD has been measured, are indicated, and a list of potential involvement in several physiological and pathophysiological processes is presented.
Figure 2

Cholesterol → CYP11A1 → CYP17A1 → 17α OH → Dehydroepiandrosterone → DHEA → 3β HSD

Pregnenolone → CYP17A1 → 17α OH

Androstenedione → 17β HSD3
Androstenedione → 17β HSD2

Testosterone → 17β HSD2

19-hydroxytestosterone → CYP19A1 → 19-OH T
19-hydroxyandrostenedione → CYP19A1 → 19-OH AD

19-oxoandrostenedione → CYP19A1 → 19-Oxo AD

19-oxotestosterone → CYP19A1 → 19-Oxo T

Estrone → 17β HSD1 → 17β Estradiol

Estradiol → 17β HSD2 → Estradiol

Dihydrotestosterone → SRD5A2
Figure 3

**HPG axis**
- Hypothalamus
  - GnRH
  - CRH
- Anterior Pituitary
  - LH
  - FSH
  - Testis
  - Ovary
- 19-OH AD
  - Sperm motility and chemotaxis

**HPA axis**
- Hypothalamus
  - CRH
- Anterior Pituitary
  - ACTH
- Cytokines
- Adrenals
  - Aldosterone
  - Cortisol
  - VIP
  - Epinephrine

**Detected 19-OH AD**
- Blood
  - Pregnancy
- Tissue
  - Hypothalamus
  - Amygdala
  - Gonads
  - Adrenals
- Disease
  - Cushing’s disease

**Potential involvement**
- Brain differentiation during critical period
- Pregnancy and parturition
- Sperm motility and chemotaxis
- High renin essential hypertension
- Prostate cancer

**Heart**
- ANP
- Renin

**Kidney**
- Blood pressure
- Angiotensin II