CHAPTER 4

Steroid Hormones and Other Lipid Molecules Involved in Human Reproduction

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Lipids serve as metabolic substrates, structural components of cellular membranes, and signaling molecules. This chapter reviews the diversity of lipid molecules that participate in signal transduction related to human reproduction, including the nomenclature, the general features of their synthesis and metabolism, the ways in which these processes are controlled physiologically, the ways in which they can be modified by pharmacologic intervention, and some disorders that interfere with normal synthesis and metabolism. The chapter emphasizes steroid hormones and eicosanoids because of their prominent roles in regulating sexual differentiation, the normal function of tissues involved in reproduction, and the pathophysiology of disorders affecting those tissues.

Steroid Hormones: Structure and Nomenclature

- *The steroid hormone backbone name is not synonymous with biological activity.*
- *The location of hydroxyl groups and whether they reside in the α (below the plane of the steroid nucleus) or β (above) configuration have major impact on the biological activity of steroid hormones.*
- *Synthetic "bioidentical" steroid hormones may contain a mixture of stereoisomers that affect potency.*

Steroid hormones and the secosteroid prohormone, vitamin D, belong to an ancient family of signaling molecules with diverse functions, including central roles in the regulation of female and male reproductive processes. The human steroid hormones are derived from cholesterol, an abundant plasma lipid and a structural component of plasma membranes, and other organelles. Seemingly subtle modifications of the four fused rings of the sterol skeleton and side chain result in molecules with different activities.

Steroid hormones share a cyclopentanoperhydrophenanthrene backbone. Each carbon in this fused-ring structure is assigned a number identifier, and each ring is assigned a letter (Fig. 4.1). The families of naturally occurring steroid hormones are named according to the saturated ring structures of the parent compound: *cholestanes*, of which cholesterol (5-cholesten-3β-ol) is a representative, have 27 carbons; *pregnanes* have 21 carbons (e.g., 4-pregnen-3-20-dione, also known by its trivial name, *progesterone*); *androstanes* have 19 carbons (e.g., 17β-hydroxy-4-androsten-3-one, or *testosterone*); and *estranes* have 18 carbons (e.g., 1,2,5[10]-estratriene-17β-ol, or *estradiol*). *Gonanes* contain 17 carbons (the cyclopentanoperhydrophenanthrene backbone) represented by synthetic progestins (e.g., desogestrel, norgestimate, gestodene). The backbone name is *not* synonymous with biological activity, which is determined mainly (but not exclusively) by which members of a family of nuclear transcription factors (the classical steroid hormone receptors) the molecules activate. For example, cortisol and progesterone are members of the pregnane family, yet they have different biological activities and act through different receptors. Synthetic molecules that activate the progesterone receptor are derived from androgens containing 18 carbons (19-nortestosterone derivatives) and are, therefore, members of the estrane family. 27-Hydroxycholesterol, a member of the cholestane family, is a selective estrogen receptor modulator.[1]

The locations of substituents in the steroid backbone are indicated by the carbon number to which they are attached. Substituents at several positions have a significant effect on metabolism and biological activity of steroid hormones, including carbons 3, 7, 11, and 17. Atoms attached to asymmetric centers are, by convention, given the designation α

Abstract

Lipids serve as metabolic substrates, structural components of cellular membranes, and signaling molecules. The steroidogenic machinery is compartmentalized at the organ, cellular, and subcellular level. Steroidogenic cells do not store premanufactured steroid hormones, and the rate-limiting step in the production of steroid hormones is the movement of substrate cholesterol to the inner mitochondrial membrane, where the first committed step in steroidogenesis takes place, which is the cholesterol side-chain cleavage reaction in which cholesterol is converted into pregnenolone. Several families of enzymes are involved in the synthesis of steroid hormones, the most important being the cytochrome P450 family and the hydroxysteroid dehydrogenases and reductases. In addition to steroid secreting glands, bioactive steroids are produced in small quantities in many cell types.

Eicosanoids are derived enzymatically from 20-carbon polyunsaturated fatty acids (e.g., arachidonic acid). They are produced by almost all cells in response to hormonal stimulation or trauma, acting as paracrine or autocrine modulators. Specificity of eicosanoid action is afforded by selective expression of biosynthetic enzymes and their receptors. Eicosanoids include products of the cyclooxygenase (COX), lipoxygenase (LOX), and P450 enzyme acting on arachidonic acid and other polyunsaturated fatty acids. Prostanoids are formed by the COX enzymes. They are essential for ovulation and have been implicated in the processes of implantation, embryogenesis, and parturition.

Keywords

Aromatase,
cholesterol,
cholesterol side-chain cleavage enzyme,
cyclooxygenase,
cytochrome P450,
disorder of sexual development,
eicosanoids,
hydroxysteroid dehydrogenase,
leukotriene,
lipoproteins,
lipoxygenase,
lysophosphatidic acid,
prostanoid,
prostaglandin,
prostacyclin,
reductase,
sex hormone binding globulin,
sphingosine-1-phosphate,
steroidogenic acute regulatory protein,
steroidogenic factor-1,
thromboxane,
vitamin D

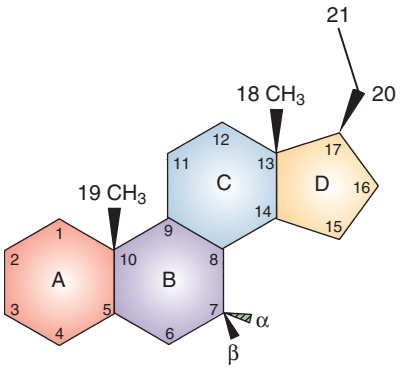

FIGURE 4.1 The steroid nucleus. Rings are identified with capital letters and carbon atoms are numbered. Substituents and hydrogens are shown projecting above *(β, solid line or filled triangle)* or below *(α, dashed line or hashed triangle)* the plane of the steroid nucleus.

if they project below the plane of the ring structure (in figures of structures, a dashed line or hatched triangle indicates the α configuration). The designation β (a solid line or filled triangle) is given to atoms that project above the plane. Hormone receptors and steroid binding proteins (e.g., sex hormone binding globulin [SHBG]) distinguish between stereoisomers. In the case of estrogen receptors, 17β-estradiol is active, but 17α-estradiol is essentially inert. In the case of androgen receptors, testosterone with a 17β-hydroxyl configuration is active, but epitestosterone with a 17α-hydroxyl configuration has little activity.

Different enzymes catalyze the oxidation or reduction of α and β hydroxyl groups and reduce the Δ^4 double bond in the steroid A ring to form 5α or 5β molecules. Such 5α-reduced steroids can be active (e.g., 5α-dihydrotestosterone, which activates androgen receptors), or inactive (e.g., 5α-dihydroprogesterone) with respect to classical steroid hormone receptor function. However, the notion that pregnane 5α-reduced compounds are inactive has been challenged by the revelation that 5α-reduced glucocorticoids may have antiinflammatory activity acting through glucocorticoid receptors.[2] Although 5β-reduced steroids are usually not capable of activating classical steroid hormone receptors, these steroids and some 5α-reduced molecules exert biological effects outside of the family of steroid hormone receptors, including modulation of neurotransmitter receptor function.

The naturally occurring steroid hormones are rarely referred to in the medical literature by their systematic names, which designate the parent structure and the number, location, and (if appropriate) orientation of substituents; instead, the trivial names are preferred (e.g., testosterone rather than 17β-hydroxy-4-androsten-3-one).

Steroid hormones used in clinical practice are derived from biological sources (e.g., conjugated equine urinary estrogens); semisynthetic, produced usually from a plant sterol starting material (e.g., stigmasterol or diosgenin) that can be chemically modified to yield a structure that is identical to the naturally occurring molecules; totally synthetic, which may result in the production of hormone isomers with different biological activity; or steroid drugs that are molecules not found in humans and animals (e.g., medroxyprogesterone acetate and norgestrel). "Bioidentical" steroid hormones produced by chemical synthesis may contain isomers that are not natural and may result in biological activities that differ from the steroid hormones produced in humans. Because of this concern, it has been argued that the term *bioidentical* steroid hormones can be misleading.

Organization of Steroidogenic Organs and Cells

- *The steroidogenic machinery is compartmentalized at the organ, cellular, and subcellular level.*
- *The biosynthesis of estrogens usually requires the concerted effort of two different organs or cell types.*

The steroidogenic machinery is compartmentalized at the organ, cellular, and subcellular levels, which has important implications for the control of steroid hormone production.[3-6] Steroid synthesis involves a series of sequential modifications of cholesterol that result in the clipping of the side chain; alterations in olefinic bonds; and the addition of hydroxyl functions, proceeding invariably (although some have argued that shortcuts do exist) from cholesterol through the pregnane, androstane, and finally, estrane families.

Specific cell types can accomplish several of these sequential steps, but rarely can they generate an estrogen from cholesterol. The requirement for cooperative efforts by two different tissues or cell types is a characteristic of estrogen biosynthesis. This joint effort enables the modulation of both androgen and estrogen production by factors that independently influence the cells involved in precursor synthesis, in addition to the cell type in which the final step, aromatization, occurs.[7] This cooperation is exemplified by estradiol synthesis in the ovarian follicle, where luteinizing hormone (LH) acts on the theca cells to stimulate production of androgen precursors and follicle-stimulating hormone (FSH) acts on granulosa cells to stimulate aromatization of these androgens into estrogens. Placental estrogen synthesis likewise requires precursors from another tissue, the fetal adrenal gland, that is under the control of fetal pituitary adrenocorticotropic hormone (ACTH). The sulfated dehydroepiandrosterone (DHEA) secreted from the fetal zone of the adrenal cortex has negligible androgenic activity in the fetus and increased solubility in plasma, so it can be efficiently transported to the placenta where cleavage of the sulfate group, followed by aromatization, occurs in the syncytiotrophoblast. Cooperative interaction among different cell types is also important in regulating the production of steroid hormones in the brain, and bioactive hormone production in endometriotic lesions, and breast and endometrial cancers.

Another example of compartmentalization of the steroidogenic machinery at the organ level is the adrenal cortex, which has histologically and functionally distinct zones that determine the relative production rates of mineralocorticoids, glucocorticoids, and adrenal androgens.[4,8] The zona glomerulosa synthesizes mineralocorticoids; the zona fasciculata, glucocorticoids; and the zona reticularis and fetal zone of the fetal adrenal cortex produce androgens. One major functional distinction between the zona glomerulosa and the zonae fasciculata and reticularis is that aldosterone synthase is exclusively expressed in the zona glomerulosa, but 17α-hydroxylase/17,20 desmolase (CYP17A1, also referred to as P450c17) is not. However, CYP17A1 is abundant in

the zonae fasciculata and reticularis. The zona reticularis expresses lower levels of type 2,3β-hydroxysteroid dehydrogenase (encoded by *HSD3B2*). Because the lyase activity of human CYP17A1 does not efficiently act on progesterone, reduced conversion of pregnenolone to progesterone resulting from the low 3β-hydroxysteroid dehydrogenase activity facilitates the conversion of pregnenolone into DHEA in the zona reticularis. Higher levels of cytochrome b_5 in the zona reticularis, which increase the lyase activity of human CYP17A1 by an allosteric mechanism, further augment the capacity to produce DHEA. The zona reticularis also has high levels of sulfotransferase. This constellation of enzymatic activities favors synthesis of DHEA sulfate.

Acquisition, Storage, and Trafficking of Cholesterol

- *Steroidogenic cells do not store premanufactured steroid hormones.*
- *Cholesterol is acquired for steroidogenesis from plasma lipoproteins, de novo synthesis, and hydrolysis of sterol esters stored in lipid droplets.*
- *Lipoprotein-derived cholesterol is the primary substrate for cells that elaborate large quantities of steroid hormone.*
- *The rate-limiting step in steroidogenesis is the movement of cholesterol to the inner mitochondrial membrane.*

Steroidogenic cells have structural features that enhance their ability to obtain and store cholesterol for use in steroidogenesis (Fig. 4.2).[5,9] Unlike protein hormone–producing cells, steroid-producing cells do not store prefabricated hormone; they synthesize the hormones on demand from cholesterol that has been acquired from the plasma, synthesized de novo, or stored in membranes, or as sterol esters in lipid droplets. Because of cholesterol's limited solubility in water (it forms aggregates at 25 to 40 nM [the critical micelle concentration]), multiple proteins are required to move the steroid hormone precursor efficiently from one cellular compartment to another.

The plasma membrane has the highest content of free cholesterol, which is derived from plasma lipoproteins and de novo sterol synthesis. This sterol pool is not static; it exchanges with plasma free cholesterol, and regularly cycles through the cell and back to the plasma membrane. During this cycling process, sterols can be diverted for use in steroid hormone synthesis or esterified and deposited in lipid droplets.

Numerous microvilli project from the plasma membrane on which lipoprotein-gathering receptors of the low-density lipoprotein (LDL) receptor family are located (e.g., LDL receptors; LDL receptor–related protein, very–low-density lipoprotein [VLDL] receptors).[5] These receptors mediate lipoprotein uptake by an endocytic mechanism that delivers the lipoproteins to the lysosomes where the apolipoproteins are degraded. The lipoprotein cholesterol esters are then hydrolyzed by acid lipase (lipase A encoded by the *LIPA* gene) to release free cholesterol. Severe acid lipase deficiency (Wolman disease) is associated with lysosomal accumulation of cholesterol esters and triglycerides, which can lead to damage of steroidogenic cells and compromised hormone production.[5] Stimulation of steroidogenic cells by trophic hormones increases the number of LDL receptors on the cell surface and accelerates the rate of LDL internalization and degradation.

Free cholesterol is released from lysosomes through a system of sterol binding proteins encoded by genes *(NPC1, NPC2)* that when mutated cause the cholesterol storage disorder, Neimann-Pick type C disease. NPC2, a soluble protein in the lysosome, delivers free cholesterol to NPC1, a membrane-associated cholesterol binding protein that controls sterol efflux from the lysosomes. Other sterol binding

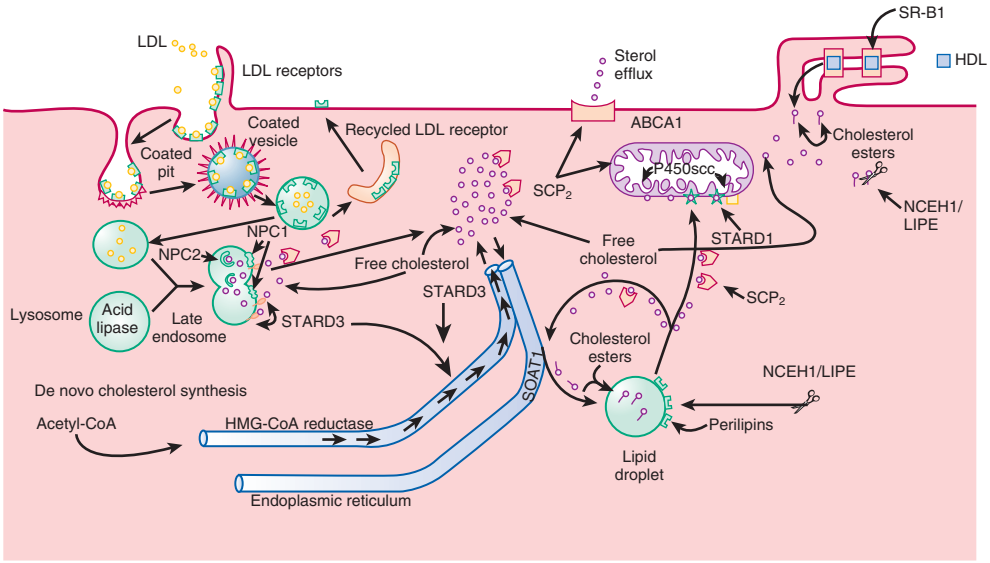

FIGURE 4.2 The acquisition, storage, and trafficking of cholesterol in steroidogenic cells. *ABCA1*, ATP-binding cassette transporter A1; *FFA*, free fatty acid; *HDL*, high-density lipoprotein; *HMG-CoA* reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; *LDL*, low-density lipoprotein; *LIPE*, hormone-sensitive lipase; *NCEH1*, neutral pH cholesterol ester hydrolase; *NPC*, Neimann-Pick type C; *SR-B1*, scavenger receptor type B; *STARD1*, steroidogenic acute regulatory protein; *STARD3*; (steroidogenic acute regulatory protein)-related lipid transfer domain 3; *SCP_2*, sterol carrier protein 2; *SOAT1*, sterol-O-acyltransferase-1; *VDAC2*, voltage-dependent anion channel 2.

proteins, including *sterol carrier protein-2* (SCP_2), STAR-related lipid transfer (START) domain protein 3 (STARD3), which is also known as metastatic lymph node 64 protein (MLN64), STARD4, and STARD5 may participate in the intracellular trafficking of cholesterol to various organelles including from endosomes to the endoplasmic reticulum, peroxisomes, and ultimately to the mitochondria.[5,10-12] In steroidogenic glands regulated by tropic hormones (e.g., ovary, testis and adrenal cortex), the delivery of cholesterol substrate to the ultimate destination, the mitochondrial cholesterol side-chain cleavage enzyme, is effected by the steroidogenic acute regulatory protein (StAR or STARD1).[5]

High-density lipoproteins (HDL) can also provide cholesterol for hormone synthesis by a pathway that differs from the "LDL pathway."[3,13] Receptors for HDL (scavenger receptor type B, class 1 [abbreviated SR-B1]) are located in closely apposed microvilli that form "microvillar channels" in which HDL particles are lodged.[3] Endothelial lipases (hepatic lipase or endothelial cell–derived lipases) may facilitate uptake of the HDL-carried sterols by steroidogenic cells, including the selective uptake the HDL sterol esters. Similar to LDL receptor expression, SR-B1 expression is upregulated in response to trophic stimulation, facilitating the usage of HDL-delivered substrate.

HDL cholesterol esters are selectively internalized by SR-B1, leaving the apolipoproteins on the cell surface. The internalized unhydrolyzed HDL cholesterol esters are then cleaved by a cytosolic, neutral pH optimum sterol esterase (also referred to as *hormone-stimulated lipase*, encoded by *LIPE*, or *neutral cholesterol ester hydrolase*, encoded by *NCEH1*), thereby releasing free cholesterol.[3,9,13]

De novo synthesis of cholesterol, a process that involves at least 17 enzymes, occurs primarily in the abundant smooth endoplasmic reticulum (SER).[5] Some steroidogenic cells have up to 10-fold more SER by volume than rough endoplasmic reticulum. In certain cells, the SER takes on unique forms, exemplified by the whorls found in testicular Leydig cells. Enzymes involved in steroid hormone formation and metabolism are also embedded in the SER. Trophic hormones that stimulate steroidogenesis generally increase both cellular cholesterol synthesis and lipoprotein uptake.

Several studies have suggested that de novo cholesterol synthesis generates important local regulators of reproductive function in addition to the substrate for steroid hormone synthesis. The biosynthetic intermediates between lanosterol and cholesterol have been shown to stimulate oocyte maturation in in vitro assays and have also been proposed to play roles in spermatogenesis.[14,15] These 4,4-dimethyl sterols, referred to as *meiosis-activating sterols*, contain 29 carbons and are found in the testis and follicular fluid in low micromolar concentrations. However, their physiological role in gamete maturation in vivo is uncertain based on results from germ cell-specific knockout mouse models (targeted deletion of cytochrome P450 lanosterol 14α-demethylase, Cyp51A1) that indicate that de novo synthesis of meiosis-activating sterols is probably not essential for reproduction.[16] The potential use of meiosis-activating sterols in clinical settings of animal and human reproduction remain subjects of debate.

The quantitative importance of circulating cholesterol carried by LDL, HDL, and other lipoproteins as steroid hormone precursor in tissues that produce large amounts of steroid hormone (e.g., the corpus luteum and adrenal zonae fasciculata and reticularis), as opposed to de novo cholesterol synthesis, is demonstrated by the fact that radiolabeled plasma cholesterol in humans is almost fully equilibrated with the steroidogenic pool of cholesterol. Additional evidence for an important role of circulating lipoprotein cholesterol in steroidogenesis comes from the study of hypobetalipoproteinemia, a disorder in which there is virtually no circulating LDL.[17,18] This rare metabolic disease is associated with reduced adrenocortical steroid production and diminished progesterone levels in the luteal phase and in pregnancy, although the lower levels of progesterone elaborated are still sufficient to achieve a term pregnancy. In the case of HDL, individuals with an SR-B1 missense variant (Pro297Ser) that reduces uptake function have increased HDL levels and attenuated adrenal steroidogenesis in response to ACTH stimulation.[13]

Individuals with familial hypercholesterolemia due to inactivating mutations in the LDL receptor have modest impairment of steroidogenic gland function, reflecting the capacity of alternative sterol acquisition mechanisms to compensate for LDL receptor deficiency. The commonly used cholesterol-lowering statins (which inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase [HMG-CoA reductase], the rate-limiting enzyme in de novo cholesterol synthesis) do not impair adrenal, testicular, or luteal steroidogenesis in adult humans despite the lowering of plasma LDL levels.[19,20]

Smith-Lemli-Opitz syndrome, an autosomal recessive disease, offers insight into the relationship of plasma cholesterol and de novo sterol synthesis for the supply of precursors for fetal steroidogenesis.[21] The disease is caused by inactivating mutations in an enzyme involved in the terminal steps of cholesterol synthesis, 3β-hydroxysteroid Δ^7-reductase (encoded by *DHCR7*). As a result, cholesterol levels are low and 7-dehydrocholesterol levels are elevated. Hypospadias or ambiguous genitalia are frequent findings in affected male neonates, reflecting diminished fetal testicular testosterone synthesis. Estrogen production during pregnancy is also reduced, due to impaired fetal adrenal hormone production. Adrenal insufficiency has been reported in some affected individuals, although others compensate with elevated ACTH secretion.[21,22] B-ring unsaturated equine-like steroids (1,3,5[10],7-estratetrenes) are produced from the 7-dehydrocholesterol that accumulates, showing that the steroidogenic enzymes do not have an absolute requirement for cholesterol as a substrate.[23] Desmosterolosis, a rare autosomal recessive disease caused by mutations in 3β-hydroxysterol Δ^{24}-reductase (encoded in *DHCR24*) is also associated with ambiguous genitalia in affected males, presumably because of impaired fetal testicular testosterone synthesis.[21]

Cytoplasmic lipid droplets represent another major depot of substrate in steroidogenic cells. As much as 80% of the total cholesterol content of steroidogenic cells is found esterified in these droplets. The sterol esters are synthesized in the endoplasmic reticulum from cholesterol acquired from lipoproteins or de novo synthesis by sterol O-acyltransferase 1 (SOAT1, previously named acyl-coenzyme A, cholesterol acyltransferase-1 or ACAT1), encoded by one of two related genes.[24] The esters generated by SOAT1 accumulate within the SER, and subsequently bud off as lipid droplets (see Fig. 4.2). A targeted mutation in the *Soat1* gene in mice results in markedly reduced sterol ester storage in the adrenal

cortex without impairment of basal or ACTH-stimulated corticosterone production by adrenal cells. These findings suggest that transit through the sterol ester pool is not part of an obligatory itinerary for steroidogenic cholesterol.

The limiting membranes of the lipid droplets contain a family of proteins called perilipins.[25,26] These proteins protect the droplet contents from hydrolysis in the basal state. They also serve as scaffolds, anchoring lipases to the lipid droplet surface, and mediating physical and functional interactions between lipid droplets and other organelles, including mitochondria. Mobilization of cholesterol ester stores occurs when cells are stimulated by trophic hormones. An adenosine 3′,5′ cyclic monophosphate (cAMP)-mediated process leads to phosphorylation of perilipins by protein kinase A and subsequent detachment of perilipins from the droplet surface, giving lipases access to the sterol esters.

The lipases that free cholesterol from lipid droplet sterol esters are hormone-sensitive lipase and neutral cholesterol esterases (see Fig. 4.2).[9,27-29] Protein kinase A activates hormone-sensitive lipase by phosphorylation of serine residues, promoting binding of the sterol esterase to lipid droplets. This enzyme's role in steroidogenesis was suggested by the reduced production of corticosterone under ACTH stimulation associated with an accumulation of lipid droplets in the adrenal cortex of mice deficient in *Lipe*. Targeted mutation of both the *Lipe* and *Nceh1* genes results in adrenal enlargement and lipid accumulation but no impairment in ACTH-stimulated corticosterone production. These different mouse models suggest a role for both hormone-sensitive lipase and neutral cholesterol ester hydrolase activities in mobilization of sterol esters. The variable impact of enzyme deficiency on steroidogenesis in these murine models may be related to genetic background or the age of mice at the time of study since lipid accumulation may have secondary deleterious effects on cell function beyond the disruption of sterol ester hydrolysis.

The size and number of lipid droplets change as the ester pool expands or contracts.[30] The quantity of sterol ester stored is determined by the availability of cholesterol to the cell through de novo synthesis, through accumulation of lipoprotein-carried cholesterol, and by the steroidogenic activity of the cell. Trophic stimulation promotes cholesterol ester hydrolysis and diverts cholesterol into the steroidogenic pool away from SOAT1, preventing re-esterification and resulting in a net depletion of cholesterol from the lipid droplets. Conversely, pharmacologic blockade of steroid hormone synthesis (e.g., with the cholesterol side-chain cleavage inhibitor, aminoglutethimide) or defects in cholesterol use for steroidogenesis (e.g., congenital lipoid adrenal hyperplasia) increase sterol ester storage by increasing the amount of cholesterol available to SOAT1.[5]

The exact intracellular itinerary of lipoprotein-derived cholesterol, free cholesterol from the plasma membrane, or free cholesterol released from lipid droplets remains to be elucidated. Much is still unknown regarding the methods in which sterol is presented to the mitochondria, which is where the first step in steroidogenesis occurs. It is likely that sterol distribution to and from organelles occurs through a dynamic vesicular–tubular late endosomal compartment and through the assistance of lipid transfer proteins.[5] The lipid transfer proteins involved in this process may include ATP binding cassette transporter G1 (ABCG1), proteins with a structure resembling StAR (STARD1), including its paralogues, STARD3, STARD4, STARD5 (see Fig. 4.2), and SCP_2.[5,10-12] The specific roles of these proteins remains to be clarified and their functions may be redundant, since knockout mouse models with the exception of STARD1 do not show strong steroidogenic phenotypes.

The mitochondria of steroidogenic cells are frequently found in close association with cytoplasmic lipid droplets, which may facilitate movement of substrate from these depots to the mitochondria. They have tubulovesicular cristae in contrast to the lamellar cristae that are characteristic of mitochondria in other cells. The remodeling of cristae in the human syncytiotrophoblast is believed to facilitate steroidogenesis. The inner mitochondrial membranes contain the cholesterol side-chain cleavage enzyme, which catalyzes the first step in cholesterol metabolism into steroid hormones leading to the formation of pregnenolone. The hydrophobic cholesterol substrate must move from the mitochondrial outer membrane across the aqueous intermembranous space to reach the inner membrane. This translocation process is the major rate-limiting step in steroidogenesis.[5] The capacity to produce large amounts of steroid hormone in rapid response to trophic stimulation requires the action of STARD1, the prototypic member of the START domain family, which greatly enhances the flux of substrate to the side-chain cleavage system (see Fig. 4.2).[5] The mitochondrial cholesterol side-chain cleavage system is also juxtaposed to downstream enzymes in the steroidogenic pathway on the endoplasmic reticulum, allowing for efficient metabolism of pregnenolone.

Regulation of Cellular Cholesterol Balance

- *Cellular uptake of lipoprotein cholesterol and de novo synthesis are tightly regulated.*
- *Tropic hormones that stimulate steroidogenesis enhance lipoprotein cholesterol uptake and de novo cholesterol synthesis.*

Cellular free (nonesterified) cholesterol balance is highly regulated by transcriptional, posttranscriptional, and post-translational mechanisms. The expression of genes involved in cholesterol biosynthesis (e.g., the gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase, *HMGCR*) and the uptake of plasma cholesterol *(LDLR)* are controlled by master transcription factors, the sterol regulatory element-binding proteins (SREBF1, which encodes SREBP-1a and SREBPB-1c, and SREBF2, which encodes SREBP-2).[5,31] SREBP-1a and SREBP-2 are the key transcription factors regulating cholesterol synthesis. The SREBPs are synthesized as inactive precursors that are bound to the endoplasmic reticulum. They interact with regulatory proteins, SREBP-cleavage activating protein (SCAP) and the insulin-induced genes 1 and 2, which inhibit SCAP. When cells are depleted of sterols, SCAP transports the SREBPs from the endoplasmic reticulum to the Golgi apparatus where SREBPs are cleaved through the action of two different proteases, releasing an NH_2-terminal domain of the transcription factors that enter the nucleus where they activate genes controlling lipid synthesis and uptake. Cholesterol loading inhibits the movement of SREBPs to the Golgi and consequently the proteolytic

processing, resulting in reduced transcription of the cholesterol synthesis and uptake genes. A micro ribonucleic acid, miR-33, derived from an intron located within the gene encoding SREBP-2, posttranscriptionally modulates expression of genes involved in cellular cholesterol balance.

Metabolites of cholesterol, the side-chain oxygenated sterols, including 22-, 24-, 25-, and 27-hydroxycholesterol, and 7-hydroxylated sterols, are endogenous regulators of cellular cholesterol metabolism acting through the liver X receptors (LXRalpha and LXRbeta, also known as NR1H3 and NR1H2, respectively).[32,33] In addition to LXRs, *steroidogenic factor 1* (SF-1, also known as NR5A1) and a related protein liver receptor homolog-1 (LRH-1, also known as NR5A2) play roles in regulating genes encoding cholesterol metabolizing enzymes including *SOAT1*, *LIPE*, and *STARD1*.

Posttranslational mechanisms influencing sterol synthesis and uptake include cholesterol-induced ubiquitination of HMG-CoA reductase, which tags the protein for degradation by proteasomes. Another posttranslational mechanism by which cells control sterol balance is the degradation of LDL receptors by proprotein convertase subtilisin/kexin 9 (PCSK9), a secreted serine protease that binds to LDL receptors on the cell surface and interferes with their recycling so that they are directed to lysosomes for degradation. Gain-of-function mutations in the *PCSK9* gene cause autosomal dominant hypercholesterolemia, while loss-of-function mutations are associated with low LDL levels. Inhibition of PCSK9 reduces LDL cholesterol in hypercholestrolemic subjects.[34]

Cells also control their sterol economy by reverse cholesterol transport (sterol efflux), mediated by members of the ATP-binding cassette subfamily A1 (ABCA1), which transfers cholesterol to plasma lipoproteins. In the primate corpus luteum undergoing functional luteolysis, a fall in STARD1 expression results in diminished progesterone production.[35,36] To maintain free cholesterol balance, expression of lipoprotein receptors is reduced (reduced uptake) and expression of ABCA1 is increased (increased efflux), perhaps by hydroxysterol activation of LXR transcription factors. Excess cholesterol not available for steroidogenesis is esterified and deposited in cytoplasmic lipid droplets. These "homeostatic" adjustments reflect, in part, changes in gene transcription, posttranscriptional, and posttranslational processes.

Overview of Steroidogenesis

- *Several families of enzymes are involved in the synthesis of steroid hormones—the most important being the cytochrome P450 family and the hydroxysteroid dehydrogenases and reductases*
- *The critical protein controlling steroidogenesis in cells that respond to tropic hormones is the STARD1.*
- *Mutations that inactivate key proteins involved in steroidogenesis cause a spectrum of phenotypes, many of them involving disorders of sexual development.*

The cellular manufacture of steroid hormones involves the action of several classes of enzymes including the cytochromes P450, hemeprotein mixed-function oxidases (named because of their distinct absorption peak at 450 nm when reduced in the presence of carbon monoxide), hydroxysteroid dehydrogenases, and reductases.[6,37]

Cytochrome P450s catalyze the major alterations in the sterol framework, cleavage of the side chain, hydroxylations, and aromatization. These hemeproteins require molecular oxygen and a source of reducing equivalents (i.e., electrons) to complete a catalytic cycle. Each member of the steroidogenic cytochrome P450 family of genes is designated "CYP," followed by a unique identifying number that usually refers to the carbon atom at which the enzyme acts.

The hydroxysteroid dehydrogenases reduce ketone groups or oxidize hydroxyl functions, employing pyridine nucleotide cofactors, usually with a stereospecific substrate preference and reaction direction. In addition to being involved in hormone biosynthesis in steroidogenic cells, this family of enzymes works with the reductases, steroid sulfotransferases, and steroid sulfatase to regulate the level of bioactive hormone in target tissues. The hydroxysteroid dehydrogenases are key determinants of the cellular response to endogenous steroid hormones and steroidal drugs.

The reductases, using the pyridine nucleotide nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor, produce saturated ring A steroids from Δ^4-steroids (again, with stereospecificity). Table 4.1 lists the key steroidogenic enzymes by class and the respective gene designation. Fig. 4.3 outlines the pathways of steroid hormone synthesis indicating where specific enzymes act.

Key Proteins in the Biosynthesis and Metabolism of Steroid Hormones

Steroidogenic Acute Regulatory Protein: The Principal Regulator of Gonadal and Adrenal Steroidogenesis

Translocation of cholesterol from the outer mitochondrial membranes to the relatively sterol-poor inner membranes is the critical step in steroidogenesis.[5,6] This translocation process occurs at modest rates in the absence of specific effectors. It is markedly enhanced by STARD1, a protein with a short biological half-life. The following evidence established STARD1 as the key mediator of substrate flux to the cholesterol side-chain cleavage system:

1. Expression of STARD1 is directly correlated with steroidogenesis.
2. Coexpression of STARD1 and the cholesterol side-chain cleavage enzyme system in cells that are not normally steroidogenic results in substantial pregnenolone synthesis above that produced by cells expressing the cholesterol side-chain cleavage enzyme system alone.
3. Mutations that inactivate STARD1 cause congenital lipoid adrenal hyperplasia, a rare autosomal recessive disorder in which the synthesis of all adrenal and gonadal steroid hormones is severely impaired before the cholesterol side-chain cleavage step.
4. Targeted deletion of the murine *Stard1* gene results in a phenotype in nullizygous mice that mimics human congenital lipoid adrenal hyperplasia.

Human STARD1 is synthesized as a 285 amino acid protein. The N-terminus of STARD1 is characteristic of proteins synthesized in the cytoplasm and then imported into mitochondria, with the first 26 amino acid residues predicted to form an amphipathic helix. Newly synthesized STARD1 preprotein (37 kDa) is rapidly imported into mitochondria and processed to the "mature" 30-kDa form.

Table 4.1 Key Human Steroidogenic Proteins and Their Genes

Protein	Gene	Chromosomal Locus	Substrates*	Major Activities	Known Deficiency States
StAR (STARD1)	*STARD1*	8p11.2	Cholesterol flux within mitochondria	Sterol delivery to P450scc	Congenital lipoid adrenal hyperplasia
P450scc	*CYP11A1*	15q23-q24	Cholesterol hydroxysterols	Cholesterol side chain cleavage	Side chain cleavage enzyme deficiency
P450c17	*CYP17A1*	10q24.3	Preg, 17OH-PregProg, [17OH-Prog]DHEA	17α-Hydroxylase/16α-hydroxylase/17,20-lyase	17α-Hydroxylase deficiency/isolated 17,20-lyase deficiency
P450c21	*CYP21A2*	6p21.1	Prog, 17OH-Prog	21-Hydroxylase	21-Hydroxylase deficiency
P450c11β	*CYP11B1*	8q21-q22	11-Deoxycortisol	11-Hydroxylase	11-Hydroxylase deficiency
P450c11AS	*CYP11B2*	8q21-q22	Corticosterone11-DOC18OH-Corticosterone	11-Hydroxylase/18-hydroxylase/18-oxidase	CMO I deficiency/CMO II deficiency
P450arom	*CYP19A1*	15q21.1	Androstenedione	19-Hydroxylase	Aromatase deficiency
P450 oxido reductase	*POR*	7q11.2	Androgens/corticoids Testosterone	17α-hydroxylase/17-20 lyase and 21-hydroxylase aromatization	Combined partial 17α-hydroxylase/17-20 desmolase and 21-hydroxylase deficiencies
3β-HSDI	*HSD3B1*	1p13	Preg, 17OH-PregDHEA, Adiol	3β-Dehydrogenase/Δ^{5-4}-isomerase	
3β-HSD2	*HSD3B2*	1p13	Preg, 17OH-PregDHEA, Adiol	3β-Dehydrogenase/Δ^{5-4}-isomerase	3β-HSD deficiency
17β-HSD1	*HSD17B1*	17q21	Estrone, [DHEA]	17β-Ketosteroid reductase	
17β-HSD2	*HSD17B2*	16q24	Estradiol, testosterone DHT, 20α-OH-prog	17β-Hydroxysteroid dehydrogenase/20α-hydroxysteroid dehydrogenase	
17β-HSD3	*HSD17B3*	9q22	Androstenedione	17β-Ketosteroid reductase	Male 17-ketosteroid reductase deficiency
17β-HSD5	*HSD17B5 (AKR1C3)*	10p14-15	Androstenedione, DHT, 3α-asdiol, 3α-androstanediol, asone, asdione	17β-Ketosteroid reductase/3α-hydroxysteroid dehydrogenase	
5α-Reductase type 1	*SRD5A1*	5p15	Testosterone, C21 steroids	5α-Reductase	
5α-Reductase type 2	*SRD5A2*	2p23	Testosterone, C21 steroids	5α-Reductase	5α-Reductase deficiency
11β-HSDI	*HSD11B1*	1q32.2	Cortisol, cortisone, corticosterone, 11-dehydrocorticosterone	11β-Ketosteroid reductase	Cortisone reductase deficiency
11β-HSDII	*HSD11B2*	16p22	Cortisol, cortisterone	11β-Hydroxysteroid dehydrogenase	Syndrome of apparent mineralocorticoid excess
Estrogen sulfotransferase	*SULT1E1*	4q13.1	Estradiol, estrone	Sulfonation	
DHEA sulfotransferase	*SULT2A1/ SULT2B1*	19q13.4	DHEA/Preg, cholesterol	Sulfonation	
Steroid sulfatase	*STS*	Xp23.3	DHEA sulfate, cholesterol sulfate	Sulfatase	Sulfatase deficiency

*Bracketed compounds are minor substrates.
Adiol, Δ^5,3β-androstanediol; *3α-Asdiol*, 3α-androstanedione; *11-DOC*, 11-deoxycorticosterone; *Asdione*, androstanedione; *Asone*, androsterone; *COA*, coenzyme A; *CMO*, corticosterone methyl oxidase; *DHEA*, dehydroepiandrosterone; *DHT*, dihydrotestosterone; *HSD*, hydroxysteroid dehydrogenase; *POR*, P450 reductase; *Preg*, pregnenolone; *Prog*, progesterone.

The preprotein has a very short half-life (minutes), but the mature form is longer-lived (hours). The START domain consists of a unique structure that accommodates a cholesterol molecule. However, the binding of one sterol molecule is not sufficient to explain STARD1 action, and it is evident that STARD1 must be a catalyst for the transfer of multiple cholesterol molecules.[5]

STARD1 contains two consensus sequences for cAMP-dependent protein kinase phosphorylation at Ser57 and Ser195. Ser195 of human STARD1 must be phosphorylated for maximal steroidogenic activity in model systems.

Tissues that express STARD1 at high levels perform trophic hormone–regulated mitochondrial sterol hydroxylations through the intermediacy of cAMP. STARD1 messenger RNA (mRNA) and protein are not present in the human placenta, an observation that is consistent with the fact that pregnancies hosting a fetus affected with congenital lipoid adrenal hyperplasia go to term. Although estrogen production

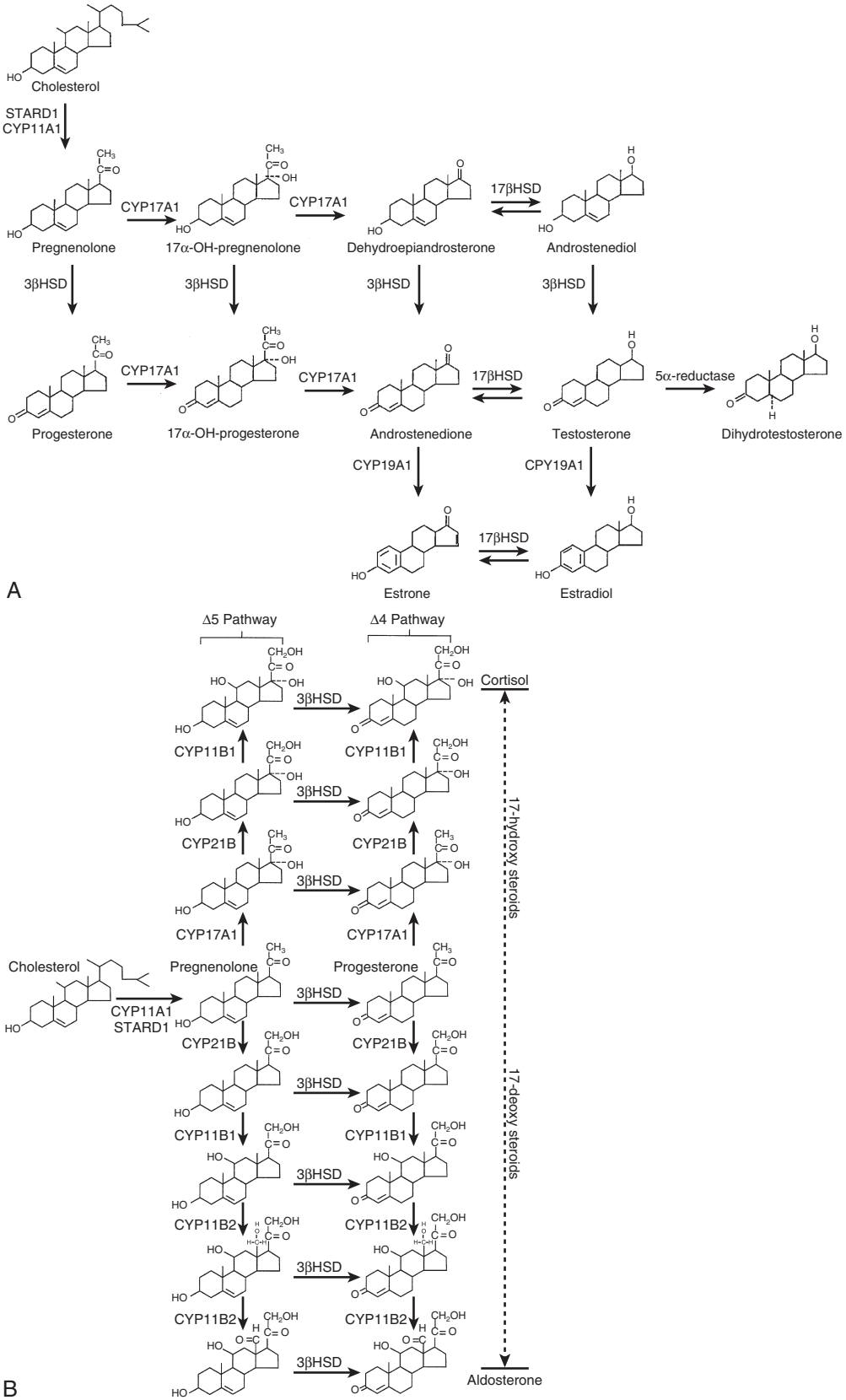

FIGURE 4.3 (A) Biosynthetic pathway for sex steroid hormones. (B) Biosynthetic pathway for adrenal steroid hormones. The 3β-hydroxysteroid dehydrogenase in the gonads and adrenal cortex is the type 2 enzyme *(HSD3B2)*, while the type 1 enzyme is responsible for this activity in the placenta. In addition, the 17β-hydroxysteroid dehydrogenases involved in the reduction and oxidation of steroids are different enzymes, as described in the text. *CYP*, Cytochrome P450; *HSD*, hydroxysteroid dehydrogenase; *STARD1*, steroidogenic acute regulatory protein.

is impaired in these pregnancies because of diminished fetal adrenal androgen production, placental progesterone synthesis is not significantly affected, indicating that the trophoblast cholesterol side-chain cleavage reaction is independent of STARD1.

The abundance of STARD1 protein in steroidogenic cells is determined primarily by the rate of *STARD1* gene transcription, which is influenced by transcription factors that control other genes encoding proteins involved in cholesterol metabolism (e.g., SREBPs, NR5A1, NR5A2, LXRs). STARD1 mRNA stability and translational mechanisms (in some species determined by the 3′-untranslated region of the transcripts) may also contribute. In differentiated cells, the *STARD1* gene is activated by the cAMP signal transduction cascade within 15 to 30 minutes. In differentiating cells (e.g., luteinizing granulosa cells), the induction of *STARD1* transcription takes hours and requires ongoing protein synthesis.

STARD1 was initially thought to stimulate cholesterol movement from the outer to the inner mitochondrial membrane as it was imported into the mitochondria. However, STARD1 protein lacking the mitochondrial targeting sequence is as effective as native STARD1 in stimulating steroidogenesis. Other STARD1 constructs engineered for prolonged tethering to the surface of the mitochondria were very active in stimulating pregnenolone production, suggesting that the residency time of the protein on the mitochondrial surface determines the duration of the steroidogenic stimulus. Recombinant human STARD1 lacking the mitochondrial targeting sequence enhanced pregnenolone production by isolated ovarian mitochondria in a dose- and time-dependent fashion, with significant increases in steroid production observed within minutes. Collectively, these findings strongly suggest that STARD1 acts on the outer mitochondrial membrane to promote cholesterol translocation. This perspective implies that import of the protein into the mitochondrial matrix, rather than being the trigger to steroid production, is actually the "off" mechanism, because it removes STARD1 from its site of action (Fig. 4.4). Consequently, ongoing production of STARD1 preprotein is required to sustain steroidogenesis.

The findings of the previously described experiments are most consistent with the idea that STARD1 enhances desorption of cholesterol from the sterol-rich outer mitochondrial membrane to the relatively sterol-poor inner membranes. The desorption process may involve a pH-dependent conformational change (molten globule transition). Even though STARD1 contains a hydrophobic pocket that binds cholesterol, sterol binding is not required for steroidogenic activity.[5]

The molecule or structure on the mitochondrial outer membrane that STARD1 acts on to promote cholesterol movement to the inner membrane has not been definitively identified. The STARD1 "receptor," which could be a protein or a lipid, is not specific for mitochondria of steroidogenic cells, since STARD1 works in the context of COS-1 cells, which are not normally steroid hormone-producing cells. Thus the specificity of the mechanism of mitochondrial cholesterol translocation is determined by whether STARD1 is expressed. Initially the translocator protein (TSPO; also known as the *peripheral benzodiazepine receptor*), which is found in the outer mitochondrial membrane of many cell types, was postulated to be a target of STARD1 because molecules that bind to TSPO stimulate steroidogenesis and

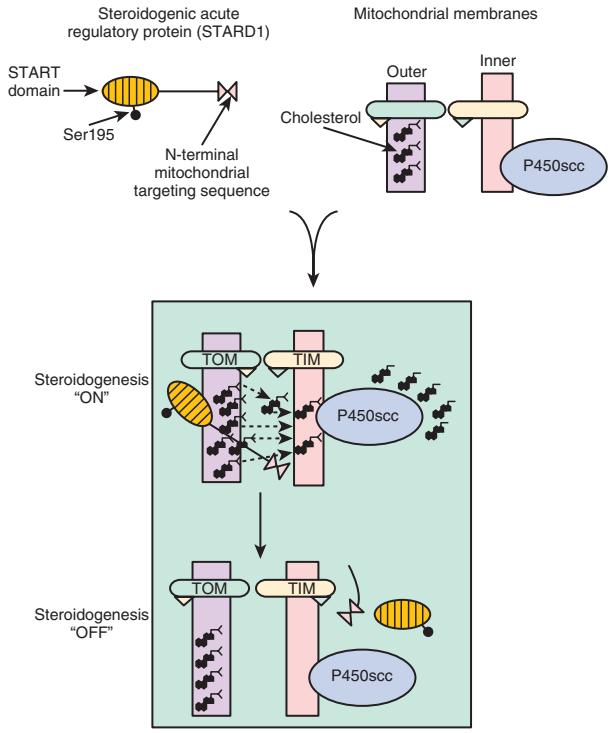

FIGURE 4.4 Structure of steroidogenic acute regulatory protein *(STARD1)* and model for its mechanism of action on intramitochondrial cholesterol translocation. StAR preprotein is targeted to the outer mitochondrial membrane where it acts to promote cholesterol movement to the cholesterol side-chain cleavage enzyme *(P450scc)*. StAR preprotein is subsequently imported by the translocator complex into the mitochondrial matrix where it is proteolytically processed to the mature 30-KDa form. *TIM*, Inner mitochondrial membrane translocators; *TOM*, outer mitochondrial membrane protein translocators; *START*, STAR-related lipid transfer/domain.

knockdown of TSPO expression in cultured cells reduced steroid synthesis in the presence of STARD1. However, the subsequent phenotyping of TSPO-deficient mice revealed that TSPO is dispensable for steroidogenesis. Despite ongoing debate in the literature over the latter findings, the weight of evidence indicates that TSPO is not required for STARD1 action.[38] Another potential candidate for the STARD1 effector molecule is the mitochondrial voltage-dependent anion channel 2 (VDAC2), which is also expressed by many different cell types and has been proposed to be a cholesterol "pore" through which outer mitochondrial membrane cholesterol could flow to the inner membrane.

As noted above, mutations in the *STARD1* gene cause congenital lipoid adrenal hyperplasia, a rare autosomal recessive disease. Exceptions occur in Japan and Korea, however, where the mutation accounts for at least 5% of all cases of congenital adrenal hyperplasia.[5,39] The pathophysiology of the disease entails a two-step process in which impaired use of cholesterol for steroidogenesis leads to accumulation of sterol esters in lipid droplets. These droplets ultimately compress cellular organelles, causing damage through the formation of lipid peroxides. This damage occurs prominently in the adrenal cortex and Leydig cells.[5,39] Mutations inactivating NR5A1 (SF-1) also cause cholesterol accumulation in Leydig cells because STARD1 and cholesterol side-chain cleavage enzyme expression are impaired.

Mutations found in the *STARD1* gene, which is composed of seven exons, include frameshifts caused by deletions or insertions, splicing errors, and nonsense and missense mutations. These mutations lead to the absence of STARD1 protein or the production of functionally inactive protein. Several nonsense mutations were shown to result in C-terminus truncations of STARD1. One of these mutations, Gln258Stop, results in the deletion of the final 28 amino acids of the STARD1 protein and accounts for 80% of the known mutant alleles in the affected Japanese population. Known point mutations that produce amino acid substitutions occur in exons 5 to 7 of the gene, the exons that encode the C-terminus. Mutations that cause partial loss of STARD1 activity (usually 20% to 30% of normal) are associated with a milder disease phenotype or "nonclassical" disease.[5]

Although affected XY subjects are pseudohermaphrodites (46,XY disorder of sexual development [DSD]) because of an inability to generate sufficient fetal testicular testosterone to masculinize the external genitalia, 46,XX subjects have normal external genitalia, develop female secondary sexual characteristics, and experience menarche. They are, however, anovulatory and unable to produce large amounts of estradiol and progesterone in a cyclic fashion. The fact that some ovarian estradiol synthesis occurs reflects the existence of STARD1-independent substrate movement to the cholesterol side-chain cleavage system.

Cholesterol Side-Chain Cleavage Enzyme (P450scc Encoded by *CYP11A1*)

Cholesterol side-chain cleavage is catalyzed by cytochrome P450scc and its associated electron transport system, consisting of a flavoprotein reductase (ferredoxin or adrenodoxin reductase) and an iron sulfoprotein (ferredoxin or adrenodoxin), encoded by the *FDX1* gene, which shuttles electrons to cytochrome P450scc.[5,6,40,41] The side-chain cleavage reaction involves three catalytic cycles: the first two lead to the introduction of hydroxyl groups at positions C-22 and C-20, and the third results in scission of the side chain between these carbons. Each catalytic cycle requires one molecule of NADPH and one molecule of oxygen so that the formation of one mole of the cleavage products, pregnenolone and isocaproaldehyde, uses three moles of NADPH and three moles of oxygen.

The slowest step of the reaction is the binding of cholesterol to the hydrophobic pocket of P450scc where the heme resides. The sterol substrate remains bound to a single active site on cytochrome P450scc for all three cycles because of the tight binding of the reaction intermediates. The dissociation constant (K_d) for binding of cholesterol, a measure of the enzyme's affinity for its substrate, is approximately 5000 nM, whereas the K_d for the binding of the intermediate product 22-hydroxycholesterol is 4.9 nM; the K_d for 20,22-dihydroxycholesterol is 81 nM. The estimated K_d for pregnenolone, the end product, is 2900 nM, which permits its dissociation from the enzyme at the end of the reaction.

Reducing equivalents are shuttled to cytochrome P450scc by ferredoxin in cycles of reduction and oxidation, facilitated by differential affinities of the proteins, depending on their state of oxidation or reduction.[41] Ferredoxin forms a 1:1 complex with ferredoxin reductase, which catalyzes reduction of the iron-sulfur protein. The reduced ferredoxin then dissociates and forms a 1:1 complex with cytochrome P450scc and is subsequently oxidized when it donates its electrons to P450scc. Oxidized ferredoxin returns to ferredoxin reductase for electron recharging. This recharging is facilitated by the fact that ferredoxin reductase has a greater affinity for oxidized over reduced ferredoxin. The binding of cholesterol to cytochrome P450scc increases its affinity for reduced ferredoxin, which enhances the shuttle of electrons to substrate-loaded enzyme.

The rate of formation of pregnenolone is determined by:

1. Access of cholesterol to the inner mitochondrial membranes
2. The quantity of cholesterol side-chain cleavage enzyme, and secondarily, its flavoprotein and iron-sulfur protein electron transport chain
3. Catalytic activity of P450scc, which can be influenced by posttranslational modification

Acute alterations in steroidogenesis generally result from changes in the delivery of cholesterol to P450scc, whereas long-term alterations involve changes in the quantity of enzyme proteins as well as cholesterol delivery.[5,6]

Mutations in the *CYP11A1* gene that result in significantly diminished cholesterol side-chain cleavage activity have been reported in association with adrenal insufficiency and 46,XY DSD (sex reversal), phenotypes that are similar to those associated with inactivating mutations in the *STARD1* gene.[5,42-44]

The discovery of mutations causing severe P450scc deficiency in some humans born at term challenges the notion that absence of P450scc activity in the fetus and placenta is incompatible with pregnancy progressing beyond the usually limited period of luteal progesterone support. Therefore, compensatory mechanisms appear to exist to maintain sufficient progestational activity, perhaps sustained corpus luteum function, to support pregnancy and fetal viability in women hosting a fetus with *CYP11A1* mutations. Another surprising and distinguishing feature of some subjects with *CYP11A1* mutations is the absence of adrenal hypertrophy, as seen in *STARD1* mutations.[5]

17α-Hydroxylase/17,20-Lyase (P450c17; CYP17A1)

P450c17 is an endoplasmic reticulum enzyme that catalyzes two reactions: hydroxylation of pregnenolone and progesterone at carbon 17 and conversion of pregnenolone into C19 steroids (in the case of the human enzyme, progesterone is also converted but to a much lesser extent).[6] The 17α-hydroxylation reaction requires one pair of electrons and molecular oxygen. The lyase reaction requires a second electron pair and molecular oxygen. The reducing equivalents are transferred to the P450c17 heme iron from NADPH by NADPH cytochrome P450 reductase (POR), an enzyme functioning in the endoplasmic reticulum.[45] The hydroxylase and lyase reactions are both believed to proceed through a ferryl oxene mechanism with the substrate bound to the enzyme in the catalytic pocket in the same orientation. P450c17 also catalyzes 16α-hydroxylation of progesterone and DHEA.

The importance of POR in steroid metabolism catalyzed by endoplasmic reticulum cytochrome P450 enzymes has been illuminated by the phenotype of individuals with POR deficiency, which causes a form of congenital adrenal hyperplasia.[45] The autosomal recessive disorder results in a steroid

profile suggestive of combined 21-hydroxylase and 17-hydroxylase/17,20-lyase deficiency, which presents as a range of phenotypes, including adrenal insufficiency, ambiguous genitalia (DSD), and Antley-Bixler syndrome-like skeletal malformation syndrome.[45,46]

Several factors determine whether substrates only undergo 17α-hydroxylation or subsequent scission of the 17,20 bond, including:

1. The nature of the substrate
2. The amount of POR and flux of reducing equivalents
3. Allosteric effectors such as cytochrome b_5
4. Posttranslational modification of P450c17 (Fig. 4.5)

Collectively, these factors determine the nature of the products produced by the enzyme, which, in the gonads and zona reticularis, favor androgens through augmentation of lyase activity. In contrast, 17α-hydroxylation required for glucocorticoid and mineralocorticoid synthesis is favored in the zona fasciculata and glomerulosa.

Human P450c17 preferentially uses Δ^5 substrates for 17,20 bond cleavage. Cytochrome b_5 (CYB5A) promotes electron transfer via POR for the lyase reaction acting as an allosteric effector and not as an electron donor per se, because the apo b_5 protein is also effective.[47] Cytochrome b_5 also increases the use of 17α-hydroxyprogesterone as a substrate for androstenedione synthesis. The distribution and regulation of cytochrome b_5 expression in the adrenal cortex, being greatest in the adult zona reticularis, supports a role for this protein in the regulation of lyase activity. The product of a second cytochrome b_5 gene (type 2 cytochrome b_5, CYB5B), found in human testis and expressed in the adrenals, also increases lyase activity.

Phosphorylation of P450c17 at serine and threonine residues by a yet-to-be-identified protein kinase appears to be necessary for maximal 17,20-lyase activity.[48] The phosphorylated P450c17 protein is evidently a substrate for protein phosphatase 2A (PP2A), since inhibitors of PP2A enhance lyase activity in cultured adrenal tumor cells.

Adrenarche, the increased production of adrenal androgens in the absence of increased production of cortisol or levels of ACTH, may result from enhanced P450c17 lyase activity due to increased expression of cytochrome b_5 or the state of P450c17 phosphorylation.

A P450c17-independent mechanism for conversion of pregnenolone into DHEA in glial tumor cell homogenates promoted by $FeSO_4$ has been described. This conversion presumably results from the fragmentation of tertiary hydroperoxides, which are probably derived from pregnenolone molecules oxygenated at carbons 17 and 20.[49] The physiological significance of this pathway, if any, to the formation of C19 steroids in the brain or other tissues is unknown.

Mutations in the *CYP17A1* gene cause combined or isolated deficiency states for each activity of P450c17.[46] Individuals with combined deficiency have a marked diminution in the production of C19 and C18 steroids, low levels of cortisol that result in elevated ACTH secretion, and excess production of steroids proximal to the P40c17 reaction. Hypertension from sodium retention and hypokalemia are a consequence of the increased production of 11-deoxycorticosterone. The inability to produce sex steroid hormones prevents adrenarche, puberty in females, and results in incomplete or absent development of male genitalia (46,XY DSD). Isolated 17,20-lyase deficiency is very rare. The documented cases result from point mutations that permit pregnenolone or progesterone to be bound and undergo 17α-hydroxylation, but prohibit the efficient receipt or usage of a second pair of electrons provided by POR to support the 17,20-lyase reaction.

Aromatase (P450aro, CYP19A1)

Aromatase, an endoplasmic reticulum enzyme, catalyzes three sequential hydroxylations of a C19 substrate by using three molecules of NADPH and three molecules of molecular oxygen to produce one molecule of C18 steroid with a phenolic A ring.[50] The first hydroxylation yields a C19 hydroxyl derivative, which is converted in a second

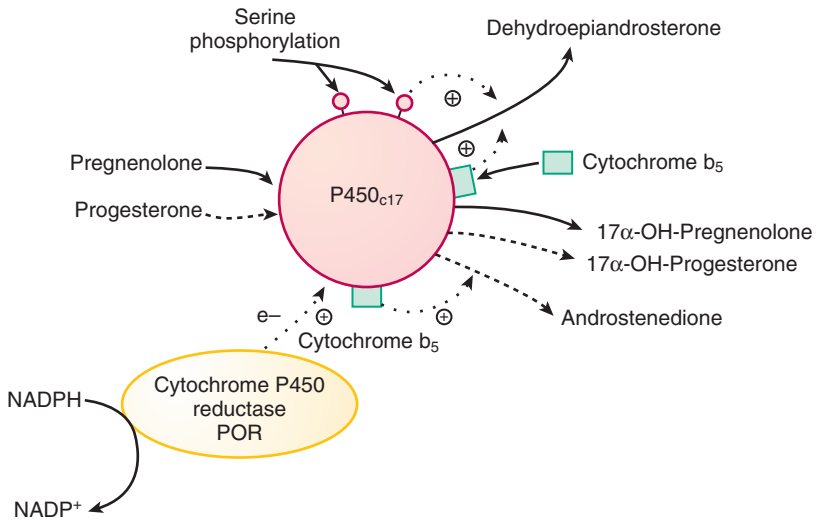

FIGURE 4.5 Factors modulating 17α-hydroxylase and 17,20-lyase activities of P450c17 *(CYP17A1)*. Phosphorylation of serine/threonine residues and cytochrome b5 stimulate *(designated by plus symbol)* lyase activity, with pregnenolone being the preferred substrate for the lyase reaction. Dephosphorylation of the serine/threonine residues by protein phosphatase 2A reduces lyase activity. Flow of reducing equivalents (e^-, electrons) from cytochrome P450 reductase *(POR)* stimulates both 17α-hydroxylase and lyase activities. *NADP*, Nicotinamide adenine dinucleotide phosphate.

hydroxylation to a gem diol that collapses to yield a C19 aldehyde. The final hydroxylation involves the formation of a 19-hydroxy-19-hydroperoxide intermediate that results in the elimination of the C19 methyl group as formic acid and concurrent aromatization. This sequence of reactions occurs at a single active site on the enzyme, with reducing equivalents transferred to P450aro by POR. The crystal structure of aromatase complexed with androstenedione revealed hydrogen bonding and tight packing of hydrophobic side chains that explain the enzyme's specificity for androgen substrates.

The aromatase protein is encoded by a single large gene, *CYP19A1*. This gene produces cell-specific transcripts from different promoters (Fig. 4.6).[51] The promoter driving ovarian aromatase expression lies adjacent to the exon encoding the translation start site (promoter IIa). In granulosa cells, FSH stimulates transcription of the genes encoding both aromatase and POR. A separate promoter lying approximately 100 kb upstream from the start of translation controls placental *CYP19A1* transcription. Expression of aromatase in adipose tissue, skin, and brain is driven from other promoters. Cytokines (including interleukin-11, interleukin-6, oncostatin-M, and leukemia-inhibiting factor) increase P450arom expression in adipose tissue via the I.4 promoter.

Several cases of aromatase deficiency have been described.[6,52-54] Pregnancies in which the fetus is affected with aromatase deficiency are characterized by low maternal urinary estrogen excretion, maternal virilization, and ambiguous genitalia or female pseudohermaphroditism (46,XX DSD) in affected genetic females. The maternal and fetal virilization in the absence of placental aromatase activity highlights the importance and efficiency of the placenta in converting maternal and fetal androgens into estrogens.

Among the mutations identified in the *CYP19A1* gene are an 87-bp insertion at the splice junction between exon 6 and intron 6, causing the addition of 29 in-frame amino acid residues, with the other mutations being mainly missense or nonsense mutations in exons 4, 9, and 10. The mutant protein with the 29 in-frame amino acid residues displayed less than 3% of the activity of normal aromatase. Compound heterozygous mutations in coding sequences found in patients with aromatase deficiency have also been shown to have minimal activity. When aromatase activity has been measured in placenta from offspring with *CYP19A1* mutations, activities have been found to be markedly reduced.

The aromatase-deficient (ArKO) mice show many of the features of human aromatase deficiency, and the consequential lack of estrogens, including a profound bone phenotype with reductions in all indices of bone mineralization.[55]

Families with autosomal dominant estrogen excess resulting from overexpression of aromatase have been reported.[56-59] The phenotypes include severe prepubertal gynecomastia in males and macromastia and premature puberty in females. The aromatase overexpression is caused in some families by heterozygous genomic rearrangements. In some cases an inversion results in a constitutively active cryptic promoter placed in control of the *CYP19A1* gene, driving excess aromatase production.

Inappropriate expression of aromatase in neoplastic and nonneoplastic tissue has also been found. In these pathological conditions, exemplified by breast cancer, there appears to be a shift in promoter use to favor the stronger gonadal (promoter IIa or I.3) over the weaker adipose tissue promoter (promoter I.4). This shift allows for activation of a cAMP-dependent signaling pathway, accounting for excessive aromatase expression and the resulting increase in estrogen synthesis.

11β-Hydroxylases (P450c11β and P450c11AS)

The human genome contains two genes that encode related mitochondrial enzymes involved in 11β-hydroxylation and aldosterone synthesis, respectively: P45011β, encoded by *CYP11B1*, and P450c11AS (also referred to as "P450aldo," "P450c18," or "P450cmo"), encoded by *CYP11B2*.[6] The encoded proteins differ in only 33 amino acid residues. Both enzymes display 11β-hydroxylase activity, but P450c11AS can also perform the two oxygenation steps at carbon 18 to produce aldosterone. They require molecular oxygen and reducing equivalents for catalysis, and the latter is shuttled to the enzymes by the adrenodoxin reductase–adrenodoxin system.

CYP11B1, a gene whose transcription is stimulated by ACTH-triggered cAMP signaling pathways, is expressed in the zonae fasciculata and reticularis of the adrenal cortex. *CYB11B1* is also expressed in human Leydig and theca cells and is responsible (along with type II 11β-hydroxysteroid dehydrogenase) for the production of 11-ketotestosterone, a molecule that activates androgen receptors.[60] In contrast, *CYP11B2* expression is restricted to the zona glomerulosa.

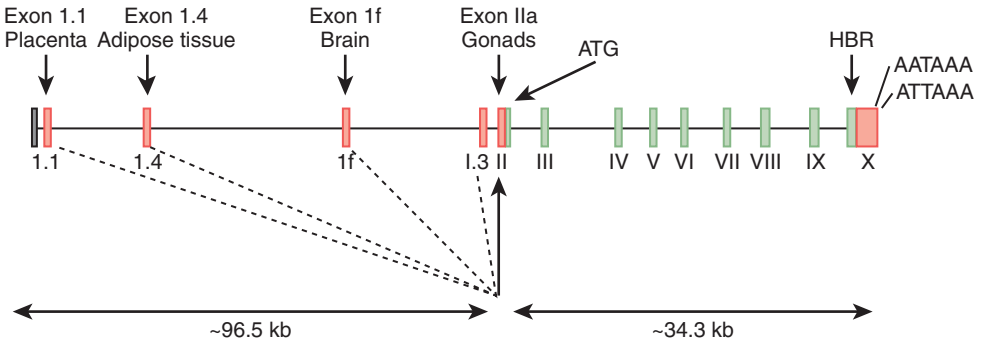

FIGURE 4.6 Structure of the human *CYP19A1* gene showing the protein coding exons indicated by Roman numerals and the location of the different 5′-UTR-coding exons and tissue-specific promoters. The P450arom heme-binding region and polyadenylation signals in exon 10 are indicated. *ATG*, Methionine codon; *HBR*, heme-binding region. *(Modified from Kamat A, Hinshelwood MM, Murry BA, Mendelson CR: Mechanisms in tissue-specific regulation of estrogen biosynthesis in humans.* Trends Endocrinol Metab *13:122, 2002.)*

Transcription of this gene is stimulated by protein kinase C signaling pathways, which are turned on by angiotensin II.

Mutations in the *CYP11B1* gene cause 11β-hydroxylase deficiency, whereas mutations in *CYP11B2* cause 18-hydroxylase or corticosterone methyl oxidase I deficiency and 18-oxidase or corticosterone methyl oxidase II deficiency.[6,61,62] Unequal crossover of the adjacent *CYP11B1* and *CYP11B2* genes creates a third hybrid gene, in which the cAMP-regulated promoter of the *CYP11B1* gene drives expression of a chimeric protein with aldosterone synthase activity. This leads to a state of glucocorticoid-suppressible aldosteronism.

11β-hydroxylase deficiency is characterized by high levels of 11-deoxycortisol and deoxycorticosterone, resulting in salt retention and hypertension. Affected females are virilized by the excess production of adrenal androgens driven by elevated ACTH levels. *CYP11B1* mutations causing 11β-hydroxylase deficiency include those resulting in nonsynonymous amino acid substitutions and a premature stop codon.

Corticosterone methyl oxidase I deficiency results from the complete absence of P450c11AS activity. Aldosterone synthesis is absent, but the production of corticosterone and cortisol is retained. Corticosterone methyl oxidase II deficiency is caused by mutations that inactivate 18-methyl oxidase while retaining 18-hydroxylase activity. This causes elevated 18-hydroxycorticosterone and low aldosterone levels.

21-Hydroxylase (P450c21,CYP21A2)

P450c21 is an adrenal endoplasmic reticulum enzyme that catalyzes the 21-hydroxylation of progesterone and 17α-hydroxyprogesterone in the pathway of mineralocorticoid and glucocorticoid biosynthesis.[6,53,55,56] The Michaelis constant (K_m) for 17α-hydroxyprogesterone (1.2 μM) is lower than that for progesterone (2.8 μM), and the apparent maximum velocity (V_{max}) for the former substrate is twice that for progesterone. The enzyme requires 1 mole of molecular oxygen and reducing equivalents (generated from NADPH through POR) to accomplish the hydroxylation of carbon 21. Mutations inactivating POR cause a partial deficiency in 21-hydroxylase activity and a partial deficiency in 17α-hydroxylase/17,20-lyase activity.[6,45] The primary regulator of *CYP21A2* expression in the zona fasciculata is ACTH.

The *CYP21A2* gene is adjacent to a pseudogene *(CYP21A1P)*, separated by the complement *C4B* gene. These genes are embedded in the human leukocyte antigen region on band 6p21.1. Frequent unequal crossovers and gene conversions make 21-hydroxylase deficiency one of the most common autosomal recessive metabolic diseases, occurring in 1:10,000 to 1:15,000 births.[63] Unequal crossover, with the complete loss of the *C4B* gene and a net deletion of *CYP21A2*, along with gene conversion events in which mutations in the pseudogene are introduced into the expressed gene, result in reduced 21-hydroxylase enzyme levels or impaired catalytic activity (Fig. 4.7). Large-scale deletions/gene conversions may extend into the adjacent gene encoding tenascin-X, which when mutated in both alleles, causes a form of Ehlers-Danlos syndrome.[64,65]

The signs and symptoms of congenital adrenal hyperplasia caused by 21-hydroxylase deficiency reflect deficits in cortisol (because of inability to convert 17α-hydroxyprogesterone into 11-deoxycortisol) and aldosterone (because of inability to convert progesterone into deoxycorticosterone). Another contributing factor is the accumulation of adrenal androgens that results from elevated ACTH levels due to the absence of cortisol-negative feedback on the hypothalamic–corticotrophin axis.

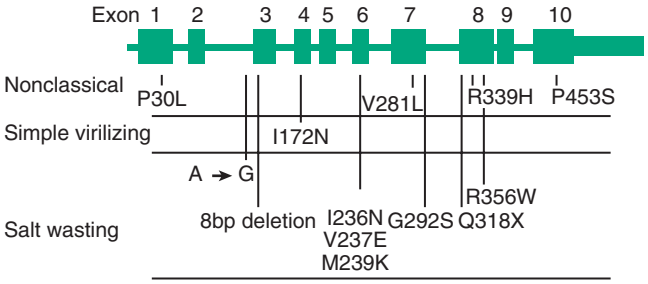

FIGURE 4.7 Structure of the *CYP21A2* gene and selected mutations causing phenotypes of 21-hydroxylase deficiency. *(Modified from White PC, Speiser PW: Congenital adrenal hyperplasia due to 21-hydroxylase deficiency.* Endocr Rev *21:245, 2000.)*

The clinical phenotypes are, however, variable and dependent on the severity of the 21-hydroxylase deficiency.[6,65] The non–salt-wasting, salt-wasting, and nonclassic forms are associated with certain mutations that affect the amount of residual 21-hydroxylase activity, with the salt-wasting form being characteristic of severe enzyme deficiency (deletions and large gene conversions). The simple virilizing (non–salt-wasting) form is associated with mutations that substantially reduce activity (e.g., the missense mutation resulting in a nonsynonymous amino acid substitution Ile172Asp), and the nonclassic (or *late-onset*) form is caused by mutations that do not severely impair the level of expression or activity of P450c21 (e.g., Val28Leu, Pro30Leu).

Hydroxysteroid Dehydrogenases and Reductases

Hydroxysteroid dehydrogenases (HSDs) or oxidoreductases catalyze the interconversion of alcohol and carbonyl functions in a position- and stereospecific manner on the steroid nucleus and side chain, using oxidized (+) or reduced (H) nicotinamide adenine dinucleotide, NAD(H), or NADP(H) as cofactors.[6,37,66,67] In some instances, HSDs display bifunctionality (e.g., they oxidize or reduce 17β and 20α oxy functions), such as HSD17B2. Although the HSDs can catalyze both the oxidation and reduction reactions in vitro under different conditions (e.g., substrate, pH and cofactor), in vivo they catalyze reactions in one direction and can be classified as dehydrogenases or reductases. The enzymes are members of the short chain dehydrogenase/reductase (SDR) and aldo-keto reductase (AKR) superfamilies.

The importance of cofactor availability to HSDs is exemplified by inactivating mutations in the hexose-6-phosphate dehydrogenase *(H6PDH)* gene, which regenerates NADPH in the endoplasmic reticulum required for the HSD11B1 reaction. Individuals with these mutations have apparent cortisone reductase deficiency due to impaired HSD11B1 activity.[6,68]

Multiple isoforms of HSDs exist, and, coupled with their tissue-specific expression, account for the ability of specific enzymes to act predominantly as reductases (ketone reduction) or dehydrogenases (alcohol oxidation). In steroidogenic tissues, HSDs catalyze the final steps in progestin, androgen,

and estrogen biosynthesis. In steroid target tissues, HSDs can regulate the occupancy of steroid hormone receptors by converting active steroid hormones into inactive metabolites or relatively inactive steroids to molecules with greater binding activity.

This is best exemplified by the human type 2 11β-HSD (HSD11B2), which controls mineralocorticoid activity in the kidney by converting cortisol (which has high affinity for both the glucocorticoid and mineralocorticoid receptors) to cortisone (which does not bind to the mineralocorticoid receptor). Thus the specificity of mineralocorticoid receptor activation is not determined by the receptor, but by activity of the HSD that removes the more abundant potential mineralocorticoid receptor ligand, leaving aldosterone as the controlling activator (Fig. 4.8).[69] Because of their tissue-specific roles in controlling the bioavailability of steroids, HSDs are interesting targets for pharmacologic manipulation.[37]

3β-Hydroxysteroid Dehydrogenase/$\Delta^{5\text{-}4}$-Isomerases

The 3β-HSD/$\Delta^{5\text{-}4}$-isomerases are membrane-bound enzymes localized to the endoplasmic reticulum and mitochondria that use nicotinamide adenine dinucleotide (NAD+) as a cofactor.[70] These enzymes catalyze dehydrogenation of the 3β-hydroxyl group and the subsequent isomerization of the Δ^5 olefinic bond to yield a Δ^4 ketone structure. They convert pregnenolone into progesterone, 3-, 17α-hydroxypregnenolone into 17α-hydroxyprogesterone, and DHEA into androstenedione.[6,70]

The dehydrogenase and isomerase reactions are performed at a single bifunctional catalytic site that adopts different conformations for each activity. The 3β-hydroxysteroid dehydrogenase step is rate-limiting in the overall reaction sequence, and the NADH formed in this reaction is believed to alter the enzyme conformation to promote the isomerase reaction.

The human genome has two active 3β-HSD/$\Delta^{5\text{-}4}$-isomerase genes: one encodes a protein predominantly expressed in the placenta, liver, breast, and brain (HSD3B1), while the other encodes a protein expressed in the adrenal cortex and gonads (HSD3B2). These two genes, each consisting of four exons, lie 100 kb apart on band 1p13.1. The human genome also contains five unprocessed pseudogenes closely related to *HSD3B1* and *HSD3B2* on band 1p13.1, with two of them lying between the expressed genes. The DNA sequences of the exons of the two active genes are very similar, and the encoded proteins differ in only 23 amino acid residues. HSD3B1, however, has a lower K_m for substrate than HSD3B2, which facilitates metabolism of lower concentrations of Δ^5 substrate. Electron microscope cytochemistry has localized HSD3B2 activity to the perimitochondrial endoplasmic reticulum. In some cell types, the enzyme appears to be localized in the inner mitochondrial membrane, positioned to act on pregnenolone produced by the cholesterol side-chain cleavage system.

Because most steroidogenic cells have a large capacity to generate progesterone when presented with exogenous pregnenolone, the 3β-HSD/$\Delta^{5\text{-}4}$-isomerases are not believed to be rate-determining enzymes. However, mutations causing deficiency of HSD3B2 cause a form of congenital adrenal hyperplasia characterized by impaired adrenal and gonadal steroidogenesis with accumulation of Δ^5 steroids in the circulation. The presence of active HSD3B1 in extraadrenal and extragonadal tissues results in the formation of some Δ^4 steroids (e.g., 17α-hydroxyprogesterone).[6]

In its severest form, HSD3B2 deficiency is associated with salt wasting because of insufficient mineralocorticoid production.[6,61,70] Kinetic analysis of mutant proteins associated with the salt-wasting and non–salt-wasting forms of the disease showed a 4- to 40-fold reduction in catalytic efficiency for the conversion of pregnenolone into progesterone. The salt-wasting form of the disease is associated with frameshift mutations resulting in protein truncation and a variety of missense mutations that affect affinity for the cofactor and protein stability. The greater instability of the mutant proteins found in subjects with salt-wasting disease compared with those proteins found in the non–salt-wasting form appears to account, in part, for the different clinical phenotypes.

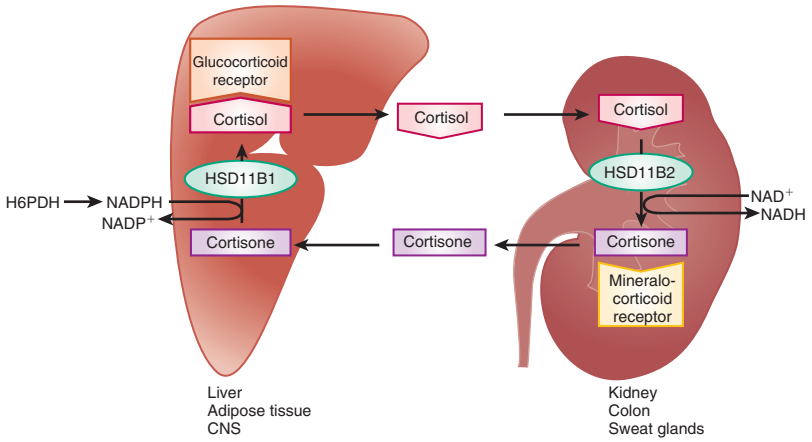

FIGURE 4.8 The roles of 11β-HSD types 1 *(HSD11B1)* and 2 *(HSD11B2)* in controlling levels of bioactive glucocorticoids. HSD11B1 reduces inactive cortisone into cortisol in liver and other tissues, whereas HSD11B2 oxidizes cortisol to cortisone. Cortisone cannot activate the mineralocorticoid receptor, thus allowing aldosterone (a steroid that is less abundant than cortisol) to specifically regulate the mineralocorticoid receptor. The supply of NADPH to HSD11B1 is a major determinant of the reaction and inactivating mutations in the H6PDH gene, which generates NADPH, causes cortisone reductase deficiency. *CNS*, Central nervous system; *HSD*, hydroxysteroid dehydrogenases; *NADPH*, nicotinamide adenine dinucleotide phosphate. *(Modified from Seckl J, Walker B: 11β-Hydroxysteroid dehydrogenase type I-A tissue-specific amplifier of glucocorticoid action.* Endocrinology *142:1371, 2001.)*

A so-called attenuated, or late-onset, form of 3β-HSD deficiency, diagnosed by steroid measurements, has been described in the literature. However, no mutations have yet been discovered in the genes encoding *HSD3B1* and *HSD3B2* in subjects with this clinical diagnosis. Mutations in the distal promoter or epigenetic factors that might alter enzyme expression cannot be excluded. The apparent reduced 3β-HSD activity could also be the result of alterations in the membrane environment that affect catalytic activity or posttranslational modifications to the enzyme that diminish its function. Mutations in *HSD3B1* have not been detected, although several sequence variants of uncertain significance have been described.

11β-Hydroxysteroid Dehydrogenases: Key Regulators of the Activity of Glucocorticoids

The biological activity of cortisol in target tissues is controlled by the action of two different 11β-hydroxysteroid dehydrogenases that are members of the short-chain dehydrogenase/reductase family (see Fig. 4.8). These enzymes catalyze the interconversion of active glucocorticoids and their inert 11-keto metabolites.[6,69] The type 1 enzyme (HSD11B1) is an endoplasmic reticulum protein with reversible oxidoreductase activity in vitro but preferentially catalyzes the reduction of the 11-keto group, using NADPH as a cofactor in vivo. This enzyme is expressed in the liver, lung, adipose tissue, brain, vascular tissue, and gonads, where it regenerates cortisol from 11-ketosteroids. In the case of the placenta, the activity ensures transport of biologically active cortisol to the fetus in the first half of pregnancy.

Targeted deletion of the type 1 enzyme gene *(Hsd11b1)* in mice results in animals with lower blood glucose levels in response to overfeeding and stress, impaired activation of gluconeogenesis, and blunted sensitivity to natural glucocorticoids. This finding substantiates a role for HSD11B1 in the amplification of cortisol and corticosterone action. HSD11B1 also plays a key role in the pharmacology of glucocorticoids. Hepatic HSD11B1 converts cortisone and prednisone, inactive prohormones, into active cortisol and prednisolone.

As noted above, mutations in the *H6PDH* gene cause a syndrome characterized by high ratios of cortisone to cortisol; impaired cortisol negative feedback resulting in elevated ACTH secretion; and increased production of adrenal androgens leading to hyperandrogenism, sexual precocity, and a polycystic ovary syndrome-like phenotype. Mice with targeted mutations in *H6pdh* share many of the phenotypes of mice with *Hsd11b1* mutations. Heterozygous mutations in the *HSD11B1* gene cause a mild form of cortisone reductase deficiency.[71]

HSD11B2, also an endoplasmic reticulum enzyme, has a higher affinity for its substrate than HSD11B1 and catalyzes the oxidation of cortisol with NAD^+ as a cofactor. It shares only modest amino acid sequence identity (21%) with HSD11B1. HSD11B2 is highly expressed in the kidney, colon, salivary glands, and placenta, all tissues that respond to aldosterone, or in the case of the placenta, tissues that act to separate the maternal and fetal endocrine systems (which is important in the third trimester). By converting cortisol and corticosterone to 11-keto compounds, HSD11B2 protects the renal mineralocorticoid receptors, which cannot distinguish cortisol or corticosterone from aldosterone, from inappropriate activation by the glucocorticoids.

Mutations that inactivate HSD11B2 produce a syndrome of apparent mineralocorticoid excess in humans, which is mimicked in the mice deficient in the enzyme that display hypertension, hypokalemia, and renal structural abnormalities. Glycyrrhizic acid, a component of licorice, and its metabolite carbenoxolone are competitive inhibitors of HSD11B2, but they also cause reduced expression of the HSD11B2 mRNA when administered in vivo. As a result, a drug-induced syndrome of apparent mineralocorticoid excess is produced.[72,73]

17β-Hydroxysteroid Dehydrogenases: Multiple Enzymes With Specific Biosynthetic and Catabolic Roles

The adrenals, gonads, and placenta reduce 17-ketosteroids into 17β-hydroxysteroids (which have greater biological potency), whereas target tissues usually oxidize 17β-hydroxysteroids, inactivating them.[6,37,74-77] In humans, these metabolic processes are mediated by at least 7 of the 14 known mammalian 17β-HSDs, designated types 1 through 14, according to the chronological order in which they were identified (Fig. 4.9). They are all members of the short-chain dehydrogenase/reductase family, except the type 5 enzyme, which is an AKR. They have different cofactor and substrate specificities, including molecules that are not steroids, subcellular locations, and tissue-specific patterns of expression.

The structures of the genes encoding the 17β-HSDs differ, and their nucleotide sequence homology is low. They can be grouped into enzymes that catalyze NAD^+-dependent oxidation (types 2, 4, 6, 8, 9, 10, 11, and 14) and those that catalyze NADPH-dependent reduction (types 1, 3, 5, and 7). Because of the broad substrate specificities, the primary roles of several of these enzymes are in basic metabolic pathways unrelated to steroid metabolism, and deficiencies of these enzymes cause metabolic disease.

The type 1 enzyme (HSD17B1) is referred to as the *estrogenic* 17β-HSD, because it catalyzes the final step in estrogen biosynthesis by preferentially reducing the weak estrogen estrone to yield the potent estrogen 17β-estradiol. The enzyme is a cytosolic protein that uses either NADH or NADPH as a cofactor. It has 100-fold higher affinity for C18 steroids than for C19 steroids. It also converts 16α-hydroxyesterone into estriol and shows modest 20α-HSD activity.

The HSD17B1 gene is expressed in granulosa cells of the ovary and the placental syncytiotrophoblast. HSD17B1 is also expressed at higher levels in breast cancer cells relative to HSD17B2, which converts estradiol into estrone. Amplification of *HSD17B1* in estrogen receptor-positive breast cancers is associated with lower survival compared with subjects without amplification. The crystal structure of HSD17B1 has been determined to a resolution of 2.2 Å with and without bound substrates, providing a molecular framework for the design of specific inhibitors which might be employed in chemotherapy of estrogen-responsive cancers.[76,77]

HSD17B2 is an endoplasmic reticulum enzyme that inactivates hormones; it preferentially oxidizes testosterone into androstenedione, and it oxidizes estradiol into estrone using NAD+ as its cofactor. However, HSD17B2 can also convert 20α-hydroxyprogesterone into progesterone.

The gene encoding HSD17B2 is expressed in liver, secretory endometrium, and the fetal capillary endothelial cells

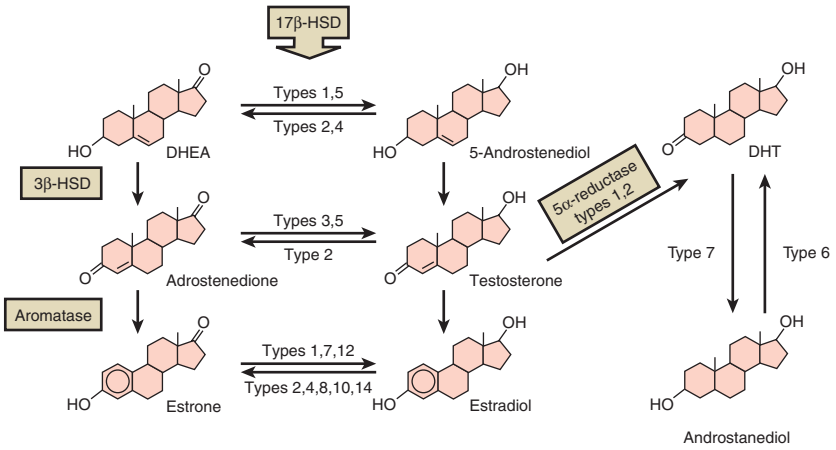

FIGURE 4.9 The family of 17β-HSDs and some of their roles in androgen and estrogen synthesis and metabolism. *DHEA,* Dehydroepiandrosterone; *DHT,* dihydrotestosterone; *HSD,* hydroxysteroid dehydrogenase. *(Modified from Luu-The V: Analysis and characteristics of multiple types of human 17β-hydroxysteroid dehydrogenase.* Steroid Biochem Mol Biol *76:143, 2001.)*

of the placenta, as well as the endothelial cells of the larger vessels. This expression pattern is consistent with its role of inactivating testosterone and estradiol. HSD17B2 in the fetal capillary endothelium protects the fetal compartment from estradiol formed in the syncytiotrophoblast and from any testosterone escaping aromatization. Its expression in the secretory endometrium permits the conversion of estradiol to estrone, whereas 20α-hydroxyprogesterone is converted back into progesterone, resulting in progestational dominance. In normal breast tissue, HSD17B2 expression predominates over HSD17B1 expression.

HSD17B3 is referred to as the *androgenic* 17β-HSD, because it catalyzes the final step in testosterone biosynthesis in the Leydig cells, reducing androstenedione to testosterone using NADPH as cofactor. It also can reduce estrone to estradiol. HSD17B3, an endoplasmic reticulum enzyme, is not expressed in the ovary, requiring androgen-producing cells of the ovary to employ another enzyme, probably HSD17B5, to synthesize testosterone.

In the absence of HSD17B3 activity, the testes are unable to convert androstenedione into testosterone, resulting in male pseudohermaphroditism (46,XY DSD) associated with 10- to 15-fold elevations in the ratio of blood androstenedione to testosterone.[6,78,79] Females with mutations in the *HSD17B3* gene are asymptomatic and produce normal amounts of estrogens and androgens. Molecular analysis of the *HSD17B3* gene in affected individuals revealed mutations that affect splicing, cause amino acid replacements in exons 9 and 10, and a small deletion leading to a frameshift. Many of the missense mutations result in proteins that are devoid of catalytic activity when expressed in eukaryotic cells.

HSD17B5, located on chromosome 10p15-14, is a member of the AKR family (AKR1C3). It has 3α-HSD and 20α-HSD activity in addition to 17β-HSD activity that produces testosterone from androstenedione. It is expressed in the adrenal cortex and may serve as the androgenic 17β-HSD of ovarian theca cells.[80,81] HSD17B5 is also expressed in prostate, mammary gland, and Leydig cells.

HSD17B6 (also known as 11-cis retinol dehydrogenase, RODH) has 3α-HSD activity and catalyzes the conversion of androstanediol to dihydrotestosterone in the prostate. It is also a 3α,3β-epimerase.

Human HSD17B7 is involved in cholesterol synthesis but also thought to produce active estrogens and inactivate androgens. It transforms estrone into estradiol and has 3-keto reductase activity for dihydrotestosterone. The gene is expressed in the ovary, breast, placenta, testes, prostate, and liver cells.

3α- and 20α-Hydroxysteroid Dehydrogenase Activities

A number of HSDs catalyze reactions at both the 3 and 20 positions of steroid hormones (e.g., AKR1C1).[6,37] The enzymes that are 3α-reductases (e.g., AKR1C2 and AKR1C4) are encoded by tandemly duplicated genes on chromosome 10p14-p15. They are expressed in liver, prostate, breast, and uterus, but some are also expressed in the testis and adrenals. Recessive mutations in one of these genes, AKR1C2, were discovered in 46,XY individuals with DSD, implicating this enzyme in a pathway of bioactive androgen biosynthesis through conversion of androsterone to androstanediol, followed by HSD17B6-mediated conversion of androstanediol to dihydrotestosterone.[82] This finding suggests that two pathways are needed for testicular androgen synthesis to support normal male sexual differentiation.

The enzymes with 20α-HSD activity that are members of the AKR family reduce progesterone to yield the inactive steroid, 20α-hydroxyprogesterone (AKR1C1). They are cytosolic proteins with a molecular weight of approximately 34 kDa, exemplified by genes expressed in human keratinocytes and cells of the liver, prostate, testis, adrenal gland, brain, uterus, and mammary gland. These enzymes prefer NADPH to NADH as a cofactor.[6,37]

Enzymes with 3α-hydroxysteroid oxidative activity that are members of the short-chain dehydrogenase/reductase family also have 20α-HSD activity and preferentially oxidize 20α-hydroxyprogesterone into progesterone.

$\Delta^{4\text{-}5}$-Reductases

The $\Delta^{4\text{-}5}$-reductases are membrane-associated enzymes that reduce the $\Delta^{5\text{-}4}$ double bond in steroid hormones by catalyzing direct hydride transfer from NADPH to the carbon 5 position of the steroid substrate.[6] They produce either 5α or 5β-dihydrosteroids.

5α-Reductases

Two different human 5α-reductases sharing 50% similarity in amino acid sequence and a molecular weight of approximately 29 kDa have been identified.[83] The genes for type 1 *(SRD5A1)* and 2 *(SRD5A2)* 5α-reductases each have five exons. The substrate-binding domain of the 5α-reductases is encoded by exon 1, and the cofactor-binding domain is encoded by exons 4 and 5. *SRD5A2* is located on chromosome 2p23, whereas *SRA5A1* is located on chromosome 5p15 with a pseudogene on Xq24-qter. A third enzyme containing a 5α-reductase domain, SRD5A3, has been identified, but its role in steroid metabolism has not been established.

SRD5A2 is predominantly expressed in male genital structures, including genital skin and prostate, where it reduces testosterone to yield the more potent androgen, 5α-dihydrotestosterone. SRD5A1, which catalyzes a similar reaction on both C21 and C19 steroid hormones, is expressed in the liver, kidneys, skin, and brain. Although this enzyme can also make 5α-dihydrotestosterone, its tissue distribution suggests that its predominant function is to inactivate steroid hormones.

SRD5A2 has an acidic pH optimum and a K_m for testosterone in the nanomolar range, whereas SRD5A1 has a broad alkaline pH optimum and a lower affinity for its substrates in the micromolar range. SRD5A2 can also be distinguished from the type 1 enzyme by its selective inhibition by finasteride with an inhibition constant (K_i) of 3 nM.

Inactivating mutations in *SRD5A2* cause male pseudohermaphrodism (46,XY DSD).[84,85] The enzyme defect is characterized by abnormal testosterone-to-5α-dihydrotestosterone ratios. Affected males have varying degrees of abnormal development of the external genitalia, ranging from mild hypospadias to severe defects in which the external genitalia are essentially female. Wolffian ducts develop normally in response to adequate levels of testosterone.

Females carrying mutations in the *SRD5A2* gene have a normal phenotype and normal menstrual cycles. They have a low incidence of hirsutism and acne, and similar to males with the disease, they have low ratios of 5α- to 5β-dihydrosteroid metabolites in the urine. The infrequency of acne in both affected males and affected females, the rarity of hirsutism in affected females, the absence of male pattern baldness, and the finding of an atrophic prostate in affected males indicates that SRD5A2 plays an important role in androgen metabolism in skin and in the androgen-dependent growth of the prostate.

Among the mutations reported are deletions that inactivate SRD5A2 and missense mutations that impair enzyme activity by affecting substrate or cofactor binding. A *SRD5A2* variant (Ala49Thr) that has increased catalytic activity has been associated with increased risk of prostate cancer.

Mutations have not been described in the human *SRD5A1* gene. However, targeted deletion of the murine counterpart results in a female phenotype of reduced fecundity and a parturition defect caused by failed cervical ripening. This defect can be reversed by administration of 5α-androstanediol.[86,87]

The only known human 5β-reductase (SRD5B1 or AKR1D1) is a member of the AKR superfamily.[88] This enzyme is involved in steroid hormone inactivation in the liver. Its reaction mechanism is similar to that described for 5α-reductase, except that an A/B *cis*-fused ring product is formed. The enzyme efficiently catalyzes the NADPH-dependent reduction of the $\Delta^{5\text{-}4}$ double bond in C27, C21, and C19 steroids to yield the 5β-dihydrosteroids with a distinct preference for C27 steroids. In keeping with this observation, mutations in the *SRD5B1 (AKR1D1)* gene that encodes this enzyme result in abnormal bile acid synthesis along with a marked reduction in the primary bile acids and 5β-reduced steroid metabolites.

Sulfotransferases

A family of enzymes introduce the sulfonate (SO_3^-) anion from an activated donor, *3′-phosphoadenosine-5′-phosphosulfate* (PAPS), to a steroid hydroxyl acceptor, inactivating the hormone.[89-91] The major enzymes performing this reaction include estrogen sulfotransferase (SULT1E1), an enzyme that sulfonates the 3-hydroxyl function of phenolic steroids, and the hydroxysteroid sulfotransferases encoded by the closely linked *SULT2A1* and *SULT2B1* genes on chromosome 19q13.4.

The SULT2A1 enzyme, also known as *DHEA sulfotransferase,* has a broad substrate range including the 3α-, 3β-, and 17β-hydroxy functions of steroid hormones. SULT2A1 enzyme is expressed at high levels in the fetal zone of the adrenal cortex, as well as in the zona reticularis after adrenarche, and in the liver, gut, and testes. This enzyme is developmentally regulated in the adrenal cortex, with expression increasing between ages 5 and 13 years in association with adrenarche. The GATA-binding factor 6 (GATA6) transcription factor, which is known to increase transcription of other genes involved in androgen biosynthesis, also activates expression of the *SULT2A1* gene.

The *SULT2B1* gene gives rise to two protein isoforms through alternative splicing. The SULT2B1a isoform sulfonates pregnenolone, whereas the SULT2B1b isoform preferentially sulfonates cholesterol. They both sulfonate dihydrotestosterone. Unlike the *SULT2A1* gene that is expressed in a limited number of tissues, SULTB1 isoforms are present in a variety of hormone-producing and hormone-responsive tissues, including the placenta, ovary, uterus, and prostate.

SULT1E1 is expressed in many tissues, including the adrenal gland, liver, kidneys, muscle, fat, and uterus. In the endometrium, progesterone increases its activity, contributing to the inactivation of estradiol in the secretory phase. The importance of estrogen sulfotransferase in modulating local levels of bioactive estrogens has been shown in mice with targeted deletions of the gene.[92] Male mice with estrogen sulfotransferase deficiency have Leydig cell hyperplasia and become infertile with age due to elevated testicular levels of bioactive estrogen. Adipose tissue mass also increases. Hydroxylated metabolites of polychlorinated biphenyls are potent inhibitors of estrogen sulfotransferase with IC50 values in the picomolar range.[93] The blockade of estradiol inactivation by these compounds may account for the reported estrogenic activity of polychlorinated biphenyls.

Mutations in the gene that encodes one of the enzymes that synthesizes the sulfate donor, PAPS (PAPS synthase type 2 [PAPSS2]), results in increased free DHEA levels, with the subsequent conversion of DHEA into biologically active androgens.[6]

Steroid Sulfatase

The sulfonate function on steroids is cleaved by steroid sulfatase, an enzyme encoded by the *STS* gene on chromosome Xp22.3.[6,94-96] This enzyme plays a key role in controlling the generation of biologically active steroids from the inactive sulfated molecules (e.g., estrone sulfate and DHEA sulfate). The sulfated steroid substrates, which are hydrophilic and not membrane-permeable, are taken up by cells via specific transport proteins, including a sodium-dependent anion transporter *(SLC10A6)*.

The syncytiotrophoblast is enriched in steroid sulfatase, which plays a key role in placental estrogen synthesis by liberating sulfonated androgen precursors produced in the fetal compartment prior to aromatization. The enzyme is also expressed in skin where it metabolizes cholesterol sulfate and sulfated estrogens.

Sulfatase deficiency is associated with marked impairment of placental estrogen synthesis during pregnancy and ichthyosis developing after birth.[95,96] It occurs mostly in males because of the X chromosome location of the sulfatase gene at a frequency of 1:2000 to 1:6000 live born males. The majority of subjects with steroid sulfatase deficiency have a deletion of the entire gene that results from recombination of repetitive elements that flank the locus. The large deletions of the *STS* gene occur in association with mutations in the adjacent Kallmann syndrome gene *(KAL1)*. Partial deletions in the *STS* gene causing enzyme deficiency have also been described.

Characteristically, in pregnancies hosting an affected fetus, maternal plasma estriol and urinary estriol excretion are quite low at approximately 5% of the levels found in normal pregnancies. Excretion of estrone and estradiol are also reduced at approximately 15% of normal. Maternal serum 16α-hydroxydehydroepiandrosterone levels are elevated, and intravenous administration of DHEA sulfate to the mother does not lead to an increase in estrogen excretion, whereas administration of DHEA does lead to increased estrogen excretion.

Steroid sulfatase is expressed in estrogen target tissues, including endometrium, bone, and breast. Increased sulfatase expression may contribute to greater bioavailability of estradiol in certain tumors, including breast and endometrial cancers. Inhibitors of sulfatase have been developed and tested in clinical trials (Irosustat).[97]

UDP-Glucuronosyl Transferases

Glucuronidation, catalyzed by a family of uridine 5′-diphospho UDP-glucuronosyl transferases, is part of the metabolic clearance mechanism for steroid hormones by the liver and extrahepatic tissues.[98-102] There are 18 known UDP-glucuronosyl transferases that have been divided into three subfamilies: UGT1A, UGT2A, and UGT2B. UGT1A enzymes are encoded by a single gene that gives rise to alternatively spliced products capable of acting on estrogens. The UGT2 enzymes are products of separate genes *UGT2A* and *UGT2B*. *UGT2A* is expressed in olfactory epithelium, and *UGT2B* is expressed in the liver, kidney, breast, lung, and prostate. At least seven members of the UGT2B family have been identified with different steroid substrate specificities. Three UGT2B enzymes, UGT2B7, UGT2B15, and UGT2B17, appear to be responsible for glucuronidation of 5α-dihydrotestosterone, androsterone, and androstane-3α-17β-diol. Of these, UGT2B15 and UGT2B17 are expressed in the prostate where they are proposed to modulate steroid action. Polymorphisms in genes encoding UDP-glucuronosyl transferases have been associated with estrogen levels, suggesting a role for these phase II biotransformation enzymes in regulating the bioactive steroid economy.

Other Steroid Hormone Metabolic Pathways

- *Alternative pathways can produce bioactive androgens in the fetal testes and polycystic ovary syndrome.*
- *Some pathways modify molecules that, although steroidal in structure, act via mechanisms that do not involve classical steroid hormone receptors.*

There are several metabolic fates of steroid molecules that have a significant role in the synthesis of bioactive hormones. A pathway for 5α-dihydrotestosterone production, referred to as a *backdoor pathway*, is one example.[82,103] Among the other fates of steroid hormones are esterification to long-chain fatty acids, formation of catechol estrogens, and the 7α-hydroxylation of androgens. Equine steroidogenic tissues also have a novel pathway for biosynthesis of estrogens, resulting in a phenolic A ring and an unsaturated B ring.

An Alternative Pathway for Androgen Synthesis

Fetal androgen synthesis in marsupials involves a novel pathway that leads from 17α-hydroxyprogesterone to 5α-dihydrotestosterone without going through androstenedione or testosterone as intermediates (Fig. 4.10). This pathway, recently identified in humans, is initiated when either progesterone or 17α-hydroxyprogesterone is 5α-reduced. The resulting 5α-C_{21} steroids are reduced by 3α-HSDs (AKR1C2/4) yielding substrates for P450c17. Androsterone produced by P450c17 can ultimately be converted to 5α-dihydrotestosterone by HSD17B6.[80,103] This pathway (the so-called backdoor pathway) is important for fetal testicular production of bioactive androgens and contributes to androgen excess in the context of 21-hydroxylase deficiency. It has been reported to be active in the polycystic ovary syndrome theca cells.

Catechol Estrogens

Catechol estrogens are generated by the actions of genes encoded by *CYP1A1* and *CYP1A2* (which catalyze 2-hydroxylation of estrogens), and *CYP1B1*, which is an estrogen 4-hydroxylase.[104-106] Although the catechol estrogens are short-lived in vivo and are postulated to have physiological functions as locally generated signaling molecules, they also yield potent genotoxic molecules implicated in carcinogenesis. The 4-hydroxyestrogens can be oxidized to quinone intermediates that react with purine bases of DNA, resulting in depurinating adducts that generate highly mutagenic apurinic sites (Fig. 4.11). Quinones derived from the 2-hydroxyestrogens produce stable DNA adducts and are presumed to be less genotoxic. Metabolism of catechol estrogens may also generate oxygen-free radicals.

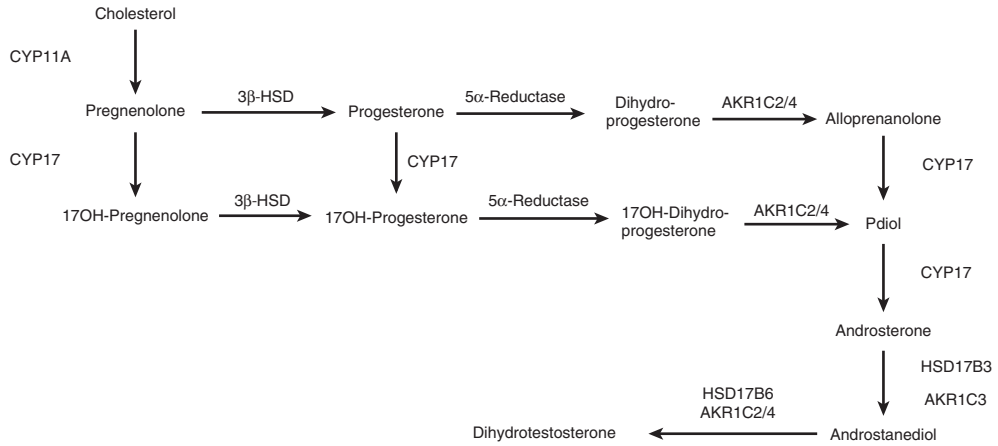

FIGURE 4.10 Alternative pathway or "backdoor pathway" for fetal testicular androgen synthesis. *AKR*, Aldo-keto reductase; *HSD*, hydroxysteroid dehydrogenase. *(Modified from Auchus RJ, Miller WL: Defects in androgen biosynthesis causing 46,XY disorders of sexual development.* Sem Reprod Med *30:417–426, 2012.)*

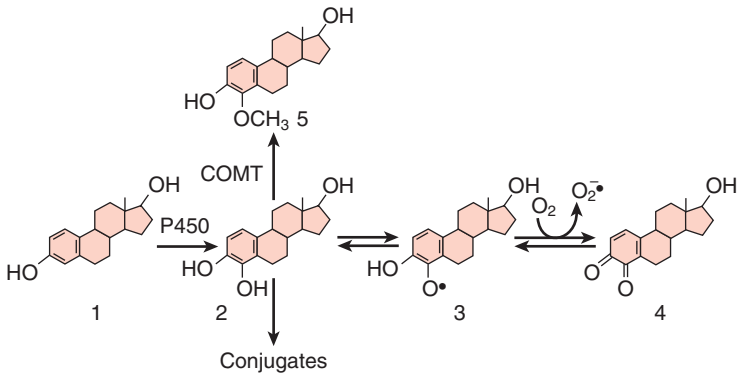

FIGURE 4.11 Metabolism of estradiol *(1)* by P450 enzymes, including P4501B1 to 4-hydroxyestradiol *(2)*. Metabolic cycling between 4-hydroxyestradiol and estradiol 3,4-quinone *(4)* can be catalyzed by P4501A1 for the oxidation step and cytochrome P450 reductase for the reduction step. The semiquinone intermediate *(3)* is a free radical that can react with molecular oxygen to form superoxide radical and quinone. 4-Hydroxyestradiol can be converted to 4-methoxyestradiol *(5)* by catechol-O-methyltransferase *(COMT). (From Liehr JG: Catechol-estrogens in the induction of tumors in the kidney of the Syrian hamster. In: Goldstein DS, Eisenhofer G, McCarty R, editors:* Advances in Pharmacology: Catecholamines. Bridging Basic Science with Clinical Medicine, *vol 42. San Diego, 1998, Academic Press, pp 824–828.)*

Catechol estrogens are methylated by catechol-O-methyltransferase, resulting in a catecholamine-like substance. The methylated catechol estrogens have antiangiogenic and antitumor activity through inhibition of hypoxia-inducible factor-1α (HIF-1α), a proangiogenic transcription factor. There is evidence that altered production of 2-methoxyestradiol may contribute to the pathogenesis of preeclampsia through its modulation of antiangiogenic factor production by the placenta.[107,108] Levels of antiangiogenic (catechol estrogens) as well as proangiogenic estrogen metabolites are correlated with luteal function.[109]

Steroid Fatty Acid Esters

Steroids esterified to long-chain fatty acids are present in blood bound to lipoproteins, or are found in tissue, particularly steroidogenic glands and fat.[110,111] These hydrophobic molecules may serve as a depot form of steroid, but they also have unique biochemical attributes. Estradiol 17-esters are produced in blood by the action of lecithin-cholesterol acyl transferase and in tissues by SOATs. The fatty acid esters of estradiol have pronounced antioxidant activity. Fatty acid esters of other steroids, including pregnenolone, testosterone, DHEA, and glucocorticoids, have also been described.

Steroid 7α-Hydroxylation

Substituents on carbon 7 of the steroid nucleus can have a significant effect on activity. A cytochrome P450 encoded by the *CYP7B* gene catalyzes 7α-hydroxylation of steroid hormones and oxysterols. The 7α-hydroxylation of DHEA produces a molecule with enhanced immunostimulatory activity, a property demonstrable in animal bioassays.[112]

B-Ring Unsaturated Steroids

The pregnant mare produces estradiol and estrone, but also B-ring unsaturated estrogens (equilin [with an 8,9 olefinic bond], equilenin [with a phenolic B ring], 17α-

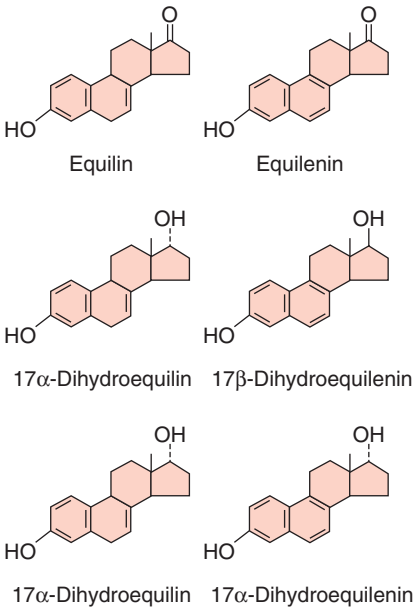

FIGURE 4.12 Structures of equine estrogens.

dihydroequilin, 17α-dihydroequilenin, 17β-dihydroequilin, and 17β-dihydroiequilenin; Fig. 4.12).[113]

These B-ring unsaturated compounds are potent estrogens in vivo. Although 7-dehydrocholesterol can be converted into B-ring unsaturated estrogens (as occurs in Smith-Lemli-Opitz syndrome), the biosynthesis of these compounds in the pregnant mare occurs by a pathway not requiring the synthesis of squalene or cholesterol, and thus does not involve 7-dehydrocholesterol. Evidently, a C25 sesterterpene pathway coexists with the normal biosynthetic route of "standard" estrogens from a cholesterol precursor.

Vitamin D Synthesis and Metabolism

- *Vitamin D_3 is produced endogenously in the skin from 7-dehydrocholesterol.*
- *Vitamin D_2 is obtained from the diet from plant sources.*
- *Vitamin D_2 and D_3 are metabolized by the same enzymes to produce biologically active compounds: 1α,25-dihydroxyvitamin D.*

Increasing epidemiological evidence indicates that in addition to calcium and bone metabolism the secosteroid, vitamin D, has important roles in immune function and reproduction. Vitamin D deficiency or deficiency of its active metabolites is associated with hypogonadism, reduced fertility, polycystic ovary syndrome, endometriosis, and leiomyomas.[114]

The impact of vitamin D on reproduction is demonstrable in genetically engineered mice.[115-117] Vitamin D receptor (Vdr) and 1α-hydroxylase *(Cyp27b1)* knockout female mice display reduced fecundity, impaired folliculogenesis, and anovulation. These reproductive abnormalities are evidently due to dysfunctional calcium homeostasis since high calcium diets partly restore fertility and increase the rate of conception in *Vdr* null mice.

Vitamin D_3 (cholecalciferol), the endogenous member of the vitamin D family in humans, is produced in the innermost layers of the skin (stratum basale and stratum spinosum) through the action of ultraviolet light on 7-dehydrocholesterol (Fig. 4.13), resulting in the opening of the steroid nucleus B ring.[115-117] Vitamin D_2 (ergocalciferol) is a plant vitamin D family member, which is metabolized similarly to vitamin D_3. Vitamin D_3 travels to the liver where it is converted into 25-hydroxyvitamin D, mainly by an endoplasmic reticulum P450, CYP2R1, a highly conserved and substrate-specific 25-hydroxylase. There are other hepatic enzymes that can catalyze 25-hydroxylation, but they have lower affinities for vitamin D molecules. The 25-hydroxylation reaction does not appear to be regulated and the formation of 25-hydroxyvitamin D molecules is determined principally by endogenous formation of vitamin D_3 or dietary intake. Rare inactivating mutations have been described in the *CYP2R1* gene leading to hypocalcemia, secondary hypoparathyroidism, and rickets. 25-hydroxyvitamin D_3 in plasma is mainly bound to vitamin D binding protein (also called group specific protein). Megalin and cubilin, endocytic receptors, participate in the uptake of vitamin D binding protein and its 25-hydroxyvitamin D_3 cargo for further metabolism. The 25-hydroxyvitamin D_3 is converted into the biologically active 1α,25-dihydroxyvitamin D_3 by the action of a mitochondrial enzyme, CYP27B1, in the kidney, but also in monocytes, maternal decidua, and placental trophoblast cells. Like mutations in *CYP2R1*, inactivating mutations of the *CYP27B1* gene are also associated with hypocalcemia, secondary hyperparathyroidism, and the clinical and radiographic features of severe rickets. The *CYP27B1* gene and the formation of 1α,25-dihydroxyvitamin D by the kidney are regulated by negative feedback by 1α,25-dihydroxyvitamin D_3, stimulated by parathyroid hormone (PTH), whose levels reflect calcium status, and inhibited by fibroblast growth factor 23 (FGF23), which reflects phosphate homeostasis.

The cholesterol side-chain cleavage enzyme, CYP11A1, can also act on vitamin D producing 20- hydroxy compounds that have no role in calcium metabolism but have antiinflammatory and antifibrotic effects.

Inactivation of 1α,25-dihydroxyvitamin D molecules is carried out mainly by a mitochondrial 24R-hydroxylase (encoded by the *CYP24A1* gene), which catalyzes both 23- and 24-hydroxylation. Mutations in the *CYP24A1* gene are associated with severe neonatal hypercalcemia, and a less severe form of hypercalcemia in adults. It should be noted that some believe that 24-hydroxylated vitamin D molecules have biological actions that are distinct from those of 1α,25-dihydroxyvitamin D_3.

Regulation of Expression of the Steroidogenic Machinery

- *NR5A1 (SF-1) is a key transcription factor regulating expression of genes encoding steroidogenic proteins.*
- *Other key transcription factors regulating genes involved in steroidogenesis include NR5A2 (LRH-1), and GATA-4 and 6.*

The regulation of expression of genes encoding proteins involved in steroidogenesis in the ovary, testes, and adrenal cortex shares a number of similarities with respect to the involvement of *cis* elements and transcription factors. SF-1, a nuclear receptor, also known as Ad4BP and by the nuclear

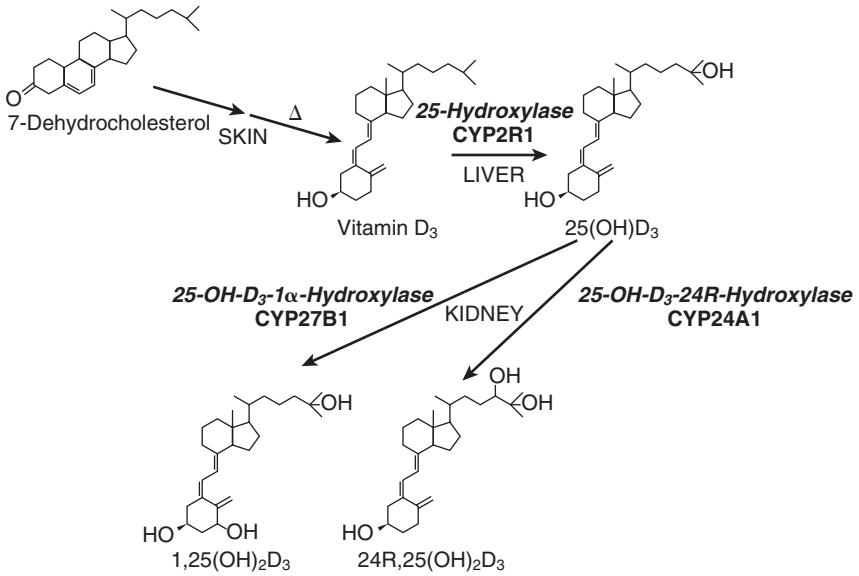

FIGURE 4.13 Metabolism of vitamin D3. 7-Dehydrocholesterol is converted to vitamin D3 in the skin triggered by ultraviolet light *(Δ)*. Vitamin D3 is transported to the liver, mostly bound to protein, where it undergoes hydroxylation at the C25-position catalyzed by CYP2R1. 25-Hydroxyvitamin D3, the major form of circulating vitamin D, undergoes hydroxylation in the kidney at either the C1 position, catalyzed by CYP27B1, or the C24 position, catalyzed by CYP24A1, resulting in 1α,25-dihydroxyvitamin D3, or 24R,25-dihydroxyvitamin D3. *(Modified from Henry HL: Regulation of vitamin D metabolism.* Best Pract Res Clin Endocrinol Metab *25:531–541, 2011.)*

receptor family designation NR5A1, is essential for development of steroidogenic glands.[118,119] Most of the genes encoding key proteins involved in steroidogenesis (e.g., *SCARB1*, *STARD1*, *CYP11A1*, *CYP11B2*, *CYP17A1*, *CYP19A1*, *CYP21A2*) contain one or more NR5A1 response elements in their proximal promoters. These elements are important for basal as well as stimulated expression of these genes, generally by a cAMP-mediated signal transduction pathway.

The importance of cAMP signaling in steroidogenic tissues is reflected in increased steroid production when specific phosphodiesterases are blocked, which elevates intracellular cAMP levels, or when mutations occur in the gene encoding the regulatory subunit of protein kinase A *(PRKAR1A)*, resulting in unrestrained protein kinase A activity.[6] There appear to be several mechanisms by which NR5A1 action is modified by cAMP, including phosphorylation of the protein by kinases, providing a link between this transcription factor and molecules that transduce signals from plasma membrane receptors.[103]

The importance of NR5A1 to the regulation of steroidogenic tissues was documented by gene targeting. Mice deficient in NR5A1 lacked adrenal glands and gonads, and males were consequently sex-reversed. Haploinsufficiency of *Nr5α1* in the mouse resulted in an impaired adrenal steroidogenic response to stress, although basal steroidogenesis was not affected due to compensatory hypertrophy. *NR5A1* haploinsufficiency has been reported in humans with primary adrenal failure and 46,XY DSD.[119] It is now evident that variation in the NR5A1 gene is associated with a range of phenotypes such as 46,XY DSD, hypospadias, anorchia, male factor infertility, and primary ovarian insufficiency. Among the *NR5A1* mutations reported are missense mutations within the DNA-binding region, a nonsense mutation, and a frameshift mutation predicted to disrupt RNA stability or protein function. Functional studies of the missense mutants (Cys33Ser, Arg84His) and of one nonsense mutants (Tyr138Stop) showed impaired activation of NR5A1–responsive target genes.

In addition to NR5A1, other transcription factors participate in the control of genes encoding the steroidogenic machinery. A related transcription factor, liver receptor homologue-1 (SF-2 or NR5A2) recognizes the same canonical DNA motif to which NR5A1 binds and may share functions with NR5A1 in certain tissues, including the adrenal cortex, testis, and ovary.[120] Both NR5A1 and NR5A2 have been crystallized and found to contain phospholipid-binding pockets, with phosphatidyl inositols being the presumed ligands. These observations suggest that phospholipids may be regulatory molecules controlling expression of genes involved in steroidogenesis.[121]

The tissue-specific regulation of genes expressed in multiple steroidogenic glands (e.g., *CYP17A1*) requires the action of other transcription factors working either independently or in concert with NR5A1 in a combinatorial fashion. In addition, the activity of NR5A1 is regulated by transcription factors that either bind to NR5A1 response elements and prevent activation of transcription (chicken ovalbumin upstream promoter-transcription factor [COUP-TF]) or bind to NR5A1 and block its ability to transactivate promoters (DAX-1, also known as NR0B1). Interestingly, expression of the latter gene is upregulated by NR5A1, so there is a complex control mechanism in place for modulating these antagonistic molecules.

Other transcription factors that are known to be important for the expression of genes involved in steroidogenesis include GATA4 and GATA6, which are members of the GATA family of transcription factors originally identified as being central to hematopoiesis and endoderm development, and LXRalpha.[122,123]

The human placenta is an outlier in many respects in terms of the regulation of steroidogenesis.[124] First, the placenta

does not express certain genes that are pivotal to gonadal and adrenal steroid hormone synthesis, including *NR5A1*, *STARD1*, *HSD3B2*, and *CYP17A1*. Moreover, HSD3B1 replaces HSD3B2 in the placenta, and a START domain protein, STARD3 (which is not subject to acute regulation), may subserve STARD1's role in cholesterol movement to the placental side-chain cleavage system.

Unlike the gonads and adrenal cortex, the placenta's capacity to produce progestins is believed to be largely determined by levels of adrenodoxin reductase (which governs the availability of reducing equivalents) and P450scc. In addition, *CYP19A1* transcription in the placenta is driven by a different promoter than that used by the gonads. Thus placental steroidogenesis is controlled in a unique way; the mechanism is more tonic, with steroidogenic capacity determined primarily by differentiation of trophoblast cells and growth of the placenta as opposed to tight regulation by trophic hormones.

Examples of Extraglandular Steroidogenesis

- *Bioactive steroids are produced in small quantities in many cell types, either de novo or by metabolism of circulating "inert" steroid molecules.*
- *The intracrine synthesis and metabolism of bioactive steroid hormones are important in normal physiology as well as neoplasia (e.g., breast cancer) and abnormal function of reproductive tissues (e.g., endometriosis).*
- *Important extraglandular sites of steroid metabolism include the liver, adipose tissue, skin, and brain.*

Although steroid hormone synthesis traditionally has been studied in the classic steroidogenic glands (ovaries, testes, adrenal cortex, and placenta), it is now evident that production of bioactive steroids, albeit at much lower levels, occurs at extraglandular sites, such as the brain, vascular tree, and adipose tissue.[125,126] Synthesis also occurs in pathological conditions affecting the endometrium (endometriosis and endometrial cancers), breast (breast cancer), and prostate (prostate cancer).[127]

Synthesis of Neurosteroids

The notion that steroid hormones could be synthesized in the central nervous system evolved from the discovery of appreciable levels of pregnenolone and DHEA and their fatty acid esters in the brains of animals, even after gonadectomy or adrenalectomy.[128] It was subsequently shown that enzymes required for steroid hormone synthesis were expressed in the brain, spinal cord, and peripheral nervous system at the mRNA and protein levels. Included in this category are STARD1, P450scc, P450c17, P450arom, P450c11β, P450c11AS, HSD17B1, HSD17B2 and 3β-HSD, 5α-reductase, 3α-HSD, and 11β-HSD activities.[129]

The enzymes and their associated activities are distributed in different brain regions and cell types, including glia (astrocytes and oligodendrocytes) and neurons. They probably can act on circulating "prohormones" as well as participate in the de novo synthesis of steroids. The expression of the steroidogenic enzymes in the brain is developmentally regulated, although little is known about the mechanisms that control expression.

The known neurosteroids may act via the classic nuclear hormone receptors, but there is also good evidence that nonclassic signaling pathways are involved, including actions on gamma-aminobutyric acid $(GABA)_A$, N-methyl-D-aspartic acid (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), glycine, serotonin, Sigma type 1, nicotinic acetylcholine, and oxytocin receptors. The actions of neurosteroids on these receptors have been implicated in stress responses, anxiolysis, seizure disorders, memory, unipolar and postpartum depression, and protection against neuronal injury. The evidence supporting these notions has mostly been derived from in vitro studies and animal studies. However, pharmacologic evidence from human studies based on synthetic neurosteroids (e.g., the short-acting anesthetic alphaxolone) substantiates the concepts derived from animal experimentation.

Steroid Hormone Metabolism in Skin

Skin is an important site of production of vitamin D precursors.[115-117] It also contributes to testosterone production in women by metabolizing prohormones, such as DHEA sulfate and androstenedione into testosterone.[130,131] The *HSD3B1*, *17BHSD3*, and *SRD5A1* and *SRD5A2* genes are expressed in skin, allowing for local formation of androgens that can activate the androgen receptors present in the stroma, sebocytes, and dermal papillae. Recent studies suggest that expression of STARD1 and aromatase in skin is correlated with skin androgen and estrogen levels.

Secretion, Production, and Metabolic Clearance Rates of Steroid Hormones

Steroid concentrations in the circulation are determined by:
- *rate of secretion*
- *rate of metabolism and precursors, and*
- *rate of extraction by tissues and metabolism.*

The concentration of a steroid in the circulation is determined by the rate at which it is secreted from glands, the rate of metabolism of precursor or prohormones into the steroid, and the rate at which it is extracted by tissues and metabolized. Steroid metabolism is influenced by sex, age, weight, and thyroid function. The *secretion rate* of a steroid refers to the total secretion of the compound from a gland per unit time.

Secretion rates have been assessed by sampling the venous effluent from a gland over time and subtracting out the arterial or peripheral venous hormone concentration. Although seemingly simple in concept, this procedure is quite challenging in practice. Much of the difficulty originates from the potential of the catheterization process to disturb gland function (e.g., endocrine changes resulting from the stress of the procedure) and the possibility of dilution or contamination from blood draining other glands. For example, the role of the postmenopausal ovary in androgen production has been challenged because of potential contamination of adrenal venous blood in ovarian venous samples.[132]

The *metabolic clearance rate* of a steroid is defined as the volume of blood that has been completely cleared of

the hormone per unit time. The *whole-body metabolic clearance rate* is usually measured, reflecting the sum of clearance rates for each tissue or organ. Experimentally, this measurement is accomplished by infusion of an isotopically labeled steroid at a constant rate.[133] At equilibrium, the concentration of the infused steroid in peripheral venous blood is constant, and the rate of clearance from the blood equals the rate of entry. The metabolic clearance rate is calculated by dividing the infusion rate by the concentration of the steroid isotope in peripheral blood, giving the metabolic clearance rate in milliliters per day or liters per day.

Most of the circulating steroids are removed from blood by the liver. Hepatic blood flow in humans is approximately 1500 L/day, so metabolic clearance rates exceeding this level generally reflect extraction of steroids by other organs in addition to the liver. The lung, with its high rate of blood flow, is another potentially important site of C21 and C19 steroid metabolism.[134,135] In addition to the amount of SHBG, therefore the amount of bound steroid, obesity, irrespective of SHBG levels, increases the metabolic clearance rate. The uptake of steroids into the liver and other organs is highly influenced by their affinity for plasma steroid–binding proteins and albumin. Binding of steroid hormones to SHBG and corticosteroid-binding globulin (CBG) reduces peripheral metabolism.[136,137] The binding of free steroids to albumin is of relatively low affinity; consequently, metabolic clearance rates of albumin-bound unconjugated steroids are relatively high compared with those of hormones, such as testosterone and cortisol, which bind, respectively, to SHBG and CBG with high affinity.[138] Sulfoconjugated steroids are an exception because they bind tightly to albumin, and as a result, are cleared very slowly from the blood. Consequently, concentrations of sulfated steroids in blood are usually several-fold higher than their respective unconjugated forms. In contrast, steroid glucuronates are weakly bound to albumin and are rapidly cleared.

The *production rate* of a steroid hormone refers to entry into the blood of the compound from all possible sources, including secretion from glands and conversion of prohormones into the steroid of interest.[133] At steady state, the amount of hormone entering into the blood from all sources equals to the rate at which it is being cleared (metabolic clearance rate) multiplied by blood concentration (production rate = metabolic clearance rate × concentration). If there is little contribution of prohormone metabolism to the circulating pool of steroid, then the production rate will approximate the secretion rate.

The fraction of prohormone that is metabolized into the steroid of interest, known as the *rho (ρ) value*, can be estimated by infusing an isotope of the prohormone at a constant rate until equilibrium is reached, then determining the blood concentrations of the unconjugated prohormone isotope and the isotopic product.[139] The amount of prohormone entering the circulation can be calculated using the ρ value and the production rate.

Table 4.2 shows the secretion, production, and metabolic clearance rates of the major steroids, and Table 4.3 provides the ρ values of selected sex steroid hormones. Note that there are some differences in these values between sexes and in the favored direction of interconversion, which reflects the integrated activities of the different 17β-hydroxysteroid dehydrogenases that selectively oxidize or reduce androgens and estrogens.

Plasma Steroid Hormone–Binding Proteins

- *Most bioactive steroid hormones in plasma are bound to specific carrier proteins and albumin.*
- *The carrier proteins determine the biologically active or "free" fraction of steroid hormone capable of entering target cells.*
- *Steroid binding proteins also influence the clearance rate of steroid hormones.*

Greater than 97% of the circulating fractions of testosterone, estradiol, cortisol, and progesterone are bound

Table 4.2 Blood Production Rates, Secretion Rates, Metabolic Clearance Rates, and Normal Serum Concentration of Sex Steroid Hormones

Steroid Hormone	Reproductive Phase	MCR (L/day)	PR (mg/day)	SR (mg/day)	Reference Values
Men	—	—	—	Testes	—
Androstenedione	—	2200	2.8	1.6	2.8–7.3 nmol/L
Testosterone	—	950	6.5	6.2	6.9–34.7 nmol/L
Estrone	—	2050	0.15	0.11	37–250 nmol/L
Estradiol	—	1600	0.06	0.05	<37–210 pmol/L
Estrone sulfate	—	167	0.08	Insignificant	600–2500 pmo/L
Women	—	—	—	Ovary	—
Androstenedione	—	2000	3.2	2.8	3.1–12.2 nmol/L
Testosterone	—	500	0.19	0.06	0.7–2.8 nmol/L
Estrone	Follicular	2200	0.11	0.08	110–400 pmol/L
—	Luteal	2200	0.26	0.15	310–660 pmol/L
—	Postmenopausal	1610	0.04	Insignificant	22–230 pmol/L
Estradiol	Follicular	1200	0.09	0.08	<37–360 pmol/L
—	Luteal	1200	0.25	0.24	699–1250 pmol/L
—	Postmenopausal	910	0.006	Insignificant	<37–140 pmol/L
Estrone sulfate	Follicular	146	0.1	Insignificant	700–3600 pmol/L
—	Luteal	146	0.18	Insignificant	1100–7300 pmol/L
Progesterone	Follicular	2100	2	1.7	0.3–3 nmol/L
—	Luteal	2100	25	24	19–45 nmol/L

MCR, Metabolic clearance rate; *PR*, production rate; *SR*, secretion rate.

Table 4.3 Mean ρ Values for Interconversion of Key Sex Steroid Hormones

Interconversion			ρ Values: Female	ρ Values: Male
Androstenedione	→	Testosterone	0.03	0.052
Testosterone	→	Androstenedione	0.122	0.076
Androstenedione	→	Estrone	0.007	0.0114
Testosterone	→	Estradiol	0.0014	0.0033
Estrone	→	Estradiol	0.041	0.05
Estradiol	→	Estrone	0.176	0.156

by plasma proteins of hepatic origin. SHBG and albumin bind testosterone and estradiol, whereas CBG and albumin bind cortisol and progesterone, and testosterone to a far lesser extent. High-affinity binding of natural steroid hormones to SHBG requires a 17β-hydoxy group and a 5α-hydrogen function. An olefinic bond in the A ring or an aromatic A ring reduces binding affinity. Consequently, SHBG has greater affinity for testosterone than it does for estradiol; 65% and 78% of circulating testosterone is bound to SHBG in men and women, respectively, whereas only 30% and 58% of estradiol in men and women, respectively, is associated with SHBG. The remainder is mostly bound to albumin.

The protein-bound steroid hormone is generally considered a reservoir, restrained from free diffusion into cells, where it can act and can be metabolized. This notion is substantiated by the discovery of variants in the *SHBG* gene that include a missense mutation (Pro156Leu) that causes abnormal glycosylation and impaired secretion. Women with this variant display symptoms of hyperandrogenemia, reflecting a greater proportion of bioavailable testosterone.[140]

Association studies and genome-wide linkage scans suggest that variation in plasma SHBG levels is influenced by polymorphisms in the *SHBG* gene, as well as several other genes at different loci in different ethnic groups.[141,142] Interestingly, the level of SHBG in plasma, as influenced by these polymorphisms, is associated with risk for developing type 2 diabetes mellitus with higher SHBG levels being protective. The molecular mechanisms underlying this association remain to be elucidated.[143]

Although SHBG is generally believed to reduce the entry of sex steroids into target tissues, it has been argued that the steroid bound to binding globulins may be selectively accumulated by some cell types by "receptors," and that target tissues also synthesize SHBG that acts locally to facilitate signal transduction by way of a cAMP mechanism.[144] However, there is no evidence for a specific SHBG receptor, suggesting that uptake of SHBG may be related to its binding to extracellular matrix elements or receptors for other proteins.

Because of the importance of plasma steroid–binding proteins in influencing the amount of bioavailable hormone, any genetic or physiological variation or pharmacologically induced change in the production of these proteins by the liver can have a significant effect on steroid hormone action and metabolism. The clinical evaluation of subjects with suspected disorders of hormone production or action may require an assessment of the level of binding protein or a measurement of the bioavailable or free fraction of hormone to clarify the basis for the clinical presentation. Conditions

Table 4.4 Factors Influencing the Binding Capacity of Sex Hormone-Binding Globulin and Cortisol-Binding Globulin

Factors and Endocrine Status	Binding Capacity: SHBG	Binding Capacity: CBG
Exogenous estrogen	↑	↑
Pregnancy	↑	↑
Exogenous androgens	↓	↓
Anabolic steroids	↓	NC
Synthetic progestins (androgenic properties)	↓	NC
Thyroid hormone (hyperthyroidism)	↑	↓
Prolactin (hyperprolactinemia)	↓	NC
Growth hormone (acromegaly)	↓	NC
Old age (men)	↑	NC
Postmenopausal	↓	↓
Obesity	↓	NC
Hyperinsulinemia	↓	↓

CBG, Cortisol-binding globulin; *NC*, no change; *SHBG*, sex hormone-binding globulin.

with increased SHBG levels are associated with increased free estradiol/testosterone ratios and those with reduced SHBG with decreased free estradiol to testosterone ratios. For example, suppression of SHBG levels associated with obesity, hyperinsulinemia, and hyperglycemia contributes to the symptoms of hyperandrogenemia associated with polycystic ovary syndrome, which is frequently accompanied by these metabolic phenotypes. Table 4.4 summarizes changes in SHBG- and CBG-binding capacity under different physiological and pathophysiological conditions and the influence of certain pharmacologic agents.

The gene encoding vitamin D binding protein *(GC)* is polymorphic with alleles showing different population distributions that are associated with skin pigmentation. Binding protein levels are increased by estrogen and are unaffected by age or body mass index (BMI).

Inhibitors of Enzymes Involved in Synthesis or Metabolism of Sex Steroids

- *Nonsteroidal and steroidal inhibitors effectively reduce production of specific steroid hormones in vivo.*
- *Some endocrine disruptors act as enzyme inhibitors and produce their biological effects by altering cellular steroid metabolism, changing the intracellular levels of endogenous bioactive steroids.*
- *Reversible enzyme inhibitors have short-lived effects, whereas mechanism-based inhibitors destroy enzyme activity and therefore require resynthesis of the enzyme to reverse the inhibition.*

The inhibition of steroidogenic enzymes has been shown to be an effective strategy for terminating pregnancy and treating disorders of excessive hormone production (including Cushing syndrome, steroid hormone–secreting malignancies, and hormone-dependent cancers of the prostate, endometrium, and breast). Inhibitors of aromatase are also used in the induction of ovulation. The inhibitors include steroid-based and non–steroid-based molecules that act as

competitive inhibitors or mechanism-based enzyme poisons (Fig. 4.14).

Endocrine-disrupting chemicals, often believed to act through direct effects on steroid hormone receptors, are now known to interfere with steroidogenic enzymes and enzymes involved in steroid metabolism. These actions may alter endogenous steroid hormone production and catabolism and lead indirectly to altered responses of hormone-responsive target tissues.[93,145]

Inhibitors of P450scc

Aminoglutethimide, a drug originally introduced as an antiepileptic, is a nonsteroidal competitive inhibitor of P450scc and P450arom.[146] The free amine of the drug (essential to the drug's inhibitory activity) interacts with the P450 heme to prevent reduction of Fe^{3+}, which is an obligatory step in the P450 catalytic mechanism. Ketoconazole and related compounds also inhibit P450scc and other P450 steroidogenic enzymes.

P450c17 Inhibitors

Ketoconazole, an antifungal agent that blocks P450s involved in ergosterol biosynthesis, is an effective inhibitor of the 17,20-lyase activity and blocks androgen biosynthesis.[146] More specific inhibitors such as the 17-heteroarylsteroid and abiraterone acetate are used to treat "castration-resistant" prostate cancer.[147] Newer steroidal and nonsteroidal compounds under development including galeterone (steroidal), orteronel, and Seviteronel/VT-464 (nonsteroidal) have greater specificity for inhibition of the lyase activity and have lesser side effects because they have a lower impact on cortisol production.[148]

P450c11 Inhibitors

Metyrapone, an 11β-hydroxylase inhibitor, reduces cortisol production with a concomitant increase in 11-deoxycortisol. It is used in diagnostic testing of the hypothalamic–pituitary–adrenal axis and has also been used to treat Cushing syndrome, but its utility in this regard is limited by side effects.[149]

Aromatase Inhibitors

Two major classes of aromatase inhibitors have been developed, the nonsteroidal imidazole and triazole analogues based on ketoconazole, and the steroidal mechanism-based inactivators ("suicide inhibitors").[150] The first class of compounds is relatively nonspecific because they can inhibit other steroidogenic P450s. Two triazole compounds that are reversible competitive inhibitors of P450arom are in clinical use (letrozole [Femara] and anastrozole [Arimidex]). These drugs bind to the iron atom of the heme protein and exclude the substrate from the catalytic pocket. As competitive inhibitors, they do not have long-lasting effects on aromatase activity and are therefore suitable for use in follicular recruitment/ovulation induction.

The steroidal mechanism–based inhibitors include 4-hydroxyandrostenedione (formestane) and 6-methylandrosta-1,4-diene-3,17-dione (exemestane [Aromasin]; Fig. 4.15). They are fundamentally innocuous by themselves but are activated by the catalytic mechanism of P450arom to produce electrophilic species, which covalently modify the active site. Inactivation requires both NADPH and oxygen. Inhibition by these drugs is long lasting because new aromatase must be synthesized to overcome the inactivation event. The mechanism-based inhibitors are selective because they only inactivate the target enzyme. In the case of 4-hydroxyandrostenedione, the ultimate electrophilic species responsible for enzyme inactivation is unknown.

5α-reductase Inhibitors

The 4-azasteroids, represented by finasteride, were developed as selective inhibitors of type 2 5α-reductase to prevent the formation of the potent androgen, 5α-dihydrotestosterone.[151] These inhibitors contain a heterocyclic A ring with a nitrogen

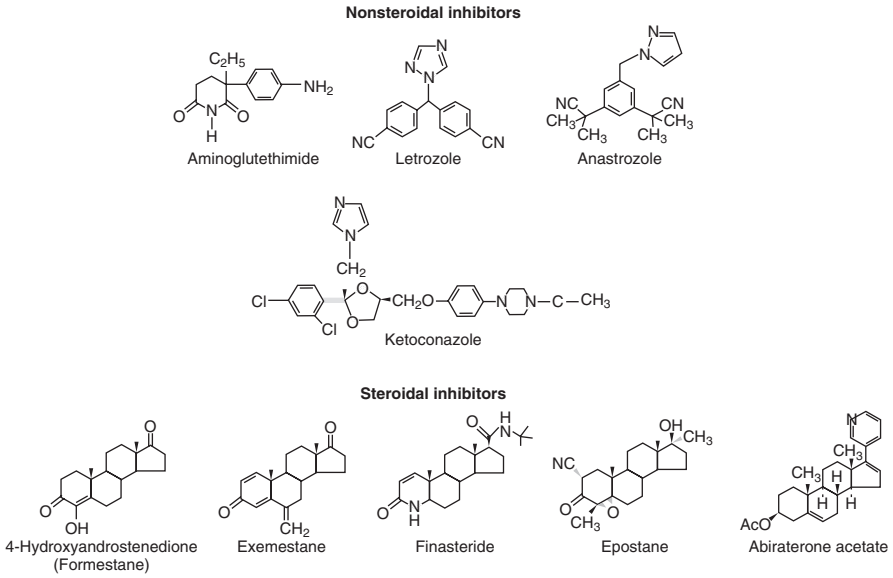

FIGURE 4.14 Structures of some nonsteroidal and steroidal enzyme inhibitors.

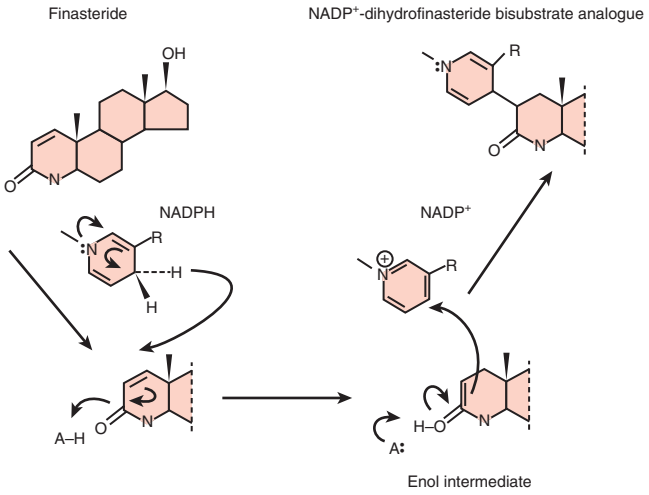

FIGURE 4.15 Inhibition of 5α-reductase type 2 by finasteride. Finasteride is reduced by 5α-reductase via an enol intermediate, which then reacts with NADP+ to produce a NADP+–dihydrofinasteride bisubstrate analogue, which is a potent enzyme inhibitor. *NADPH*, Nicotinamide adenine dinucleotide phosphate. *(Modified from Strauss JF III, Penning TM: Synthesis of the sex steroid hormones: molecular and structural biology with application to clinical practice. In: Fauser BCJM, Rutherford AJ, Strauss JF III, et al, editors.* Molecular Biology in Reproductive Medicine. *New York, 1999, Parthenon, pp 201–232.)*

substitution at C4. The potent competitive inhibition of SRD5A2 (K_i = 3 nM) seen with finasteride was originally attributed to the ability of this compound to produce a mimetic of the enolate transition state. It is now evident that finasteride acts as a mechanism-based inactivator of 5α-reductase to form an isocitrate dehydrogenase (NADP+)–dihydrofinasteride bisubstrate analogue with a K_i of 10^{-13} M (see Fig. 4.15).

Other steroidal-based inhibitors include steroid acrylates, which contain a carboxylic acid substituent at C3. The carboxylate moiety again mimics the enolate transition state. Interestingly, these compounds are potent noncompetitive inhibitors because they form an abortive enzyme—NADP+–acrylate complex. Dual-enzyme 5α-reductase inhibitors (e.g., GI198745) that are under development can suppress dihydrotestosterone production by 99% 24 hours after oral administration.

3β–Hydroxysteroid Dehydrogenase/ Δ^{5-4}-Isomerase Inhibitors

Compounds originally developed to target 3β-HSD were derivatives of 2α-cyanoketone (2α-cyano-4,4,17α-trimethylandrost-5-en-17β-ol-3-one).[152,153] The subsequently developed compounds trilostane and epostane are relatively specific competitive inhibitors for blocking steroidogenesis in the adrenal cortex and placenta, respectively.[136]

Other Enzyme Targets for Reducing Sex Steroid Production

Inhibition of HSD17B1 and steroid sulfatase is an approach to the reduction of bioavailable estradiol levels.[37,76,77,154] Tibolone, a drug used in Europe for hormone replacement therapy, has the interesting property of inhibiting sulfatase activity in breast cancer cells, but not in bone cells, a tissue-selective action that could reduce estradiol bioavailability in breast cells, but not in bone cells.[155] As noted previously, hydroxylated metabolites of polychlorinated biphenyls are potent inhibitors of estrogen SULT1E1, and thus increase the bioavailability of estradiol.[93,145]

Eicosanoids: Other Bioactive Lipids Playing a Role in Reproduction

- *Eicosanoids include products of the COX, LOX, and P450 enzymes acting on arachidonic acid and other polyunsaturated fatty acid substrates.*
- *Eicosanoids are not stored in cells but formed and released to act locally in response to chemical and physical stimuli.*
- *Peroxidation products of these substrates, isoeicosanoids, are eicosanoid isomers that may reflect quantitatively lipid peroxidation in vivo. More controversially, they have the capacity to act as incidental ligands at eicosanoid receptors although whether they do at concentrations formed in vivo is unknown.*

The study of the prostaglandin family of lipids emerged from reproductive biology. von Euler first described the presence of substances in semen that induced uterine contractility.[156] The active substances were named *prostaglandins* based on the belief that they were produced by the prostate gland. The subsequent discovery of the widespread occurrence of prostaglandins, their broad range of biologic activities, chemical structures, and biosynthetic pathways, and determination of their relevance to the mechanism of action of nonsteroidal antiinflammatory drugs (NSAIDs) was recognized by the Nobel Prize being awarded to the pioneers in prostaglandin research, Bergström, Samuelsson, and Vane in 1982.[157,158]

Prostaglandins and related lipid mediators are collectively referred to as *eicosanoids*. Eicosanoids (from *eicosi*, a Greek root meaning "20") are derived enzymatically from 20-carbon polyunsaturated fatty acids, principally arachidonic acid in mammalian species. They are produced by almost all cells in the body in response to hormonal stimulation or mechanical trauma, acting as paracrine or autocrine modulators of cellular function in diverse tissues (including the reproductive system) at extremely low concentrations. Tissue specificity in eicosanoid action is afforded by selective expression of biosynthetic enzymes and membrane receptors for eicosanoids. The active eicosanoids are short-lived because they are susceptible to spontaneous or metabolic inactivation.

Eicosanoid Structure and Nomenclature

The term *eicosanoid* refers to both prostaglandins (PG) and thromboxanes (TX) (products of the COX pathway), and leukotrienes (products of the 5-LOX pathway, abbreviated as LT). All PGs have a hairpin configuration and contain a cyclopentane ring with two side chains oriented in *trans* positions relative to the cyclopentenone ring. Each group of prostaglandins is allocated a letter (e.g., A, E, F), which denotes functional groups at carbons 9 and 11 of the ring structure.

The degree of unsaturation of the side chains is indicated by the subscript numeral after the letter; thus PGE_1, PGE_2, and PGE_3 have one, two, and three double bonds, respectively. In the case of $PGF_{2\alpha}$, the subscript α denotes the stereochemistry of the 9-hydroxyl group in the cyclopentane ring.

A similar system is used for nomenclature of TXs that have oxygen-bridged six-member rings in place of the five-member ring in PGs. All LTs have three conjugated double bonds, and the subscript numeral (e.g., the 4 in LTA_4 and LTC_4) denotes the total number of double bonds in the molecule. LTA_4 is an unstable epoxide and serves as a parent molecule in the biosynthesis of LTB_4 (hydrolysis product), LTC_4, LTD_4, and LTE_4 (peptide derivatives).

Biosynthesis of Eicosanoids

Similar to steroid hormones, eicosanoids are not stored, but they are rather synthesized de novo when cells are activated by hormonal or mechanical stimuli. The first obligatory (and often rate-limiting) step in eicosanoid biosynthesis is the release of arachidonic acid from membrane phospholipid stores (Fig. 4.16).[159,160] This process is exquisitely regulated by a host of enzymes, particularly several types of phospholipase A_2 (PLA_2),[161] and it appears to be coordinated with the induction of downstream eicosanoid biosynthetic enzymes.[160,162-164]

There are at least three types of PLA_2 involved in arachidonic acid release during different stages of cell activation.[160] Under basal conditions, the Ca^{2+}-independent PLA_2 ($iPLA_2$) is the dominant phospholipase involved in the liberation of arachidonic acid and other polyunsaturated fatty acids from membrane phospholipids.[160] $iPLA_2$ is primarily involved in membrane remodeling and usually does not cause significant eicosanoid production, because its activity in arachidonic acid release is balanced by acylase enzymes. However, the activity of $iPLA_2$ may become significant when acylase enzymes are downregulated. For example, mice heterozygous for acyl-coenzyme A synthetase 4 deficiency accumulated high levels of uterine PGs, had abnormal uterine cysts, and reduced fertility.[165]

When cells are activated, such as by receptor ligation or calcium ionophore stimulation, intracellular calcium levels rise and the Ca^{2+}-dependent cytosolic PLA_2 ($cPLA_2$) becomes involved.[160] The activity of $cPLA_2$ far exceeds that of acylase enzymes, and as a result, there is rapid accumulation of free arachidonic acid to be acted on by either constitutive or inducible eicosanoid-synthesizing enzymes.

Under certain situations of sustained cellular activation, secreted PLA_2 ($sPLA_2$) family members may also participate, further increasing the supply of free arachidonic acid to meet the need of eicosanoid-synthesizing enzymes. $cPLA_2$ plays a key role among these enzymes because cells lacking $cPLA_2$ are generally incapable of eicosanoid biosynthesis.[161]

After its release from membrane phospholipid stores, arachidonic acid is transformed enzymatically to various eicosanoids through multiple pathways (Fig. 4.17). One pathway is initiated by the enzyme PG H synthase (PGHS, also known as COX)[166] and results in the production of PGs, TX (TA_2), and prostacyclin (PGI_2). These COX-derived products are also referred to as *prostanoids*.

The initial cyclized fatty acid derivative formed in this reaction is PG G_2 (PGG_2). The peroxide moiety at the 15-carbon position of PGG_2 is subsequently reduced to an alcohol group to form PGH_2 because of the inherent peroxidase activity of PGHS (see Fig. 4.17). Both PGG_2 and PGH_2 are unstable intermediates with very short half-lives.

The conversion of PGH_2 to individual PGs is relatively tissue-specific, depending on the local expression of specific PG-synthesizing enzymes.[166,167] For example, TXA_2 is synthesized in platelets and macrophages, whereas PGI_2 is the dominant COX product of macrovascular endothelial cells.[157,167]

The second major pathway of arachidonic acid metabolism is initiated by LOXs. Similar to COX, LOX enzymes are dioxygenases that catalyze the insertion of molecular oxygen into arachidonic acid to form a hydroperoxy derivative.[168] The insertion of molecular oxygen can occur at several carbon positions (e.g., 5, 12, and 15) along the fatty acid chain. These determine the specificity of the enzymes and their names (e.g., *5-LOX* and *12-LOX*).[168] 5-LOX is of particular importance because its product, 5-hydroperoxyeicosatetraenoic acid (5-HPETE), serves as a precursor for the LT family of eicosanoids, which are involved in host defense and immediate-type hypersensitivity reactions.[157,169] Other eicosanoids potentially formed from LOX pathways include lipoxins and related compounds, which can be formed, at least in vitro, by the interaction of neutrophils with other cells in the vascular compartment. Although they have been proposed to play a role in the resolution of inflammatory

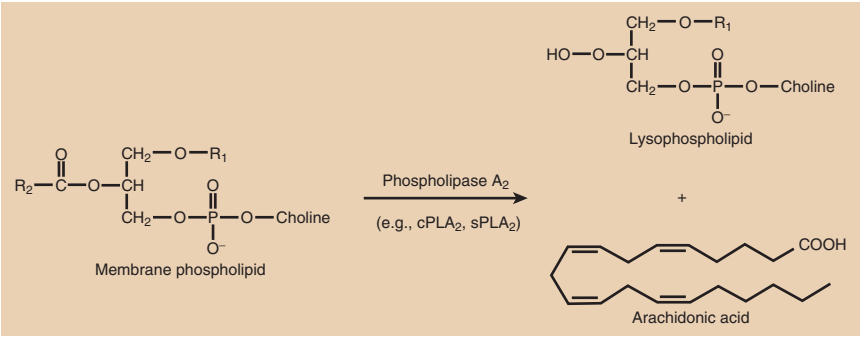

FIGURE 4.16 Release of free arachidonic acid from membrane phospholipid stores is the first step in eicosanoid biosynthesis. Arachidonic acid *(R_2-COOH)*, located at the sn-2 position of phospholipids (phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol) is mainly cleaved by phospholipase A_2 enzymes when cells are activated. R_1 is often a C16 or C18 saturated or monounsaturated fatty acid in acyl linkage.

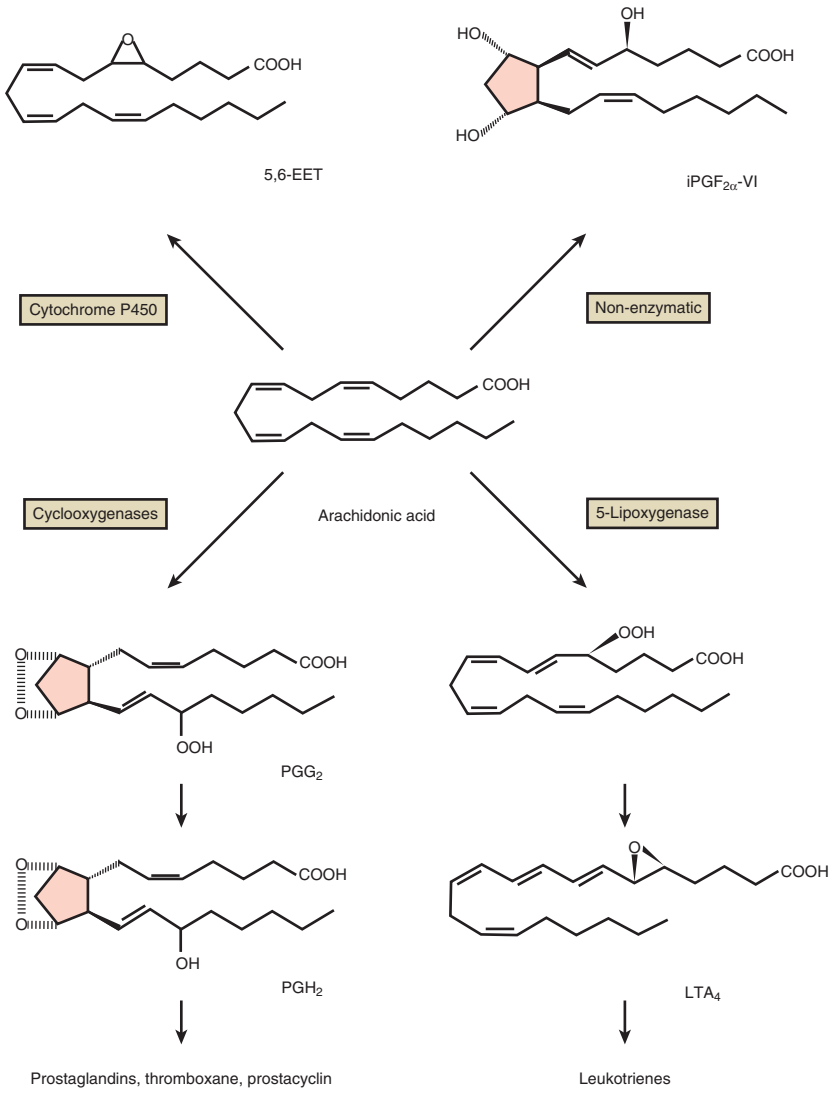

FIGURE 4.17 Major pathways of eicosanoid biosynthesis from arachidonic acid. Arachidonic acid is converted by two forms of cyclooxygenase (formally known as prostaglandin *H synthase*) to PGH_2 and then to prostaglandins, thromboxane, and prostacyclin; by lipoxygenases to leukotrienes (depicted) and other products, such as hydroxy-eicosatetraenoic acids (not shown); by cytochrome P450 enzymes to epoxy-eicosatrienoic acids (EETs; 5,6-EET is shown as an example). Arachidonic acid can also be converted nonenzymatically to isoprostanes through free radical–catalyzed peroxidation and cyclization (there are 64 potential isomers of isoprostanes[22,23]; the structure of isoprostane isomer VI of prostaglandin F2 alpha *[iPGF$_{2\alpha}$-VI]* is shown as an example). In contrast to the enzymatic pathways, formation of isoprostanes can occur on phospholipids, and release of free arachidonic acid is not a prerequisite.[24]

responses,[170] there is little evidence for their formation at biologically relevant concentrations when rigorous analytical approaches are applied either in humans or experimental systems. Although synthetic versions of such "specialized proresolving lipid mediators" have been shown to promote resolution of inflammation in some experimental settings, their importance as endogenous mediators remains to be established.

Arachidonic acid can also be metabolized by an additional pathway through the activity of cytochrome P450 enzymes.[17] In contrast to the COXs or LOXs, cytochrome P450 enzymes catalyze the monooxygenation (insertion of one atom of oxygen from O_2) of arachidonic acid, with hydroxy or epoxy derivatives of arachidonic acid as their major products.[171] Cytochrome P450 products modulate nephron ion transit and renovascular tone.[171-173] Some cytochrome P450–derived epoxy metabolites of arachidonic acid are elevated in women with pregnancy-induced hypertension (PIH),[174] and others may have antiinflammatory activities.[175]

Lastly, peroxidation of arachidonic acid and cyclization to PG-like compounds called *isoprostanes* can also occur nonenzymatically by free radical–catalyzed reactions.[176,177] In contrast to the enzyme-produced eicosanoids, isoprostanes can be formed in situ on phospholipids and then released by phospholipases.[178] Isoprostanes may serve as incidental ligands for eicosanoid receptors under certain conditions.[177,179]

In the clinical setting, urine and plasma isoprostane levels have proven to be reliable markers of lipid peroxidation and oxidant stress in vivo.[176,177] For example, elevated levels of urinary isoprostanes were detected in women with android obesity[180] and in individuals with alcohol-induced liver injury.[181]

Both conditions are associated with increased oxidant stress and inflammation, as determined by other independent markers.

Major Products of the COX Pathway: Prostaglandins, Thromboxanes, and Prostacyclin

- *Prostanoids are those eicosanoids formed by the COX enzymes; they act on G-protein-coupled membrane receptors.*
- *COX-2 is the major but not the exclusive source of prostanoids evoked by inflammatory cytokines and mitogens; NSAIDs targeting specifically COX-2 reduce but do not eliminate gastrointestinal (GI) adverse effects observed with mixed inhibitors.*
- *GI adverse effects have been attributed to blockade of COX-1 dependent thromboxane formation by platelets and cytoprotective effects of COX-1 derived PGE_2 and PGI_2 by gastroduodenal epithelium. However, suppression of COX-2 derived products may play some role, for example, in ulcer healing.*
- *Suppression of COX-2 derived PGI_2 and PGE_2 accounts for the cardiovascular hazards attributed to NSAIDs.*

Two COX enzymes that initiate the formation of prostaglandins (see Fig. 4.17), encoded by separate genes, have been identified in vertebrates.[166,182] One COX enzyme (COX-1) is largely constitutively expressed, whereas the other (COX-2) is more often inducible by cytokines, growth factors, and hormones. This categorization as constitutive COX-1 and inducible COX-2 is useful but is not always the case (e.g., COX-2 is constitutively expressed in some organs, such as the kidney glomerulus[183]).

The subsequent conversion of PGH_2 to individual prostanoids is relatively tissue-specific and is catalyzed by the corresponding isomerases and synthases (Fig. 4.18).[182] For example, TXA_2 synthase is expressed in platelets and macrophages, PGI_2 synthase is found in endothelial cells, and $PGF_{2\alpha}$ synthase is abundant in the uterus.[157]

There are at least two types of glutathione-dependent PGE_2 synthases.[162-164,185] One PGE_2 synthase, cPGES, is a constitutively expressed cytosolic enzyme functionally coupled to COX-1; the other is a microsomal and inducible enzyme, mPGES-1, which appears to be coupled to COX-2.[162-164,185] Another enzyme, mPGES-2, found in bovine endometrium and other sites, is less well characterized, and its role in PGE_2 synthesis is unclear.[186]

Major Products of the Lipoxygenase Pathway: The Leukotrienes and HETEs

- *LOX products formed by neutrophils promote neutrophil activation and migration and interact with the vasodilator properties of PGE2 and PGI2 to promote inflammation.*
- *Several LOX products also promote smooth muscle cell contraction. Blockade of leukotriene receptors has established efficacy in asthma, and there is research interest in the role leukotrienes may play in promotion of atherothrombotic disease.*
- *Besides promoting inflammation, some leukotriene-like compounds are formed in a transcellular manner when*

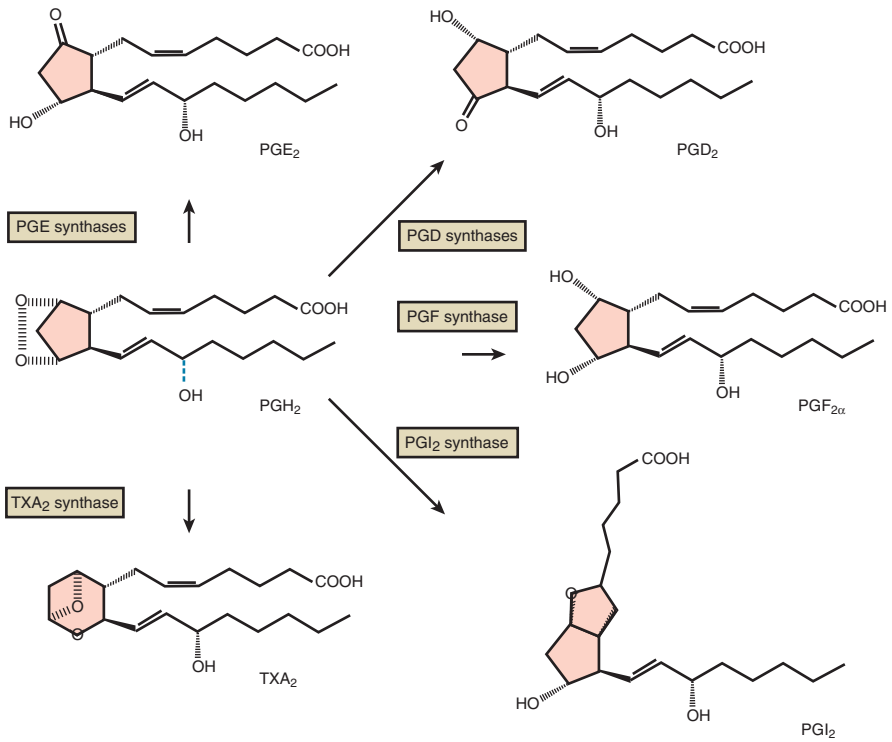

FIGURE 4.18 Biosynthesis and structures of prostanoids from prostaglandin *(PG)* H_2 *(PGH_2).* Formation of PGE_2 is catalyzed by PGE synthases, of which at least three have been identified.[8-10,30,31] Formation of PGD_2 is catalyzed by at least three different PGD_2 synthases.[184] Formation of $PGF_{2\alpha}$ is catalyzed by PGF synthase, a member of the aldo-keto reductase family of enzymes. Thromboxane A_2 *(TxA_2)* and PGI_2 are formed by TxA_2 synthase and PGI_2 synthase, respectively. Both enzymes are cytochrome P450 enzymes associated with microsomal membranes.

neutrophils and platelets are mixed and activated in vitro. Synthetic versions of these compounds promote resolution of inflammation in some settings in model systems. However, their importance as endogenous mediators remains to be established.

Products of the 5-LOX pathway with well-defined biologic activities are mainly produced by inflammatory cells.[157,187-189] The activation and biochemical cascade of the 5-LOX pathway in leukocytes is particularly well understood, because it gives rise to the potent LT family of lipid mediators that have chemotactic or smooth muscle–contracting properties.[2]

Biosynthesis of LTs is initiated by the formation of 5-HPETE from arachidonic acid by 5-LOX in a Ca^{2+}-dependent process that involves translocation of both 5-LOX and $cPLA_2$ to the nuclear envelope.[157,190,191] The activity of 5-LOX also requires the cooperation of an accessory protein, 5-LOX–activating protein (FLAP), in intact cells.[192]

Some 5-HPETE molecules can escape from the active site of the 5-LOX enzyme and are ultimately reduced by cellular peroxidases to the corresponding 5-hydroxy derivative of arachidonic acid (5-HETE). Other 5-HPETE molecules may be further converted to the labile epoxide known as *leukotriene A₄* (LTA_4) by a secondary dehydrase activity contained in 5-LOX (Fig. 4.19).[189] LTA_4 undergoes one of several potential transformation routes depending on the cellular context.

Hydrolytic attack of LTA_4 by LTA_4 hydrolase produces LTB_4,[184] which is a potent chemotactic agent for neutrophils. The conjugation of LTA_4 with glutathione is catalyzed by LTC_4 synthase, thereby forming LTC_4 at the nuclear envelope.[193] Sequential cleavage of the peptide moiety of LTC_4 by extracellular peptidases produces LTD_4 and LTE_4 (see Fig. 4.19).

The three cysteinyl LTs, LTC_4, LTD_4, and LTE_4, are the major constituents of the *slow-reacting substance of anaphylaxis* released from antigen-sensitized guinea pig lungs that produced slow and sustained smooth muscle contraction of intestinal smooth muscle strips, observed more than 60 years ago.[187] LTA_4 can also serve in vitro as a precursor for the transcellular biosynthesis of lipoxins.[191,194]

LOXs with catalytic oxygenase specificity at different carbon positions of arachidonic acid, include 12R-LOX, 12S-LOX, 15-LOX-1 and 15-LOX-2, and 8-LOX, encoded by different LOX genes.

Transport and Metabolism of Eicosanoids

- *Eicosanoids may be subject to active transport out of cells to act on membrane receptors. Although some can activate nuclear receptors, the evidence that they do activate the receptors at concentrations formed endogenously is weak.*
- *The capacity of tissues to form eicosanoids, measured by such approaches as serum concentrations of TxB_2, exceeds by orders of magnitude actual biosynthetic rates in vivo. Thus measurements of primary eicosanoids are highly subject to sampling artifacts. This discrepancy also emphasizes the important distinction between what an eicosanoid "can" do and what it does at the trace (fM-pM) concentrations formed in vivo.*
- *Urinary concentrations of metabolites, formed particularly in the liver, represent a time integrated, noninvasively acquired but indirect approach to assessing eicosanoid formation in vivo.*

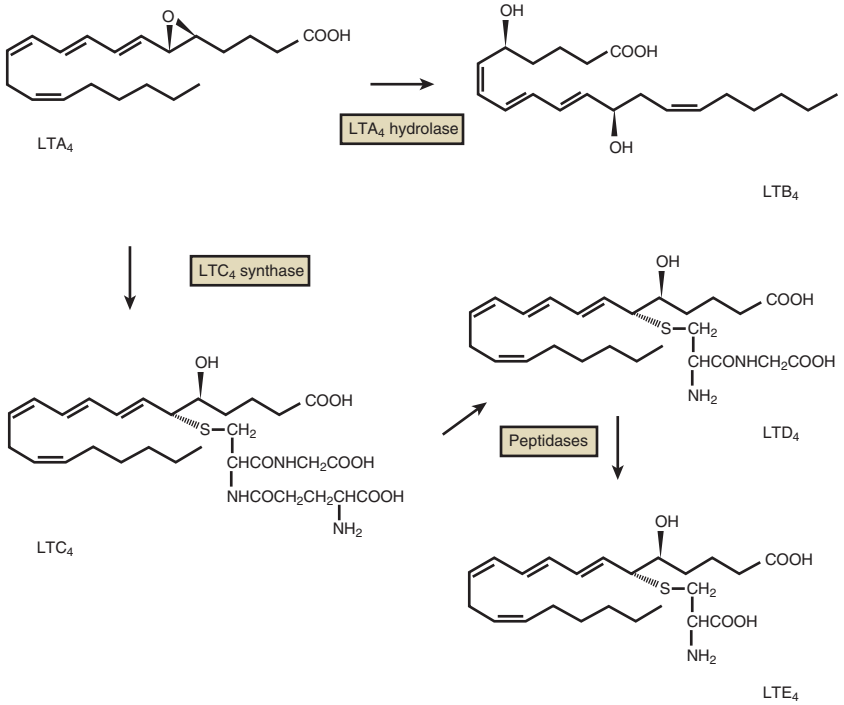

FIGURE 4.19 Structures and biosynthesis of leukotrienes from LTA_4. LTA_4 is formed by 5-lipoxygenase and is prone to spontaneous hydrolysis. Hydrolytic attack by LTA_4 hydrolase produces LTB_4, a dihydroxy derivative of arachidonic acid with specific stereochemistry of the hydroxy groups. Conjugation of LTA_4 with glutathione is catalyzed by LTC_4 synthase. Sequential cleavage of the oligopeptide moiety in LTC_4 by extracellular peptidases yields LTD_4 and LTE_4.

Although eicosanoids are lipid compounds, they do not permeate the cell membrane freely. A PG transporter (PGT), belonging to the organic anion transporter polypeptide family, has been identified and is found in a limited range of cells where it is subject to humoral and physical stimuli.[195] PGT mediates the cellular uptake of most prostanoids but not PGI_2.[195] Vascular expression of PGT appears to be responsible for clearing most classes of PGs when these compounds are infused into an animal and enter the pulmonary circulation.[195] On the other hand, multidrug-resistance protein 4 (MRP4) functions as a PG efflux transporter.[196] In the LT pathway, newly synthesized LTC_4 is transported outside of the cells by transporters such as multidrug-resistance protein 1 (MRP1).[197]

A hallmark of eicosanoids is their short lifespan. Products such as PGI_2 and TXA_2 are chemically unstable and are degraded spontaneously through hydrolysis in aqueous solutions, particularly at neutral to acid pH. PGI_2 undergoes hydrolysis to form 6-keto $PGF_{1\alpha}$, a stable and inactive product, whose metabolite, 2,3-dinor-6-keto-$PGF_{1\alpha}$, has been used as a surrogate marker for systemic PGI_2 biosynthesis in vivo. Similarly, TXA_2 rapidly undergoes facile hydrolysis to form the inactive product TXB_2, which is subject to beta oxidation. Plasma or urinary TXB_2 metabolites have served as useful indices of TXA_2 formation in vivo.

The most important catabolic step for PGs is the conversion of the 15-hydroxy group to a 15-keto group by a NAD(+)-dependent 15-hydroxyprostaglandin dehydrogenase (15-OH-PGDH).[198] 15-OH-PGDH is a cytosolic protein with highest concentrations in the lungs, the placenta, the spleen, and the kidney cortex.[47] Both PGE_2 and $PGF_{2\alpha}$ are excellent substrates for 15-OH-PGDH.[198]

The second notable step in the degradation of PGs is reduction of the double bond at position 13 by $\Delta^{13\text{-}14}$-PG reductase, which is highly specific for 15-keto PGs; this enzyme has a tissue distribution similar to that of 15-OH-PGDH. The metabolites then undergo both beta (i.e., carboxyl chain) and omega oxidation, resulting in urinary excretion of the final products.

Pharmacologic Regulation of Eicosanoid Synthesis

- *COX-2 tends to be the more regulated isoform in its expression, while COX-1 is ubiquitously expressed and often evident under basal conditions. However, such distinctions are relative and not absolute.*
- *The crystal structures of the two COXs are almost superimposable, but a distinction is in the hydrophobic pocket, which permits access of the lipid substrate to the active site. This is more accommodating in COX-2, which has a wider substrate specificity. It also has a side pocket that has been the structural basis for synthesis of COX-2 specific inhibitors.*
- *Besides NSAIDs developed specifically to inhibit COX-2, some of the older drugs, such as diclofenac and meloxicam, exhibit similar specificity.*

The discovery of the COX-2 enzyme and its physiologic and pharmacologic characterization in the early 1990s hailed a new era in eicosanoid research.[199] COX-2 expression is readily inducible by cytokines, growth factors, and hormonal stimulation.[166] The two COX enzymes within a given species share 60% to 65% sequence identity in their primary structures.[166]

Both enzymes are membrane-anchored proteins, localizing primarily to the luminal surface of the endoplasmic reticulum and the nuclear envelope.[166] The crystal structures of COX-1 and COX-2 are remarkably similar, as are their catalytic mechanisms and kinetics. However, two subtle structural and biochemical differences have been noted between the two isozymes. First, the active site of COX-2 is larger and more accommodating than COX-1; this property was exploited by the pharmaceutical industry for developing selective COX-2–specific NSAIDs. Second, the gross kinetics (e.g., K_m, V_{max}) for COX-1 and COX-2 are similar, but COX-2 competes more efficiently with COX-1 in intact cells when the release of arachidonic acid is limiting.[160,182] Thus, when COX-1 and COX-2 are both present in the same cells, catalysis of arachidonic acid (provided exogenously or through activation of cellular lipases) is favored by COX-2.[160] COX-1 appears to function independently only when COX-2 is absent or inhibited.[160,182,200]

It has been proposed that this difference in sensitivity to arachidonic acid concentration is related to the sensitivity of their heme groups to lipid peroxide–catalyzed oxidation, and thus activation of the COX enzymes.[201] The concentration of lipid peroxide required to activate COX-1 is 10 times more than that required to activate COX-2 in the presence of low concentrations of arachidonic acid.[201]

Aspirin and traditional NSAIDs (e.g., indomethacin) function as nonselective inhibitors of both COX-1 and COX-2,[166] and their mechanisms of action had been extensively investigated, even before the discovery of COX-2. Low doses (<100 mg/day) of aspirin, such as those used for cardioprotection, preferentially target COX-1. Long-term use of traditional NSAIDs in managing inflammatory disorders is associated with significant adverse effects, such as gastrointestinal bleeding (Fig. 4.20).[157,166,182,199] COX-2 is the COX isoform most responsible for producing PGs that contribute to the inflammatory process, offering a rationale for developing selective inhibitors targeted at COX-2.

Although such a strict functional segregation between a proinflammatory role for COX-2 and one focused on "housekeeping" PGs required for maintaining normal physiologic (e.g., mucosal protection in the stomach and normal kidney function) for COX-1 is imperfect, it posited that the GI adverse effects associated with traditional NSAID use would result from their inhibition of the COX-1 enzyme, whereas their antiinflammatory efficacy would be attributable to coincidental inhibition of COX-2 (see Fig. 4.20). This principle forms the basis for the development and marketing of the selective COX-2 inhibitors known as the coxibs (e.g., celecoxib, rofecoxib, and valdecoxib) for treating the arthritides[157,202]; indeed, randomized trials revealed that such drugs were less likely to cause serious GI adverse effects than mixed NSAID inhibitors, albeit that they did not avoid them completely.

COX-1 and COX-2 genes and enzymes are present even in zebrafish[157,203] suggesting that both enzymes have fundamental physiologic roles to play. Both COX-1 and COX-2 gene–disrupted mice display multiple developmental and physiologic defects, including abnormalities in steps of reproduction.[204-207] Also, the administration of the selective COX-2 inhibitors to healthy human subjects suppressed

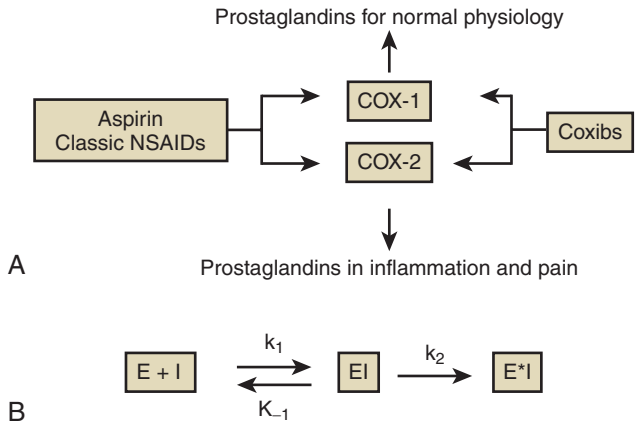

FIGURE 4.20 Mechanism of action of aspirin, traditional nonsteroidal antiinflammatory drugs *(NSAIDs)*, and coxibs. (A) Aspirin and traditional NSAIDs inhibit both COX-1 and COX-2, whereas coxibs preferentially inhibit COX-2. (B) All COX enzyme *(E)* inhibitors *(I)* exhibit one of three kinetic models of inhibition: (1) rapid, reversible binding to form EI (e.g., ibuprofen); (2) rapid, lower-affinity reversible binding, followed by time-dependent, higher-affinity, slowly reversible binding (e.g., flurbiprofen and coxibs); (3) rapid, reversible binding, followed by covalent modification (acetylation) of a serine residue in COX (aspirin). The mechanism for the time-dependent inhibition is not well understood, but it may involve conformational change of the enzyme *(E*I)*. Coxibs confer their selectivity for COX-2 by causing time-dependent inhibition of COX-2, but not COX-1 enzymes.

systemic PGI_2 biosynthesis.[208] This finding combined with extensive studies in mice provided a mechanistic basis for the interpretation of evidence now from 10 randomized trials that COX-2 inhibitors confer a cardiovascular hazard. Some of the older NSAIDs, such as diclofenac, also are selective inhibitors of COX-2, and epidemiological evidence is consistent with these drugs also conferring a cardiovascular risk.

Eicosanoid Receptors

- *Eicosanoids activate membrane G-protein–coupled receptors (GPCRs) and their deletion in mice has revealed a remarkably diverse and often contrasting biology.*
- *Antagonists and agonists of these receptors have been widely used in model systems, and in several cases, they have progressed to evaluation in humans.*
- *There is considerable interest presently in E prostanoid receptor antagonists as modulators of immunological function.*

An explosion of knowledge regarding the expression, structure, signaling pathways, and physiologic functions of eicosanoid receptors has occurred in the past 20 years.[209,210] The existence of membrane receptors for eicosanoids was supported by early pharmacologic evidence[211] and was consistent with the notion that eicosanoids are locally released paracrine mediators acting on self and neighboring cells.

The first eicosanoid receptor to be cloned was the human TX receptor in 1991.[212] There are 13 distinct eicosanoid receptors cloned and characterized, 9 for COX-derived prostanoids and 4 for LTs (Table 4.5).[157,209,210] All 13 eicosanoid receptors characterized so far are rhodopsin-like, seven transmembrane domain–containing, GPCRs.[209,210] The nine G-protein–coupled prostanoid receptors, conserved in mammals from mouse to human, are the TX receptor (TP), the PGI_2 receptor (IP), the $PGF_{2\alpha}$ receptor (FP), two PGD receptors (DP1 and DP2), and four PGE_2 receptors (EP1, EP2, EP3, and EP4). These receptors are encoded by separate genes. In addition, there are several splice variants for the EP3, FP, and TP receptors, which differ only in their C-terminal tails (Fig. 4.21).[209,213-216]

Table 4.5 Summary of Eicosanoid Receptor Types and Properties

Receptor	Cognate Ligand	Property*	Intracellular Signaling
IP	PGI_2	Relaxing	↑ cAMP
TP	TXA_2	Contractile	↑ Ca^{2+}
FP	$PGF_{2\alpha}$	Contractile	↑ Ca^{2+}
DP1	PGD_2	Relaxing	↑ cAMP
DP2 (CRTH2)	PGD_2	Chemoattractant	↑ Ca^{2+}
EP1	PGE_2	Contractile	↑ Ca^{2+}
EP2	PGE_2	Relaxing	↑ cAMP
EP3	PGE_2	Inhibitory	↓ cAMP
EP4	PGE_2	Relaxing	↑ cAMP
BLT1	LTB_4	Leukocyte Restricted	↑ Ca^{2+} ↓ cAMP
BLT2	LTB_4	Widely distributed	↑ Ca^{2+} ↓ cAMP
CysLT1	LTD_4	Lung SMC, macrophages	↑ Ca^{2+}
CysLT2	LTC_4, LTD_4	Spleen, Purkinje fibers, etc.	↑ Ca^{2+}

*Smooth muscle activities.
cAMP, Cyclic AMP; *PGI_2*, prostacyclin; *SMC*, smooth muscle cells.

The second PGD receptor, DP2 (also known as CRTH2),[217] induces intracellular calcium mobilization and is a chemoattractant receptor for type 2 T-helper cells.[217] DP2 is also found on human eosinophils where both PGD_2 and 15-deoxy-PGJ_2 (a degradation product of PGD_2) serve as activation ligands at nanomolar concentrations.[218,219]

The remaining eight prostanoid receptors fall into three categories according to their functional properties and the secondary messenger systems to which they couple. IP, DP1, EP2, and EP4 are smooth muscle "relaxing" receptors that signal predominantly through a G_s-mediated increase in intracellular cAMP. The FP, TP, and EP1 receptors form the "contractile" subgroup and transduce their signals through a G_q-mediated increase of intracellular calcium. The EP3 receptor is regarded as an "inhibitory" receptor, because it is coupled to G_i and causes inhibition of cAMP production.

Several aspects of prostanoid receptor biology and pharmacology require comment. First, the existence of four subtypes of PGE_2 receptors is quite remarkable because all other prostanoids (except PGD_2) have only a single receptor. Sequence-based phylogenetic tree analysis of the prostanoid receptors reveals that the four PGE_2 receptors were the first to evolve because homology among these subtypes is less than that among receptors of different classes of prostanoids.[209,210] Thus the four PGE_2 receptors must have evolved to play distinctive physiologic functions, and other prostanoid receptors are likely derived from functionally related PGE_2 receptor subtypes through gene duplication.[210]

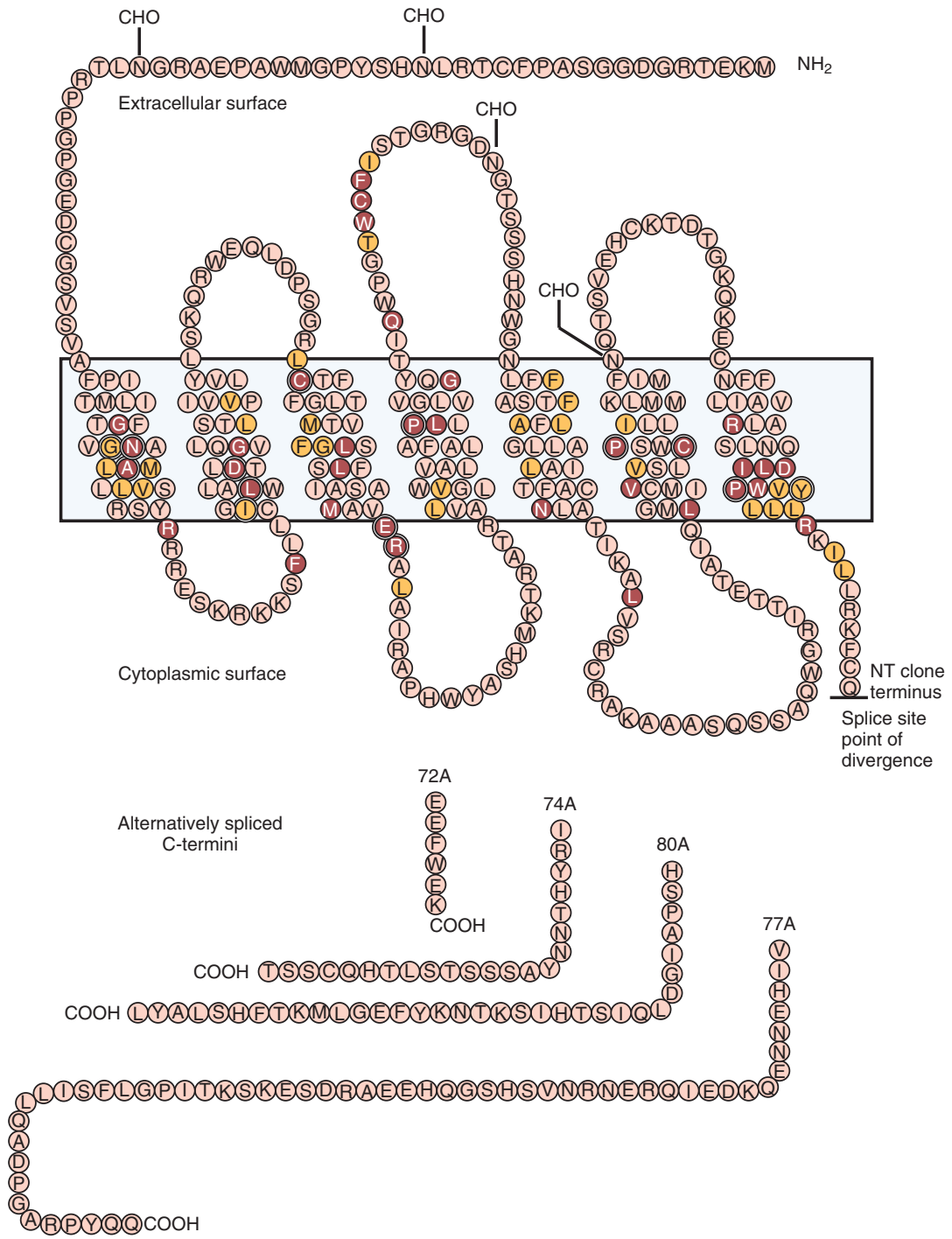

FIGURE 4.21 Schematic representation of five rabbit EP3 receptor splice variants differing only in their intracellular carboxyl termini. The carboxyl-variable tails range from 56–amino acid residues for clone 77A to none for the NT (no-tail) clone. Conserved residues in the EP3 receptor are also identified after EP3 receptor sequences from various species are aligned with those of other prostanoid receptors. Conserved residues are indicated by yellow circles, and invariant residues are indicated by red circles. Residues with *bulls-eye symbols* are conserved across the entire superfamily of G-protein–coupled receptors; those without this inset are unique to the prostanoid receptors. The amino acid sequences of each splice variant are represented by a one-letter amino acid code. *(From Breyer RM, Bagdassarian CK, Myers SA, Breyer MD: Prostanoid receptors: subtypes and signaling.* Annu Rev Pharmacol Toxicol *41:661-690, 2001, with permission.)*

Second, although there is high ligand selectivity for each of the prostanoid receptors, cross-activation by prostanoids other than the cognate ligand may occur; coupling to alternative downstream signaling pathways at different concentrations of the same ligand may also occur.[210,216] Third, the multiplicity in prostanoid receptor subtypes and signaling pathways, coupled with their differential tissue expression, explains why the same PG at different concentrations or in different tissues can sometimes produce opposing effects.

Among the four membrane LT receptors characterized to date, two are receptors for LTB_4 (BLT1 and BLT2) and two are for the cysteinyl LTs (CysLTl and CysLT2). The high-affinity ligand-binding BLT1 is primarily expressed on leukocytes,[220] whereas BLT2 has a wider tissue distribution and binds LTB_4 with lower affinity.[221] Both receptors signal through intracellular calcium mobilization and through inhibition of adenylyl cyclase.

The existence of distinct cysteinyl LT receptors has been supported by pharmacologic evidence.[222] CysLT1 has been the primary target for the development of anti-LT drugs for asthma symptom management. Molecular characterization of CysLTl confirmed it to be a GPCR, with LTD_4 as its preferred endogenous ligand.[223] CysLTl is expressed on lung smooth muscle cells and tissue macrophages, and activation of the receptor results in an elevation of intracellular calcium.[223]

CysLT2, originally defined by pharmacologic methods in trachea and pulmonary vein preparations, is expressed in the spleen, Purkinje fibers of the heart, the adrenal gland, and the microvasculature of certain organs.[224,225] Both LTD_4 and LTC_4 are equipotent agonists for this receptor, which also increases intracellular calcium when activated.[224] Other CysLT receptors may exist but still await definitive characterization.

Lipoxins have the capacity to activate the lipoxin A4 receptor (ALX)/formyl peptide receptor (FPR2), GPR 32, the aryl hydrocarbon receptor, and the cysteinyl leukotriene receptor.[226] It is unknown which if any of these receptors are activated by endogenous concentrations of lipoxins in vivo.

Several eicosanoids have been shown to have the capacity to activate nuclear receptors. However, whether they have that capacity at concentrations formed in vivo is controversial.

For example, PGI_2 formed from COX-2 has been proposed to act as a natural ligand for PPARδ to mediate processes ranging from colorectal carcinogenesis,[232] to apoptosis,[233] to embryo implantation.[227-231,234] However, the relative importance of eicosanoids signaling through nuclear receptors under physiologic conditions remains speculative compared to the well-established signaling via eicosanoid G protein receptor-coupling.

Eicosanoids and Reproduction

- *COX-2 deletion reveals its multiple roles in development.*
- *Prostanoids have been implicated in implantation, embryogenesis, and parturition.*
- *E prostanoid receptor activation is fundamental to closure of the ductus arteriosus (DA).*
- *Both COX and P450 products have been implicated in stem cell replication.*
- *Low-dose aspirin may have value in some patients with preeclampsia, either as a preventative therapy or as a hypotensive when given in the evening.*

The role of eicosanoids in reproductive processes is one of the most intensely investigated of their biologic functions. In fact, the uterine-contracting property of PGs led to their discovery. The activity of PGs in stimulating uterine contractility and inducing cervical ripening rendered them and their synthetic analogues attractive candidates for labor induction and as abortifacients.[235-237] For example, misoprostol, a PGE_1 analogue, has been used alone or in combination with mifepristone for first-, second-, and third-trimester abortions.

Traditional NSAIDs, via their capacity to inhibit eicosanoid biosynthesis, block ovulation in several mammalian species,[238] and PGE_2 and $PGF_{2\alpha}$ are well known as effective luteolytic agents within the veterinary community.[239,240] PG biosynthesis is involved in maintaining DA patency during fetal life, and in anecdotal reports, NSAID use during pregnancy was associated with increased risk of premature DA closure and fetal demise.[241,242] Advances in eicosanoid pathway molecular biology have significantly improved our understanding of the role PGs play in reproduction. Much of this knowledge has emerged from studies using knockout mice. Table 4.6 summarizes the major reproductive steps where eicosanoids are known to play a physiologic role or have been shown to have therapeutic relevance.

Eicosanoids and Ovulation

An essential role for PGs in ovarian function was established many years ago,[238] and the intricacies of their mechanism of action have been delineated (see Chapter 8). A series of elegant studies established that induction of COX-2 in the preovulatory follicles of the rat ovary was an obligatory downstream event in the LH surge–stimulated ovulation process.[243-246]

In contrast to the COX-1 enzyme, which was constitutively expressed in thecal cells and corpora lutea, LH-induced COX-2 enzyme was present in granulosa cells of the rat preovulatory follicles.[243-246] This observation was later

Table 4.6 Physiologic Roles and Therapeutics of Eicosanoids in Reproduction

Function	COX Enzyme	Eicosanoids	Receptor	Therapeutics
Ovulation	COX-2	PGE_2	EP2	
Luteolysis		$PGF_{2\alpha}$	FP	
Fertilization	COX-2			
Implantation	COX-2	PGI_2	PPARΔ	
Decidualization	COX-2	PGI_2	PPARΔ	
Dysmenorrhea	COX-2	$PGE_2/PGF_{2\alpha}/PGI_2$		NSAIDs/coxibs
Ductus arteriosus remodeling	COX-2	PGE_2	EP4	
Preeclampsia		PGI_2/TxA_2		Low dose aspirin
Cervical ripening				PGE analogues
Preterm labor	COX-2	$PGE_2/PGF_{2\alpha}$		NSAIDs/coxibs
Labor	COX-1	$PGF_{2\alpha}$	FP	
Erectile dysfunction				PGE_1

COX, Cyclooxygenase; *NSAIDs*, nonsteroidal antiinflammatory drugs.

extended to other mammalian species, including the cow and mare.[247] Interestingly, the time required for COX-2 induction in preovulatory follicles after the LH surge differed considerably in various species (2 to 4 hours in the rat, 18 hours in the cow, and 30 hours in the mare), yet induction of COX-2 expression always occurred approximately 10 hours before ovulation in each of these mammals.[247]

LH surge–induced COX-2 expression in the granulosa cells of macaques, a primate species, has also been established using reverse transcriptase–polymerase chain reaction and immunocytochemistry methods.[248] Furthermore, follicular administration of indomethacin in the rhesus monkey was demonstrated to significantly, although not completely, inhibit oocyte release from the ovary.[249]

Thus, in primate, rodent, and domesticated animal species, the induction of COX-2 seems to serve as an "alarm" signal for ovulation.[247,250] Ovulation, initiated by the LH surge that leads to a cascade of proteolytic events that culminate in follicle rupture and release of the ovum, has often been compared to an inflammatory response.[251] In this regard, it is fitting that COX-2, rather than COX-1, should be implicated in this process.

The predominant role of COX-2 in the ovulation process was ratified by studies using COX-1– and COX-2–deficient mice. Mice lacking the COX-2 enzyme had multiple reproductive failures, including ovulation defects, whereas COX-1–deficient mice had impaired parturition, but ovulated normally.[208,252] Biochemical studies showed that exogenous gonadotropins (pregnant mare serum gonadotropin [PMSG] and human chorionic gonadotropin [hCG]) stimulated a fourfold increase in ovarian PGE_2 levels in wild-type and COX-1–deficient mice 8 hours after hormonal challenge; COX-2–deficient mice showed no such increase.[252]

Examination at the cellular level showed that the primary defect responsible for failed ovulation in COX-2–deficient mice was impaired expansion of the cumulus oophorus. This impairment possibly originated from an alteration in the proteoglycan contents of the cumulus oophorus and follicular wall.[252] Whether COX-2 deficiency might also have affected the activation of proteolytic enzymes that participate in the breakdown of the follicular wall is not yet known. Indomethacin treatment, however, did cause oocyte retention but did not prevent follicular rupture in the rhesus monkey ovary.[249]

The critical role of COX-2 in ovulation has also been corroborated by pharmacologic studies using coxibs as selective inhibitors of this enzyme in wild-type animals. Administration of celecoxib to mice[253] and NS-398 to rats[254] decreased, but did not abolish, ovulation in the treated animals. NS-398 also reduced PGE_2 synthesis in isolated LH-stimulated rat preovulatory follicles in vitro.

The degree of inhibition of PGE_2 synthesis by NS-398 in this assay was similar to that achieved by indomethacin, supporting the conclusion that COX-2 mediated the previously observed ovulation blockade by traditional NSAIDs.[238] Finally, delayed follicular rupture in humans by the selective COX-2 inhibitor rofecoxib was observed in a randomized double-blind study,[255] supporting a link between NSAID use and reversible female infertility originating from ovulation interference.[255]

Several lines of evidence suggest that PGE_2 is the principal COX-2 product that mediates the ovulation process.[256] First, ovarian PGE_2 production is increased in response to the LH surge. Second, application of exogenous PGE_2 to gonadotropin-primed, COX-2–deficient mice corrected the anovulatory phenotype.[252] Administration of $PGF_{2\alpha}$ in the same experiment also slightly improved the ovulation outcome, but this was most likely attributable to cross-activation of PGE receptors by $PGF_{2\alpha}$ because no ovulatory abnormality was detected in FP-deficient mice.[257]

Third, a moderate reduction in the ovulation rate has been detected in two independently generated EP2-deficient mouse lines,[258,259] but no apparent ovulation defect has been seen in other prostanoid receptor–mutant mice. EP2-deficient mice had severely compromised fertility, which was attributed partially to an ovulation defect and predominantly to fertilization failure.[258,259]

A role for EP2 in ovulation and fertilization is supported by its specific expression in the cumulus cells of the preovulatory follicles, where it is upregulated in response to gonadotropin stimulation.[259] The impaired fertilization of EP2-deficient mouse eggs was shown to result from abortive cumulus expansion in the oviduct,[259] a phenomenon also observed in the preovulatory follicles of COX-2–deficient mice.[252] These studies suggested that EP2 may be essential for the late-stage events of ovulation by inducing ordered cumulus expansion after ovulation to ensure successful fertilization; however, it is relevant but not indispensable for follicular rupture and ova release.

Contrary to these initial findings, another follow-up study of adult COX-2-deficient and EP2-deficient mice concluded that the ovulatory process, *not* follicular growth, oocyte maturation, or fertilization, is primarily affected by these deficiencies.[260] This study also showed that severely compromised ovulation, seen in adult COX-2–deficient and EP2-deficient mice, was not manifested in immature mice with those same deficiencies. This finding suggests that the process of ovulation may be more dependent on PGs in adult mice.[260]

If EP2 is the receptor subtype physiologically responsible for initiating PGE_2-induced ovulation in follicles, then the incomplete penetration of the ovulation phenotype in EP2-deficient mice could indicate compensatory responses from other EP receptor subtypes in this process. In addition to EP2, EP4 mRNA and protein are detected in mouse and monkey oocytes.[256] More recent data have implicated multiple EP receptors in regulating proteolysis in the primate periovulatory follicle.[261]

Eicosanoids and Corpus Luteum Function and Luteolysis

Ovarian PGE_2 levels were slightly suppressed in both COX-1– and COX-2–mutant mice 24 hours after hormonal stimulation.[252] This discovery suggested that while COX-2–derived PGs are responsible for ovulation, both isozymes may contribute to PG production during formation of the corpora lutea. However, neither COX isoform is indispensable for granulosa cell development or corpora lutea formation, because ovarian morphology and steroid hormone production in COX-deficient mice were similar to those of wild-type mice 24 to 30 hours after gonadotropin stimulation.[252] There was also no apparent difference in estrous cyclicity between wild-type, COX-1–deficient, and COX-2–deficient mice.[252]

The role of $PGF_{2\alpha}$ as a physiologic luteolysin has been well established in many nonprimate mammalian species

that depend on the presence of the uterus for corpus luteum regression. Indeed, this finding proved to be a major breakthrough in the livestock industry, where $PGF_{2\alpha}$ and its synthetic analogues are routinely used to regulate the breeding of domesticated animals.

The involvement of PGs in the luteolysis of primate species is not as well defined (see Chapter 8). Unlike in other mammals, the primate corpus luteum undergoes luteolysis in the absence of the uterus. There is, however, evidence to suggest that locally produced PGs in the ovary may act as mediators to promote luteolysis. For example, $PGF_{2\alpha}$ is produced by the human corpus luteum, and specific receptors for $PGF_{2\alpha}$ are present in human luteal tissue.[262] High doses of $PGF_{2\alpha}$ injected directly into human corpus luteum in vivo also caused premature regression of the corpus luteum and shortening of cycle length.[263]

On the other hand, results from PG synthesis–inhibitor experiments in the rhesus monkey have been equivocal.[264,265] Also, it is not known whether PG levels change significantly in the primate ovary or corpus luteum during the luteal phase.[266] Thus, although there is considerable evidence that PGs of ovarian or luteal origin may participate in the induction of corpus luteum regression in primates, further studies will be required to conclusively resolve this question.[266]

Eicosanoids and Fertilization, Implantation, and Decidualization

The role of PGs in oocyte maturation and fertilization is supported by the phenotype of COX-2–deficient mice (see Chapter 8).[208] In addition to an ovulation defect, the few ovulated eggs from COX-2–deficient female mice recovered after PMSG and hCG challenge were totally incapable of being fertilized.[208] In accordance with this observation, immunostaining detected COX-2 enzyme in wild-type mouse cumulus cells enclosing ovulated eggs.[208] Thus local COX-2–derived PGE_2 in the cumulus cells may function as an autocrine factor to activate EP receptors and induce cumulus cell expansion necessary for successful fertilization.

Because COX-2–deficient mice are defective in ovulation and fertilization, the implantation and decidualization processes in these animals could be investigated only by embryo transfer (from wild-type mice) and by a procedure involving intraluminal infusion of oil to induce artificial decidualization.[208,234,267]

Initial published studies showed that 50% of the transferred embryos were successfully implanted in the uteri of wild-type mice at day 5 after pseudopregnancy, but fewer than 2% of the embryos implanted in the uteri of COX-2–deficient mice.[208] Similarly, induction of artificial decidualization in wild-type mice caused a 16-fold increase in uterine wet weight but did not produce any appreciable weight gain in COX-2–deficient mouse uteri.[208] The failure of implantation and decidualization in COX-2–deficient mice is not secondary to steroid hormone insufficiency from ovarian dysfunction, because supplementation of progesterone and estradiol did not rescue the phenotype.[208] COX-1, on the other hand, is not required for embryo implantation and uterine decidualization.[208]

A specific role for COX-2 (but not COX-1) in these processes is also supported by the dynamic regulation of COX-2 expression in the mouse uterus. Although COX-2 is normally expressed minimally in the uterus, it is robustly induced at 2 hours after artificial decidualization and its expression returns to basal levels within 8 hours. COX-1, in contrast, is constitutively expressed in the mouse uterus and barely changes its expression during decidualization.[208]

A critical role for COX-2 in implantation was disputed in a separate study conducted by Cheng and Stewart.[267] Their investigation into the role of COX-2 in embryo implantation and uterine decidualization concluded that COX-2 has a role in mediating the initial uterine decidual response but is *not* essential for embryo implantation, sustained decidual growth, or embryo development throughout the remainder of pregnancy.[267]

PGI_2 may be the eicosanoid that mediates COX-2–dependent embryo implantation and uterine decidualization.[208,234] Researchers have proposed that the effect of PGI_2 in these processes is mediated by the nuclear receptor PPARδ. PGI_2 is the most abundant prostanoid formed at implantation and decidualization sites, and PGI_2 synthase transcripts are also present and upregulated at the implantation sites between day 5 and day 8 of pregnancy.[208,234]

The finding in earlier studies that two PGI_2 analogues (carbaprostacyclin [cPGI] and iloprost) activated PPARδ[268] and that IP-deficient mice had normal fertility[269] led to the hypothesis that endogenous PGI_2 mediates embryo implantation and decidualization through PPARδ.[208,234] The coordinated uterine expression of PPARδ and COX-2 during decidualization supports this contention. The hypothesis was also supported by the finding that PGI_2 agonists such as cPGI and iloprost (as well as a classic PPARδ-selective agonist, L-165,041) restored implantation and decidualization in COX-2–deficient mice.[208,234] Moreover, PPARδ was found to be essential for spontaneous and preimplantation PGI_2-stimulated embryo development and blastocyst hatching.[270] The implantation of cultured embryos was enhanced by PPARδ activation.

Nevertheless, many questions remain to be answered about the validity of this hypothesis. First, varied phenotypes of PPARδ-deficient mice have been reported.[271] Second, cPGI and iloprost have been shown to activate PPARδ,[208,234,268] but one cannot assume with certainty that endogenous PGI_2 will do the same. Indeed, cicaprost, another PGI_2 agonist for membrane IP, was not an activator of PPARδ,[268] suggesting that caution should be exercised when extrapolating data from PGI_2 analogues. Third, it is possible that implantation and decidualization could be mediated by COX-2 and PPARδ as separate and redundant pathways. To further muddy the waters, more recent studies suggest that IP receptors play an important role in preimplantation embryo development and mediate the embryo's response to exogenous PGI_2.[272] A PPARδ agonist had no effect in IP receptor knockout mice, suggesting that the nuclear receptor pathway may be downstream of IP activation. Clearly, more research is needed on this topic. Even if the PGI_2-PPARδ hypothesis is accepted at face value, the mechanism and downstream events of this signaling pathway in the uterus remain to be established. No specific defect in the expression of implantation-related genes or steroid responsiveness has been found in the uterus of COX-2–deficient mice.[208] The direct causes of impaired implantation and decidualization failure in these animals are still unknown. Clearly, however, other eicosanoids are needed for the implanted embryos to

develop to term. Thus administration of cPGI was shown to improve implantation and decidualization in COX-2–deficient mice, but the subsequent growth of the implanted embryos in these mice was not comparable to that implanted in the wild-type mice.[208,234] Interestingly, coadministration of cPGI with PGE_2 markedly improved embryonic and decidual growth in COX-2–deficient mice.[234]

Eicosanoids and Menstruation and Dysmenorrhea

Abnormal PG biosynthesis is involved in the pathogenesis of human dysmenorrhea, a gynecologic disorder that is defined as the occurrence of pain associated with menstruation. Elevated levels of $PGF_{2\alpha}$ and PGE_2 occur in the endometrium and menstrual fluid of women who have dysmenorrhea with an elevated ratio of $PGF_{2\alpha}$ to PGE_2.

A markedly increased capacity for PGI_2 synthesis has been detected in endometriosis tissue. This elevation is considered to cause hyperalgesia and to contribute significantly to dysmenorrhea in endometriosis.[273] Furthermore, intrauterine administration of $PGF_{2\alpha}$ induces uterine contractility and dysmenorrhea-like pain.

Cumulative data of clinical trials have indicated that 80% of patients with significant primary dysmenorrhea experience adequate pain relief with the use of traditional NSAIDs.[274] Both celecoxib and valdecoxib, two COX-2–selective inhibitors, have also been used for the treatment of primary dysmenorrhea.[275,276] However, valdecoxib was removed from clinical use in 2005 because of an association with elevated risk of Stevens-Johnson syndrome and cardiovascular hazard, whereas celecoxib remains in use.

Eicosanoids and Parturition and Preterm Labor

There has been a great deal of interest in the role that PGs play in term and preterm labor (see Chapter 11) and their clinical use in induction of labor.[277] Aspirin-like drugs were shown to delay parturition in humans more than 30 years ago,[278] and NSAIDs are also well known to be effective agents in attenuating the progression of term and preterm labor in various species.[279,280] PGE_2 and $PGF_{2\alpha}$, produced by both maternal and fetal tissues during pregnancy,[281] stimulate uterine contractions in vitro and in vivo; they also promote the coordinated inflammatory response that results in dilation and thinning of the cervix.[281] Studies with COX-1– and prostanoid receptor–deficient mice have provided additional insight into the role and mechanism of action of PGs in the process of parturition and preterm labor.

FP-deficient female mice became pregnant, but they did not deliver fetuses normally at term.[257] Induction of the oxytocin receptor at term, an event believed to trigger parturition, did not occur in the FP-deficient mouse uterus.[257] The phenotype of impaired parturition was due to the failure of maternal progesterone levels to decline and could be corrected by ovariectomy. These observations suggest that $PGF_{2\alpha}$ induces labor through its luteolytic actions in rodents. Its uterotonic action in the myometrium is apparently not essential for parturition. A similar parturition defect was characterized in COX-1–deficient mice, and administration of $PGF_{2\alpha}$ was sufficient to correct this defect.[282,283]

Although studies of FP-deficient mice revealed that the primary target of $PGF_{2\alpha}$ action with regard to parturition is the corpus luteum, which is not reflective of the process of parturition humans, there are relevant changes in uterine prostanoid synthesis and metabolism including a 40-fold induction of COX-1 in late gestation.[257] In addition, PGF synthase and a $PGF_{2\alpha}$-metabolizing enzyme, 15-OH-PGDH, were also time-dependently regulated in the pregnant mouse uterus with a dramatic increase in PGFS and a decrease in 15-OH-PGDH toward late gestation.[284,285]

The lack of a parturition phenotype in PG receptor–deficient mice other than FP knockout mice suggests that PGs are less important in promoting cervical ripening and myometrial contraction in the mouse. This hypothesis is supported by the finding that the luteolytic effect alone of $PGF_{2\alpha}$ made its function indispensable for murine parturition. This role contrasts greatly with what is believed to occur in human labor. Various PGs are produced in human myometrium, with PGI_2 being the highest.[286] Recently, PGI_2 was paradoxically shown to prime pregnant human myometrium for an enhanced contractile response in parturition via induction of genes encoding for contractile proteins via the IP receptor.[287,288] EP1, EP3, and FP receptors are also present in human myometrial tissue,[289] as are the enzymes that generate PGs, including COX-2 and PGE synthase isoforms.[290]

Increased PG production is also associated with cervical ripening, and a topically applied PG product, containing either dinoprostone or misoprostol, is the most popular means to soften and dilate the cervix in human labor induction.[291] Local administration of PGs to the cervix in pregnant women results in clinical, histologic, and biochemical changes that are consistent with those observed during physiologic cervical ripening. The mechanism of action of PGs in human cervical ripening is not known but may include induction of major matrix degrading enzymes and alterations in the expression of genes encoding proteins that modify proteoglycan function. The lack of a phenotype in COX and PG receptor knockout mice in cervical ripening and uterotonic responses may indicate a species difference, or it may suggest compensation in these tissues between the two COX enzymes.

Although COX-1 is involved in term labor, COX-2 induction is likely to be primarily responsible for PG-mediated preterm labor. COX-2, but not COX-1, is induced during inflammation-mediated preterm labor elicited by lipopolysaccharide (LPS) administration.[292] In a murine model, the COX-2–selective inhibitor SC-236 was found to be more effective than the COX-1–selective inhibitor SC-560 in stopping LPS-prompted preterm labor and increasing uterine PG synthesis.[292] Furthermore, COX-1–deficient mice, which showed a delay in the onset of term labor, exhibited no delay in the onset of preterm labor after LPS treatment.[292]

Although it is likely that COX-2–derived $PGF_{2\alpha}$ acted as a luteolysin in the mouse model of LPS-induced preterm labor, it is not known whether the uterotonic effect of $PGF_{2\alpha}$ and other COX-2–derived PGs may also have played a role in this setting. The latter possibility may be particularly relevant to human preterm labor, because indomethacin, an inhibitor of both COX-1 and COX-2, has been used successfully in treating human preterm labor when given systemically and when delivered locally through the vaginal route.[293,294]

The clinical utility of indomethacin as a tocolytic agent, however, is tempered by concerns over fetal and neonatal

complications, such as constriction of the DA.[293,294] The adverse effects associated with indomethacin use in preterm labor have been mainly linked to inhibition of COX-2 based on mouse studies using selective COX-1 and COX-2 inhibitors.[295,296] COX-1–selective inhibitors, should they be developed clinically, would be expected to produce fewer adverse effects than traditional NSAIDs when used as tocolytic agents.[294-296] Studies in animals and pregnant women comparing indomethacin and coxibs produced mixed results when fetal ductus blood flow was used as a measure of potential adverse effects.[294]

Eicosanoids and Ductus Arteriosus Remodeling

As mentioned earlier in the chapter, one of the complications of using NSAIDs such as indomethacin to treat preterm labor is the induction of premature closure of the fetal DA.[241,297] The DA is a large fetal vessel that shunts deoxygenated blood away from the pulmonary circulation to the descending aorta and to the umbilicoplacental circulation, where oxygenation occurs. In neonates, rapid remodeling of the DA leads to its closure after adaptation of spontaneous breathing in newborn infants. Although patency of the DA in utero is essential for proper fetal growth and development, failure of the DA to close after birth, called *persistent patent DA*, compromises postnatal health by causing circulatory complications such as pulmonary hypertension and congestive heart failure.[297]

Prostaglandins are intimately involved in DA function and its perinatal remodeling, and details of their mechanism of action are emerging. The finding that indomethacin administration induced premature DA closure in fetuses[241,297] suggested that fetal PGs are essential for maintaining DA patency. However, mice lacking both COX-1 and COX-2 are unable to make any PGs and die postnatally with patent DA,[298,299] indicating that fetal-derived PGs are not necessary for maintaining DA patency in utero. Such PGs do, however, play an indispensable role in DA remodeling after birth.

How can this phenotype be reconciled with the results of pharmacologic inhibition studies? Researchers have proposed that PGE_2 in the fetal circulation (supplied in part by the placenta) maintains dilation of the DA in utero and that COX-2 in the DA produces constrictor PGs that are important for DA contraction after birth.[299] Thus indomethacin-induced premature DA closure in fetuses may have reflected inhibition of dilatory PGE_2 synthesis in the placenta without sufficient inhibition of ductal COX-2 to attenuate DA contraction. Although COX-1 deficiency alone did not affect perinatal DA remodeling, it was found to exacerbate the phenotype of patent DA on the background of COX-2 deficiency.[299]

In other studies, mice deficient in the smooth muscle relaxant receptor EP4[300,301] or the PGE_2-metabolizing enzyme 15-OH-PGDH[302] also failed to survive postnatally due to patent DA. EP4 expression in DA and 15-OH-PGDH expression in the fetal lung increase dramatically just before birth, supporting the conclusion that these proteins play an important role in perinatal DA remodeling.

The phenotypes of EP4-deficient and 15-OH-PGDH–deficient mice, together with the results of pharmacologic studies, support alternative models for the DA remodeling process.[301-303] Signals through EP4 have two essential roles in DA patency and remodeling, namely vascular dilation and intimal cushion formation (ICF). The vascular smooth muscle–relaxing ability of PGE_2 acting via EP4 maintains patency during fetal life. When PGE_2 levels plummet at birth due to rapid and efficient metabolism by late gestation–induced 15-OH-PGDH activity, the vasodilatory role is curtailed, and functional DA closure is triggered rapidly by an increase in oxygen tension. ICF occludes the vascular lumen and results in permanent closure after birth. ICF within the DA is a result of an increase in vascular smooth muscle cell migration and proliferation and the production of hyaluronic acid (HA) under the endothelial layer, along with decreased elastin fiber assembly. PGE_2 via EP4 signaling during late gestation controls a gene, *HAS2*, which regulates HA synthesis and ICF.[303]

Eicosanoids and Preeclampsia

The possible involvement of PGs in preeclampsia and the potential therapeutic efficacy of low-dose aspirin in their prophylactic treatment have received considerable attention. Preeclampsia occurs in 10% of pregnancies and are recognized as important, prevalent sources of risk to both mother and fetus (see Chapter 27).

Although the exact cause of the disease is unknown, dysregulated production of PGI_2 and TxA_2 has been postulated as one of many potential etiologic factors.[304] Decreased urinary PGI_2 metabolites are found to precede the development of preeclampsia,[305] and increased TxA_2 metabolite excretion occurs in patients with severe preeclampsia.[306,307]

Such changes may predispose to vasoconstriction of small arteries, activation of platelets, and uteroplacental insufficiency—clinical outcomes that are associated with PIH and preeclampsia. In a murine model of enhanced TXA_2 activity, transgenic overexpression of TP in the vasculature resulted in intrauterine growth retardation that was rescued by timed suppression of TXA_2 synthesis with indomethacin.[308]

Many clinical trials have been conducted to evaluate the use of low-dose aspirin in the prevention of preeclampsia. Some randomized studies indicated a beneficial effect for women at increased risk, other trials did not show a positive effect of low-dose aspirin use on reducing the incidence of preeclampsia or on improving perinatal outcomes in pregnant women at high risk for preeclampsia.[309-311] Based on recent evaluation of clinical trials, the US Preventive Task Force recommended daily low dose aspirin for women at high risk for preeclampsia.[312,313]

Eicosanoids in Male Reproduction

Although PGs are critically involved in multiple steps of female reproduction, as previously discussed, their physiologic role in male reproduction is not well understood—though it appears to be less remarkable, based on gene knockout studies. No discernible male reproductive phenotype has been noted in any of the COX-deficient or prostanoid receptor–deficient mice.[205,206]

On the other hand, a number of studies have described androgen-dependent regulation and distinctive tissue distribution patterns of COX-1 and COX-2 enzymes, as well as PG-synthesizing enzymes, in the male reproductive tracts

of rodents and humans.[314-317] Specific functions have also been documented for COX-2–derived PGD_2 and PGE_2 in mediating cytokine production in Leydig cells and in regulating apoptosis in the rat epididymis.[318,319] Although they are not definitive, such studies suggest that PGs may be synthesized and functional in a regulated manner in the male reproductive organs, even though their physiologic roles in these sites are dispensable.

In the future, a better understanding of the eicosanoid network in the male reproductive system may lead to new PG-based therapies or provide mechanistic insight about existing therapies. For example, highly specific expression of COX-2 has been found in the distal end of the rat vas deferens.[314] Because the distal vas comprises an extensive submucosal venous plexus connected to the penile corpora cavernosa, PGs from the vas may play a role in erection.[314] In this context, it is notable that intracavernosal injection of PGE_1 has been used clinically as an effective therapy for erectile dysfunction in man.[320,321] While PGs were originally isolated in large amounts in male seminal vesicles, their role at this site has remained enigmatic. The recent finding that ovarian PGs may act as sperm guidance factors via an insulin and FOXO transcription factor pathway could indicate a synergistic role with male accessory gland-derived PGs.[322]

Other Lipid Mediators: Lysophosphatidic Acid and Sphingosine-1-Phosphate

Besides eicosanoids, other lipid mediators have been implicated in reproductive function, such as lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P).[323,324]

LPA is a lipid-signaling molecule and an intermediate in the de novo biosynthetic pathway of phospholipids consisting of a glycerol backbone, a phosphate head group, and a long-chain fatty acid (usually oleic acid or palmitic acid), most commonly in acyl linkage (see Fig. 4.17).[325] LPA generation is complex, proceeding by at least two pathways: conversion from lysophospholipids or via phosphatidic acid. Several phospholipase activities are necessary, including phospholipase A_1 (PLA_1)/PLA_2 plus lysophospholipase D (lysoPLD) and phospholipase D(PLD). Additional extracellular phospholipases, such as secretory PLA_2 ($sPLA_2$-IIA), membrane-associated PA-selective PLA_1 ($mPA\text{-}PLA_1$), and lecithin–cholesterol acyltransferase (LCAT), can also be involved.[326] LPA signaling is mediated primarily by six members of the GPCR family currently referred to as LPA_1, LPA_2, LPA_3, LPA_4, LPA_5, and LPA_6.[327]

The lipid mediator S1P is a bioactive sphingolipid derived from the abundant phospholipid sphingomyelin. Sphingomyelinases generate ceramide, which is cleaved by ceramidases to sphingosine, followed by phosphorylation by sphingosine kinases to S1P (Fig. 4.22).[328] S1P signals extracellularly via transmembrane receptors but also has intracellular targets.[329,330] There are five S1P receptors, also members of the GPCR family, referred to as $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$.[331] S1P stimulation of these receptors elicits a battery of downstream effects, including inhibition of cAMP and activation of mitogen-activated protein kinases, phospholipase C, and PI3 kinase to evoke a broad range of cellular activities.[331]

LPA and S1P in Reproductive Function

LPA and S1P are two lysophospholipids that play important roles in reproduction acting via their respective GPCRs.[332] Most of the LPA receptor genes have been disrupted in mice, with some affecting reproductive function. LPA_3 (also

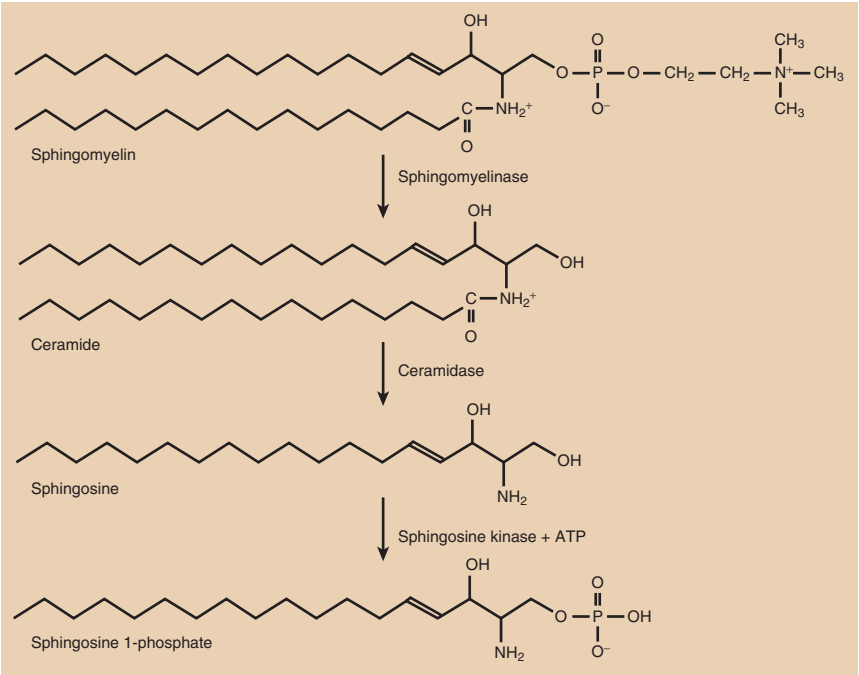

FIGURE 4.22 Structures and biosynthesis of a representative sphingosine-1-phosphate *(S1P)* lipid mediator from sphingomyelin. Sphingomyelinase removes the phosphorylcholine head group to yield ceramide. Ceramidase cleaves the amide bond removing one aliphatic chain to generate sphingosine. In the presence of adenosine triphosphate *(ATP)*, sphingosine kinase will phosphorylate sphingosine to generate S1P.

known as *Edg7*) exhibits a female reproductive phenotype.[333,334] LPA_3 mRNA has been detected in oviduct, placenta, and uterus, but not in ovary and oocytes, with expression highest early in pregnancy, at approximately embryonic day 3.5 in mice. LPA_3 is regulated positively by progesterone and negatively by estrogen.[333,334] LPA_3-deficient females produce small litters and show a prolongation of pregnancy by approximately 1.5 days (normal gestation, 19.5 days).[333] The mice show no obvious defects in ovulation, ovum transport, or blastocyst development. However, the defects were related to delayed implantation and altered crowding or positioning of embryos, which led to delayed embryonic development and death, accounting for the reduced litter size. The observed phenotypes were the result of maternal LPA_3 signaling and were not due to embryo LPA_3 signaling.[333] Strikingly, the phenotypes were similar to those seen with $cPLA_2$ female knockout mice and rodents treated with indomethacin.[335-337] Remarkably, LPA_3-deficient female uteri had markedly reduced COX-2 expression and PGE_2/PGI_2 levels at E3.5, thus linking LPA signaling to PG biosynthesis and fertility control.[333] Overall, this particular study raises speculation that therapeutic manipulation of LPA_3 signaling could influence the low implantation rate that is a major drawback during infertility treatments using assisted reproductive technologies.

LPA_1, LPA_2, and LPA_3 are differentially expressed in male testes with the latter two in the basal regions of seminiferous tubules, primarily in immature germ cells (spermatogonia and spermatocytes) and LPA_1, showing stage-specific expression in germ cells.[338] Triple knockout mice exhibit male reproductive defects including alterations in mating behavior and heterogeneic spermatogenic disruption resulting in sterility.[338] Many questions remain to be answered on the mechanisms for these effects.

Of the "newer" LPA receptors LPA_4, LPA_5, and LPA_6, there is no evidence for the involvement of these signaling pathways in reproductive function. LPA_4 has been detected in ovaries, uterus and placenta, but only one of three research groups reported a reduced litter size in mice deficient for LPA_4, but this was apparently attributable to other nonreproductive mechanisms.[339]

Clues to the roles of S1P in reproductive function have come from various sources. Based on expression studies of the five receptor subtypes that bind S1P, it is known that only three ($S1P_1$, $S1P_2$, and $S1P_3$) show widespread distribution in mice, with expression in gonadal tissues and in the uterus during decidualization.[331,340] $S1P_1$ and $S1P_2$ colocalize with COX-2 at the maternal/fetal interface throughout pregnancy, suggesting a link between sphingolipid and PG signaling and indicating that S1P coordinates uterine mesometrial angiogenesis during implantation.[340]

Because $S1P_1$-deficient mice die in mid-embryogenesis as a result of complications of vasculogenesis, it has not been possible to decipher a role for this particular signaling pathway in reproductive function.[331] Both $S1P_2$- and $S1P_3$-null mice show no obvious phenotypes with the exception of slightly smaller litter sizes.[327,331] However, when both receptors are knocked out, there is infertility.[327] The specific roles of each S1P receptor subtype in female reproductive function will still require more study.

A role for S1P in the preservation of female fertility is being recognized.[341] Programmed cell death (apoptosis) is an established paradigm in the mammalian female germline. Ceramide generated by membrane cleavage of sphingomyelin by sphingomyelinase or via de novo biosynthesis by ceramide synthase is translocated from cumulus cells to adjacent oocytes to induce germ cell apoptosis.[342] This is prevented by S1P, a ceramide metabolite within the same pathway or by acid sphingomyelinase (ASM) deficiency. The therapeutic management of infertility by S1P in premature menopause and in female patients with cancer appears promising, but awaits further study.[341]

Sphingolipids also seem to play a role in male germ cell apoptosis.[343,344] Ceramide induces an early apoptotic pathway event in male germ cells that is partially suppressed by S1P. However, although maintenance of normal sphingomyelin levels in testes and normal sperm motility are dependent on ASM, testicular ceramide production and the ability of germ cells to undergo apoptosis do not require ASM.

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