Novel Insights into the ACTH-induced and Basal Steroidogenesis in Adrenocortical Cells

by

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Abstract

Adrenocortical cells — the corticosteroids-producing cells of the adrenal glands — are rich in cholesterol containing lipid droplets (LDs) and mitochondria, and both are steroidogenically interconnected. The former serves as the precursor substrate for corticosteroids biosynthesis whereas the later represents the site where corticosteroidogenesis begins. The corticosteroidogenesis has been studied extensively; however, a number of fundamental questions related to adrenocorticotrophic hormone (ACTH)-induced and basal steroidogenesis remain unclear, including cholesterol transport to the inner mitochondrial membrane (IMM) where steroidogenesis initiates and the role of intracellular cholesterol pool. Prohibitin-1 (PHB1) is an evolutionarily conserved protein that localizes to the IMM, where it functions as a lipid and protein chaperone. Recently, our laboratory has discovered that PHB1 plays a role in testicular Leydig cell steroidogenesis, involving cholesterol homeostasis and mitochondrial biology pertaining testosterone production. Thus, the present study investigates the role of PHB1 in corticosteroidogenesis (*in vitro* using human HAC15 and mouse Y-1 cell culture models), as the fundamental aspects of steroidogenesis related to cholesterol handling and mitochondrial biology are similar in different steroidogenic cell types. Manipulation of PHB1 level was found to affects mitochondria, lysosomes and lipid droplets characteristics, as well as corticoid production by adrenocortical cells. Mechanistically, I found that PHB1 modulates PKA and ERK signaling in adrenocortical cells. A consistent finding of PHB1's role in steroidogenesis in adrenocortical cells from two different species, as well as previously in testosterone producing murine Leydig cells imply that its role in steroidogenesis is likely conserved. Moreover, as the framework of corticosteroidogenesis is built around cholesterol — the precursor substrate for steroid hormones, including its cellular uptake by adrenocortical cells, mobilization and trafficking to the IMM, I explored a potential role of intracellular cholesterol pool in corticosteroidogenesis *in vitro* using a simple approach that appears to be not exploited before. Cholesterol deprivation induced steroidogenic events/factors (as determined by their marker proteins) and increased basal steroidogenesis in adrenocortical cells. Data obtained suggest that cholesterol plays an important role in basal steroidogenesis. In summary, my findings provide new insights into ACTH-induced and basal steroidogenesis in adrenocortical cells.

Publications

1. Bassi G*, Sidhu SK*, Mishra S. The expanding role of mitochondria, autophagy and lipophagy in steroidogenesis. *Cells*. 2021 Jul 22;10(8):1851. doi: 10.3390/cells10081851. PMID: 34440620; PMCID: PMC8391558. (* indicates equal contributions).

2. Bassi G, Sidhu SK*, Mishra S. The intracellular cholesterol pool in steroidogenic cells plays a role in basal steroidogenesis. *J Steroid Biochem Mol Biol*. 2022 Mar 24;220:106099. doi: 10.1016/j.jsbmb.2022.106099. Epub ahead of print. PMID: 35339650. (* performed the majority of the experiments related to Y-1 adrenocortical cells).

Table of Contents

List of Figures

List of Tables

Chapter 3

List of Open Access and Copyright Materials

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List of Abbreviations

ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of variance
ATCC	American type culture collection
ATG7	Autophagy related 7
CAH	Congenital adrenal hyperplasia
cAMP	Cyclic adenosine monophosphate
CARC	Inverted-CRAC motif
CD	Cholesterol depleted
CHX	Cycloheximide
CO_2	Carbon dioxide
CRAC	Cholesterol recognition amino acid consensus
CRH	Corticotropin releasing hormone
CYP	Cytochrome P450
Db-cAMP	Dibutyryl cyclic adenosine monophosphate
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DMEM	Dulbecco's modified Eagle's medium
Drp1	Dynamin-related protein 1
eCG	Equine chorionic gonadotropin
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ER	Endoplasmic reticulum
ERK1/2	Extracellular stress-regulated kinase 1/2
Erlin-1	Endoplasmic reticulum lipid raft-associated protein 1
Ermin-2	Endoplasmic reticulum lipid raft-associated protein 2
Fabp4	Fatty acid binding protein-4
FBS	Fetal bovine serum
Fig	Figure
FSH	Follicle stimulating hormone
GPCR	G-protein coupled receptor
h	Hour
HAC15	Human adrenocortical cell line
hCG	Human chorionic gonadotropin
HDL	High density lipoprotein
HPA	Hypothalamic pituitary-adrenal axis
HSL	Hormone sensitive lipase
i-AAA	Intermembrane AAA protease
IGF	Insulin-like growth factor
IMM	Inner mitochondrial membrane
LC3	Microtubule-associated protein 1A/1B-light chain 3
LC3-I	A cytosolic form of LC3
LC3-II	LC3-phosphatidylethanolamine conjugate
LDL	Low density lipoprotein
LDs	Lipid droplets

LH	Luteinizing hormone
M	Molar
m-AAA	Matrix AAA protease
MAPK	Mitogen-activated protein kinase
MC2R	Melanocortin-2 receptor
MEC-2	Mechanosensory protein 2
Mfn1	Mitofusin-1
Mfn2	Mitofusin-2
mg	Milligram
min	Minute
ml	Milliliter
MLN64	Metastatic lymph node 64
mM	Millimolar
NC	Normal culture
nm	Nanometer
OMM	Outer mitochondrial membrane
Opa1	Optic atrophy-1
PAGE	Polyacrylamide gel electrophoresis
PARL	Presenilins-associated rhomboid-like protein
PE	Phosphatidyl ethanolamine
p-ERK1/2	Phosphorylated extracellular stress-regulated kinase 1/2
PHB	Prohibitin
PHB1	Prohibitin-1
PHB2	Prohibitin-2
Phe	Phenylalanine
PKA	Protein kinase A
PM	Plasma membrane
PM	Plasma membrane
p-PKA	Phosphorylated protein kinase A
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation assay buffer
rpm	Revolution per minute
SCC	Side chain cleavage
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
Ser	Serine
SER	Smooth endoplasmic reticulum
shControl	Short hairpin scrambled control
shRNA	Short hairpin RNA
SLP2	Stomatin-like protein-2
SR-B1	Scavenger receptor B1
StAR	Steroidogenic acute regulatory protein
START	StAR-related lipid-transfer
TBS	Tris buffered saline
TEM	Transmission electron microscopy
Tg	Transgenic

Thr	Threonine
Tyr	Tyrosine
Y-1	Mouse adrenocortical cell line
YME1L	An ATP-dependent metalloprotease
µg	Microgram

CHAPTER 1

Literature Reviews

Sections of this literature reviews have been published as a review article in the journal *Cells* titled “The expanding role of mitochondria, autophagy and lipophagy in steroidogenesis”, Bassi G*, Sidhu SK*, Mishra S. 2021 Jul 22;10(8):1851.
(* indicates equal contributions)

1.1 Adrenal glands – General anatomy and histology

The adrenal glands are an integral part of the endocrine system. There are two adrenal glands, which are anatomically located on the top of each kidney. Thus, the name, adrenal or suprarenal glands [1,2]. An outer connective tissue called capsule encloses the adrenals. Structurally, the adrenal gland has two distinct regions — the outer adrenal cortex and the central adrenal medulla, which have different embryonic origin and distinct functions to perform (Fig. 1). The cortex comprises approximately 90% of the adrenal weight and area, whereas the inner medulla comprises about 10%. The adrenal cortex produces many steroid hormones, such as glucocorticoids (e.g., corticoids and corticosterone), aldosterone and the adrenal androgens [2,3].

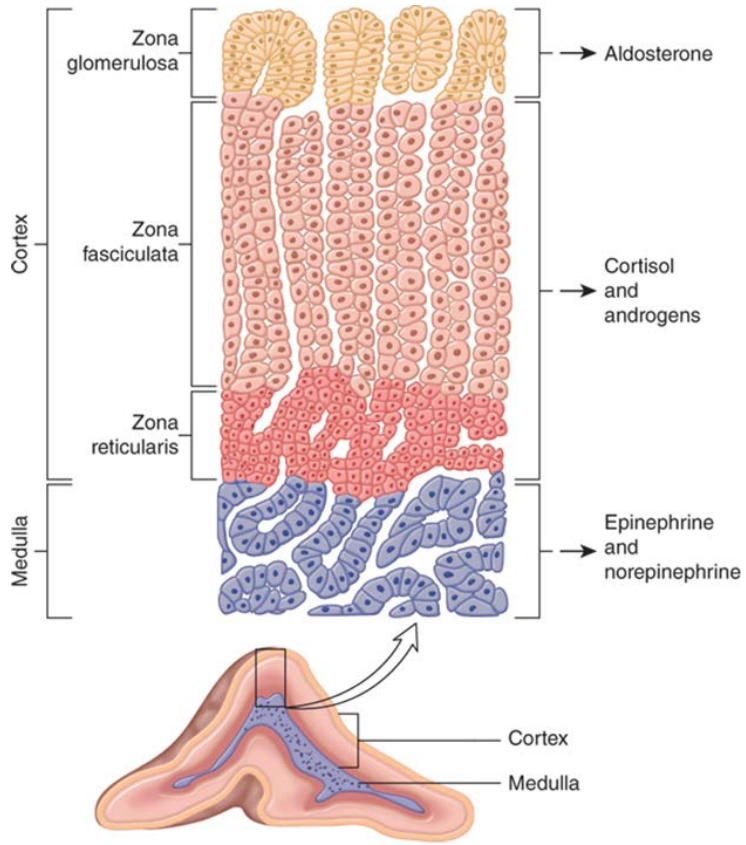


Figure 1.1. Schematic showing histological zonation of adrenal cortex and their main hormones products in human. Adapted and modified from OpenStax College - Anatomy & Physiology, Connexions Web site. http://cnx.org/content/col11496/1.6/, Jun 19, 2013., CC BY 3.0, https://commons.wikimedia.org/w/index.php?curid=30148154.

Histologically, adrenocortical cells of the cortex are divided into three zones — an outer glomerulosa, an intermediate fasciculata, and inner reticularis (Fig. 1.1). The zona glomerulosa constitutes about 15% of adrenal cortex, which lacks a well-define structure, its cells are relatively poor in their lipid content, and are scattered underneath the adrenal capsule [2,3]. The zona glomerulosa cells produces aldosterone in response to renin-angiotensin system and by potassium [2]. This zone do not express the gene *CYP17* encoding 17α-hydroxylase — a steroidogenic enzyme required for 17α-hydroxypregnenolone and 17α-hydroxyprogesterone synthesis, which are the precursor for cortisol and androgens [2,3]. The zona fasciculata constitutes about 75% of the cortex. The cells of the zona fasciculata are organized in columns and are relatively larger in size, which are also known as ‘clear cells’ because of their higher lipid contents, and produces cortisol and androgens [2]. The cells of the zona reticularis, which surrounds the medulla are compact, lack significant lipid content, and produces cortisol and androgens like fasciculata. Both zones (fasciculata and reticularis) are regulated by ACTH, which is produced from the corticotrope cells of the pituitary gland in response to corticotropic releasing hormone (CRH) from the hypothalamus [2,3]. Moreover, both zones do not express the gene *CYP11B2* encoding P450 aldosterone synthase and therefore cannot convert 11-deoxycorticosterone (also known as 21-hydroxyprogesterone) to aldosterone [2]. It is postulated that the zona fasciculata cells can respond acutely to ACTH stimulation with increased cortisol production, whereas the reticularis cells maintain basal glucocorticoid secretion and that induced by prolonged ACTH stimulation. Dysregulation of adrenal steroid production leads to classic endocrinopathies such as Cushing syndrome (an endocrine disorder that occurs when the body makes too much of the hormone cortisol over a long period of time), Additions disease (an endocrine disorder that occurs when the body doesn't produce enough of certain hormones, such

as cortisol and aldosterone), hyperaldosteronism, and syndromes of congenital adrenal hyperplasia (CAH) [2,3].

The brownish central part of adrenals is called medulla. It is embryologically derived from ectodermal neural crest cells, which later differentiate into precursor chromaffin cells in response to cortisol secreted by cortex and are located in clusters [3]. Medulla is very important part of sympathetic division of autonomic nervous system. It produces adrenaline and noradrenaline in response to stress that regulate the “flight-or-fight” response. Amongst the many diseases that could affect medulla, pheochromocytoma, neuroblastomas, paragangliomas are most prevalent ones [3,4].

1.2. Corticosteroids and corticosteroidogenesis

Corticosteroids are an important class of steroid hormones, which are synthesized and produced by the adrenal glands of vertebrates, in response to steroidogenic stimuli, as well as the synthetic analogues of these hormones [3]. There are two main classes of corticosteroids, glucocorticoids and mineralocorticoids (Fig. 1.2). The importance of corticosteroids is evident from their wide-ranging essential functions in the body physiology, including carbohydrate metabolism, stress response, regulation of salt balance, and immune functions [2,3]. In addition, adrenal glands produce adrenal androgens, such as dehydroepiandrosterone (DHEA) [2,3].

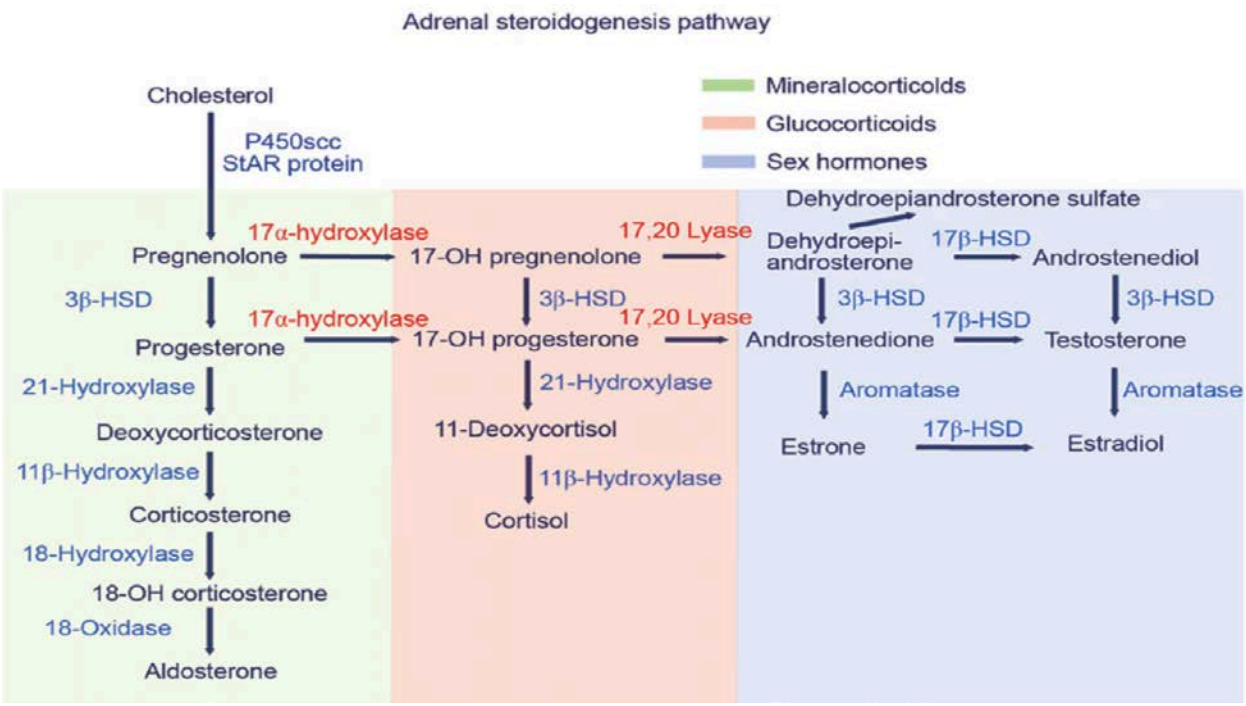


Figure 1.2. Schematic showing main steps of corticosteroidogenesis in three different zones of adrenal cortex and their main steroid hormone products. Adapted from Xu et al. (2016). Molecular Medicine Reports. 15. 10.3892/mmr.2016.6029.

1.2.1. The biosynthesis of corticosteroids

An overarching commonality among different steroid hormones, including corticoids, is that they all are produced from cholesterol. Most of the steroidogenic enzymes belong to the family of cytochrome P450 oxygenase, which are located in the mitochondrial and smooth endoplasmic reticulum (SER) membranes. Consequently, steroidogenesis is compartmentalized in the mitochondria and the SER [5].

The conversion of cholesterol to pregnenolone, which is the first step in steroidogenesis, occurs in the mitochondria (Fig. 2). This involves cleavage of the cholesterol side chain, which is mediated by the enzyme P450scc [2,5]. Next step in cortisol synthesis is 17α-hydroxylation of pregnenolone by the enzyme 17α-hydroxylase to form 17α-hydroxypregnenolone, which is subsequently converted to 17α-hydroxyprogesterone. Both steps occur in the SER. This involves conversion of its 5,6-double bond to 4,5-double bond by the enzyme 3β-hydroxysteroid

dehydrogenase [3,5]. An alternate pathway for cortisol synthesis involves pregnenolone to progesterone to 17α-hydroxyprogesterone; however, this pathway is less important in the zonae fasciculata and reticularis [3,5]. Subsequent step involves the 21-hydroxylation by 17α-hydroxy-progesterone to form 11-deoxycortisol in the SER, which is further hydroxylated within mitochondria by 11β-hydroxylation to produce cortisol. Moreover, the zona fasciculata and zona reticularis also produce 11-deoxycorticosterone, 18-hydroxydeoxycorticosterone, and corticosterone.

1.2.2. The biosynthesis of adrenal androgens

The biosynthesis of adrenal androgens from pregnenolone and progesterone requires 17α-hydroxylation and thus does not occur in the zona glomerulosa, which lacks this enzyme [2,3]. The major pathway from adrenal androgen involved conversion of 17α-hydroxypregnenolone to DHEA (a c-19 steroid) and its sulfated form DHEA sulfate (Fig. 2). Thus, 17α-hydroxypregnenolone undergoes removal of its two-carbon side chain at the C17 position by microsomal 17,20-desmolase, yielding DHEA with a keto group at C17 [3,5]. DHEA is then converted to DHEA sulfate by a reversible adrenal sulfokinase (Fig. 1.2).

The other major adrenal androgen, androstenedione, is produced mostly from DHEA, mediated by 17β-hydroxysteroid dehydrogenase (17β-HSD), and possibly from 17α-hydroxyprogesterone, also by 17β-HSD [5]. Androstenedione can be converted to testosterone, although adrenal secretion of this hormone is minimal. The adrenal androgens, DHEA, DHEA sulfate, and androstenedione, have minimal intrinsic androgenic activity, and they contribute to androgenicity by their peripheral conversion to the more potent androgens testosterone and dihydrotestosterone. Although DHEA and DHEA sulfate are secreted in greater quantities,

androstenedione is qualitatively more important, because it is more readily converted peripherally to testosterone [2,3].

1.3. Regulation of synthesis and secretion of corticosteroids

1.3.1. The hypothalamic-pituitary-adrenal (HPA) axis

The major regulator of corticoids and adrenal androgen production is ACTH produced from the pituitary gland, which in turn regulated by the hypothalamus and the central nervous system via neurotransmitters and CRH and arginine vasopressin (AVP), including stress responsiveness of the HPA axis (Fig. 1.3) [6].

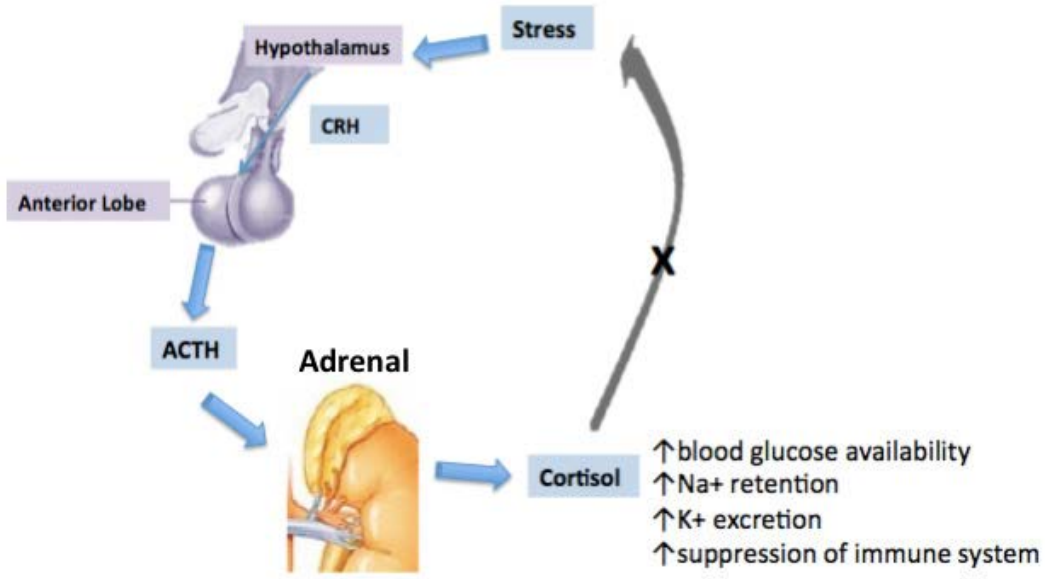


Figure 1.3. Schematic showing the main component of the HPA and its mode of action in the regulation of biosynthesis and secretion of corticosteroids. Adapted from Hine J, Schwell A, Kairys N. (https://www.ncbi.nlm.nih.gov/pubmed/28139270) under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

In addition, feedback inhibition by corticoids at the hypothalamus and pituitary plays a crucial role in ACTH secretion (Fig. 1.3) [6]. In adrenocortical cells, ACTH stimulation induces rapid synthesis and secretion of adrenal steroids. In addition, ACTH increases RNA and protein synthesis of steroidogenic enzymes, as well as StAR [7]. Chronic stimulation by ACTH leads to

adrenocortical hyperplasia and hypertrophy, whereas ACTH deficiency has opposite effects (i.e., decreased steroidogenesis and adrenocortical atrophy).

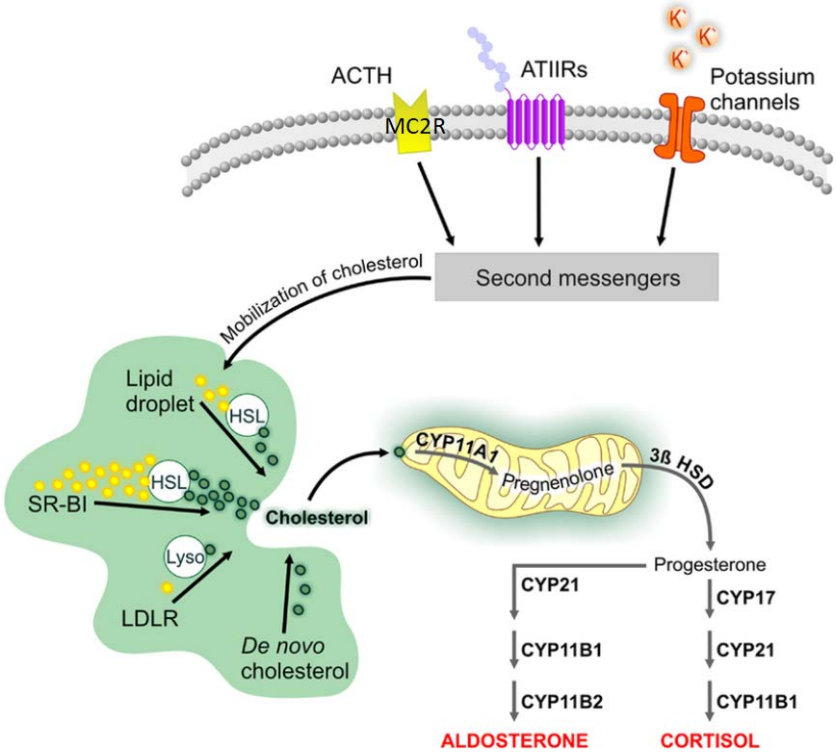


Figure 1.4. Schematic showing characteristic features of steroidogenic (adrenocortical) cells and the mechanism of steroidogenic stimulation leading to corticoids biosynthesis and secretion. Adapted and modified from Martinez-Arguelles DB and Papadopoulos V (2015) Mechanisms mediating environmental chemical-induced endocrine disruption in the adrenal gland. *Front. Endocrinol.* 6:29. doi: 10.3389/fendo.2015.00029 under the terms of the Creative Commons Attribution License (CC BY).

ACTH action on adrenocortical cells is mediated by its binding to cognate receptors, melanocortin-2 receptor (MC2R), a member of the G-protein coupled receptor (GPCR) family present on the plasma membrane (Fig. 1.4). ACTH binding to MC2R leads to the activation of cAMP-PKA signaling pathways and subsequently several phosphoprotein kinases, as well as phosphorylation and acute regulation of StAR [7,8]. ACTH action leads to increased free cholesterol availability for steroidogenesis thorough modulation of proteins involved in intracellular cholesterol homeostasis, such as cholesterol uptake, cholesterol ester hydrolase, and

cholesteryl ester synthase [7,8]. Subsequently, StAR protein transport free cholesterol from the cytoplasm through the outer mitochondrial membrane (OMM); however, its further transport to the IMM, where steroidogenesis begins, remains unclear.

Tropic hormone-induced steroidogenesis in the adrenals and the gonads have been studied extensively. However, our knowledge of the context-dependent potential contributions of cell-intrinsic events/factors (e.g., varying intracellular cholesterol pool) remain limited. New evidence demonstrating the importance of autophagy and lipophagy in steroidogenesis [9-11] in combination with our previous knowledge of cholesterol homeostasis and mitochondrial dynamics in steroidogenic cells [12] have created an opportunity to explore these aspects and attain new insights. For example, preclinical models mimicking congenital adrenal hyperplasia (CAH) phenotype provides an excellent example to investigate cell-intrinsic processes and the physiological needs of cell type-specific steroidogenesis on the varying impacts of the loss-of-function mutation in StAR.

The characteristic features of lipoid CAH are hypertrophied adrenals with enlarged lipid droplets because of high ACTH level and renin activity, and substantially reduced serum levels of steroid hormones [13]. Bose et al [14] proposed a two-hit model to explain the pathophysiology of lipoid CAH. According to this model, the loss of StAR activity due to naturally occurring mutation is the first hit, causing reduced steroidogenesis and consequently an increase in trophic hormones (i.e., ACTH, LH and FSH) [14,15]. Higher trophic hormones lead to increased production of secondary messenger and consequently increased cholesterol uptake and biosynthesis by adrenocortical cells. This imbalance in cholesterol accumulation and utilization leads to the second hit — mitochondrial damage due to lipotoxicity and the loss of residual steroidogenic capacity [14], which explains the phenotypic manifestation in different

steroidogenic cell types/tissues. For instance, earlier manifestation of the loss of StAR function in steroidogenic cells of the fetal testes and after birth in adrenocortical cells correlates with the initiation of steroidogenesis in both tissues, respectively [15]. On the other hand, the fetal ovary does not make steroids and remains unstimulated and therefore remain normal until puberty. Together, this evidence suggests that a coordination between the upstream stimuli and downstream functions are important to maintaining cholesterol homeostasis and mitochondrial functions in steroidogenic cells [16]. Of note, the StAR-knockout mouse model recapitulates the phenotype of StAR deficiency in humans. Thus, the phenotypic manifestation of StAR deficiency precisely correlates with the sensitivity of steroidogenic cells to respective tropic hormones and their steroidogenic activity during different stages in life.

1.4. Steroidogenic cells and steroidogenic mitochondria

A cell is classified as ‘steroidogenic’ if it expresses the enzyme P450 cholesterol side chain cleavage (P450scc, encoded by the gene *Cyp11A1*) and therefore can catalyze the first reaction of steroidogenesis (i.e., the conversion of cholesterol to pregnenolone) [16]. Many cells can transform steroids produced in other cells (e.g., adipocytes), but only cells expressing P450scc are steroidogenic [12]. As P450scc resides in the IMM, the mitochondria of steroidogenic cells can be called ‘steroidogenic mitochondria’ by the same token, because of their distinct and inherent ability to begin steroidogenesis, which is not shared by the mitochondria from non-steroidogenic cells. However, as IMM is cholesterol-poor, there is a prerequisite for steroidogenesis by P450scc, i.e., for a cholesterol import to the mitochondria, which is mediated through the START domain containing protein (e.g., steroidogenic acute regulatory (StAR) protein in steroidogenic adrenocortical and gonadal cells and STARTD3, also known as metastatic lymph node 64 (MLN64) protein, in placental cells [12]. Thus, P450scc is

essential, but not sufficient, to initiate steroidogenesis by itself because of its physical location in the IMM and is assisted by proteins involved in cholesterol transport to the mitochondria. Consequently, unlike many metabolic pathways (e.g., glycolysis, citric acid cycle, fatty acid synthesis), the committed step in steroidogenesis is not an enzymatic reaction (i.e., the P450scc-mediated conversion of cholesterol to pregnenolone, which was previously thought to be one) [16], but rather, cholesterol transport to the mitochondria that is mediated by the StAR protein [12]. In addition to P450scc, 11-hydroxysteroid dehydrogenase and aldosterone synthase in adrenocortical cells localize to the IMM, and 3β-hydroxysteroid dehydrogenase has been reported to be present in the mitochondria [17]. In fact, 3β-HSD was first isolated from the mitochondria [18]. Furthermore, the mitochondria in steroidogenic cells are responsive to the actions of trophic hormones, and steroidogenic cells have evolved a variety of ways to acquire and mobilize cholesterol for the maintenance of basal, acute, and chronic steroidogenesis. Thus, it is a combination of interconnected features spanning different cellular compartments, which define the cell type-specific function of a steroidogenic cell and the steroidogenic mitochondria, including cholesterol handling for steroidogenic need (Fig. 1.4) [16].

1.5. Cholesterol – its importance as a starting substrate and need for cholesterol import to mitochondria

Cholesterol, which is an essential component of all animal cell membranes, has many characteristics. On the one hand, cholesterol plays a critical role in determining the biochemical and biophysical properties of cellular membranes, whereas on the other hand, cleavage of its six-carbon unit side chain from cholesterol molecule (at the beginning of enzymatic step in the steroidogenic pathway) in combination with its oxygenation at certain residues changes its chemical properties and functions [19]. This change in chemical properties dramatically shifts its

biological role from membrane structure-function to cell signaling and transcriptional regulation [19]. Notably, the cholesterol content of the plasma membrane and different subcellular organelle membranes differ substantially. For example, relative to the ER and mitochondria membranes, the cholesterol content of the plasma membrane is approximately 40-fold higher [20]. As cholesterol levels are low in the mitochondrial membranes, cholesterol must be transported to the IMM for the initiation of steroidogenesis. In addition, the OMM and IMM must be supplied with cholesterol for the membrane's biochemical and biophysical characteristics at large. Thus, the mitochondrial membranes in steroidogenic cells are distinct from non-steroidogenic cell types in relation to their dual requirements of cholesterol. First, the very low levels of cholesterol in the IMM allow to control steroidogenesis through the regulation of cholesterol transport to the IMM (including the acute regulation in response to steroidogenic stimulation). Second, because of very low levels of cholesterol contents of the mitochondrial membranes, even small changes can have substantial impact on the biophysical and functional characteristics of the membrane and likely to alert the mitochondria to changes in cholesterol content. Consistent with this notion, many studies have suggested that mitochondrial membrane cholesterol can influence mitochondrial function (independent of steroidogenesis) and may contribute to the pathology of diseases related with mitochondrial abnormalities. However, our current understanding of the mechanisms involved remain unclear. As steroidogenesis is essential to life, it is not surprising that many pathways for cholesterol supply to the steroidogenic mitochondria have been described. Thus, deficiency in one pathway can be taken care by other pathways to ensure normal steroidogenesis. However, our current understanding of cholesterol delivery from the OMM to the IMM in general and its distribution between them when cholesterol levels changes substantially in particular are largely unknown. This scenario

raises an obvious question about steroidogenic cells because in these cells, cholesterol serves as the substrate for the synthesis of all steroid hormones [21]. To overcome such a challenging scenario, the steroidogenic cells have become highly evolved to perform cholesterol uptake, mobilization, and trafficking to the mitochondria to perform steroidogenic functions.

1.5.1. Different sources of cholesterol for steroidogenesis

Cholesterol serves as a precursor substrate for all steroid hormones, including adrenocorticoids and sex steroids. The precursor cholesterol for steroidogenesis is known to come from at least three different sources, including the mobilization of cholesterol from the intracellular lipid droplets (LDs), the uptake of circulating lipoproteins containing cholesterol esters, and the *de novo* synthesis of cholesterol, which have been described extensively in many review articles [22,23]. Plasma lipoproteins are the major source of adrenal cholesterol [24]. For example, low-density lipoprotein (LDL) accounts for about 80% of cholesterol delivered to the adrenal gland for steroidogenesis. Moreover, *de novo* synthesis of cholesterol from acetate does occur in the adrenal glands; however, its contribution (compared with other sources of cholesterol, such as cholesterol uptake by adrenocortical cells) to total steroid hormones produced is relatively less. A readily available pool of free cholesterol in the adrenal exist, which serve the basal and acute production of steroid hormones in response to stimuli. In addition, steroidogenic stimulation increases hydrolysis of stored cholesterol esters to free cholesterol, increase uptake from plasma lipoproteins, and increased cholesterol biosynthesis within the gland. The acute response to a steroidogenic stimulation is mediated by the StAR protein, which facilitate cholesterol transport across outer mitochondrial membrane (OMM) [25]. However, our understanding of the relative contributions of various sources of cholesterol to the different stages of steroidogenesis (i.e., basal, acute, and chronic) in different steroidogenic cell types

remains limited. It is likely that these processes work in a coordinated manner to maintain the physiological needs of different steroid hormones (which vary substantially) in a context-dependent manner [16]. For example, the mobilization of cholesterol from LDs may play a major role in the acute response to trophic hormones, whereas the *de novo* synthesis of cholesterol may be primarily involved in basal and chronic steroidogenesis, whereas the cellular uptake of cholesterol in replenishing the depleted, readily available pool due to the acute response, and in the maintenance of a chronic response. For the same reason, the instantaneous contribution of cellular uptake and the *de novo* synthesis of cholesterol for acute steroid production in response to trophic hormones is expected to be minimal [16,26].

Furthermore, the steroid hormones are not stored in secretory vesicles like peptide hormones, but released into the blood upon their biosynthesis [27]. This instant set-up between the biosynthesis and release of steroid hormones is expected to require an arrangement to maintain the readily available cholesterol pool within steroidogenic cells, because the cholesterol contents of mitochondrial membranes, especially the IMM, where steroidogenesis begins, is insufficient to support steroidogenesis [27]. Emerging evidence suggest that mitochondrial dynamics, autophagy, and related lipophagy play crucial roles in intracellular cholesterol uptake and in the maintenance of cholesterol homeostasis in steroidogenic cells, and consequently support steroid hormone production to maintain physiological functions (Fig. 1.5).

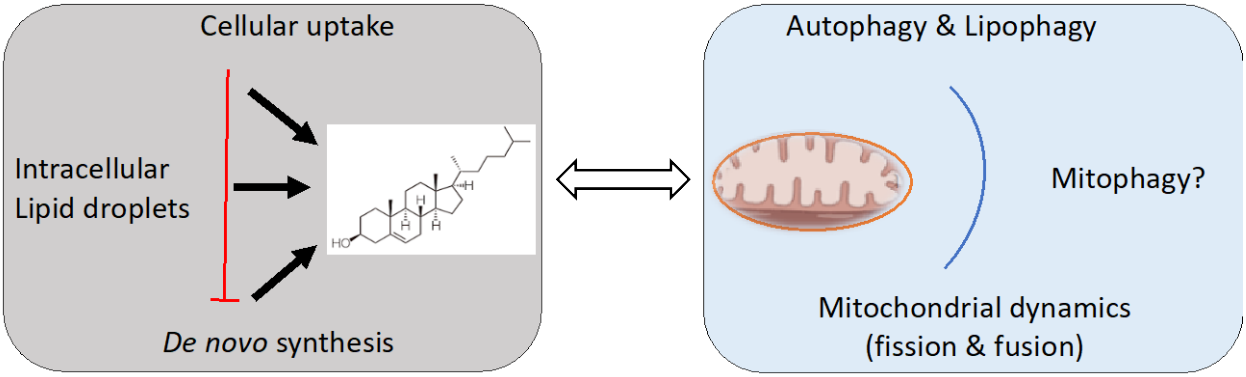


Figure 1.5. Schematic showing cell-intrinsic events involved in the instant synthesis and secretion of steroid hormones, as well as in the maintenance of cholesterol homeostasis in a steroidogenic cell. Modified from Bassi et al. *Cells*, 2021 under the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1.5.2. Heterogeneity in membrane cholesterol distribution and cellular compartmentalization of steroidogenesis

Cholesterol constitutes approximately 30–40% of total cellular lipids, and nearly 60-80% of total cellular cholesterol is present in the plasma membranes [28]. In addition to the plasma membranes, cholesterol concentrations are high in the Golgi apparatus. In contrast, cholesterol concentrations are very poor in the endoplasmic reticulum and mitochondrial membranes. Thus, cholesterol concentration is highly heterogeneous in cellular membranes, ranging from 0.5-1% in the SER to 50-60% in the plasma membranes, and 0.1% to 0.2% in the IMM and OMM, respectively [28]. In cellular membranes, cholesterol influences many biophysical and biochemical aspects of membranes and membrane protein functions. Such heterogeneity in cholesterol composition in different subcellular membranes (e.g. ER and PM) and within organelle membranes (e.g. IMM and OMM) would imply that cholesterol distribution and homeostasis must be carefully regulated in relation to membrane-specific functions. As IMM, where steroidogenesis begins, is cholesterol-poor in comparison to other subcellular membranes and compartments, it is predictable that the delivery of cholesterol to the IMM may serve as a critical regulatory step for steroidogenesis. As expected, many studies have established that this is indeed the case [29-31]. Moreover, the low basal levels of cholesterol in mitochondrial membranes make the mitochondria sensitive to changes in cholesterol content, which can have a substantial impact on various non-steroidogenic functions of the mitochondria, as IMM is considered to be the most protein-enriched membrane in the cell. This would require a strict regulation of cholesterol handling in steroidogenic cells to protect mitochondria and

mitochondria-mediated non-steroidogenic vital cellular functions. Thus, it is possible that the growing number of mitochondrial attributes and other cellular events (i.e. autophagy, lipophagy and mitochondrial dynamics) that are linked with cholesterol mobilization and trafficking in steroidogenic cells provide some flexibility to carry on steroidogenic and non-steroidogenic functions successfully under varying conditions and meet the diverse physiological needs of different steroid hormones [16,26]. Thus, a possibility exists that autophagy, lipophagy and mitochondrial dynamics that have been implicated in supporting steroidogenesis protect the steroidogenic mitochondria from the potentially damaging effects of cholesterol, remove damaged mitochondria, and recycle biomolecules, including the maintenance of cholesterol homeostasis [16,26]. It is likely that blocking or interfering with these protective mechanisms would lead to mitochondrial dysregulation and impaired steroidogenesis. Thus, an execution of the cell type-specific function of mitochondria (e.g., steroidogenesis) would necessitate a protection of the cell-neutral functions of mitochondria in an integrated fashion [16,26]. Consistent with this notion, it is important to note that these activities (i.e., autophagy, lipophagy, mitochondrial dynamics), which have been implicated in steroidogenesis, are also operative in non-steroidogenic cells with cell type-specific mitochondrial functions, such as brown adipocytes, cardiomyocytes, hepatocytes [32-34]. In addition, the compartmentalization of intracellular cholesterol in lipid droplets (LDs) in steroidogenic cells may not only provide a readily available pool for steroidogenesis, but also allows its portioning from the structural membrane cholesterol, and in protecting the functional integrity of mitochondria and other organelles. Notably, once cholesterol is imported into the mitochondria, the steroidogenic process proceeds uninterrupted. Thus, the mitochondria may be seen as a portioning point between the processes involved in maintaining the readily available pool of precursor substrate

cholesterol and the multi-enzymatic steps of steroidogenesis in the IMM and SER. This arrangement makes sense and may have evolved to protect both cell-specific and cell-neutral mitochondrial functions from the potential disruptive effects of high cholesterol levels in steroidogenic cells [16,26]. For example, increased mitochondrial cholesterol levels have been reported to decrease membrane fluidity [32-34], which can affect the function of mitochondrial membrane proteins [35], including different transporters for metabolites [36,37]. In addition, functional changes associated with increased mitochondrial cholesterol are similar to the effect of lipotoxicity in many cell types, including increased reactive oxygen species production and a pro-oxidative environment [38-40], increased opposition to mitochondrial membrane permeabilization, and decreased oxidative phosphorylation [41,42]. Thus, steroidogenesis involved a coordination between different cellular compartments. As such, steroidogenesis may be conceptualized as a compartmentalized process that involves a fine coordination of events in the different cellular compartments (e.g., the cytoplasm, mitochondria, and SER) to operate the cell-specific needs of the steroidogenic mitochondria without affecting the mitochondria's cell-neutral global functions.

Furthermore, despite the cholesterol-centric arrangement of steroidogenic cells, whether cholesterol in itself plays a role in the regulation of steroidogenic events is unexplored. In addition, the precise relationship between trophic hormone-induced steroidogenic events under a varying intracellular cholesterol pool (e.g., sufficiency and insufficiency) is largely unknown. It is likely that the trophic hormone-induced cell-intrinsic steroidogenic events might vary substantially depending on the available intracellular pool of steroidogenic cholesterol. In other words, a pre-existing cholesterol pool might modify trophic hormone-induced steroidogenic events, and thus, both could modulate each other in a context-dependent manner. Moreover, the

intracellular cholesterol pool might play a role in basal steroidogenesis, which is currently unexplored. This gap in knowledge is likely due to inherent challenges associated with it because of the integral nature of cholesterol in steroidogenesis, as well as being a critical molecule for cellular membranes. For example, perturbing the cholesterol availability using genetic and chemical approaches may not be helpful in deciphering the steroidogenesis-specific function of cholesterol. Moreover, blocking cholesterol transport to the mitochondria or its subsequent utilization for steroidogenesis has its own challenges and pathological consequences, which are well-documented, including within the *StAR* and *Cyp11A1* knockout mouse models [43,44] and in the loss of function mutations in the *StAR* gene in humans [45,46].

1.6. Autophagy in steroidogenesis

Autophagy is an evolutionarily conserved process in cell physiology, from organisms such as yeasts to mammals, which raises the question of whether autophagy's steroidogenic role, which has been reported in many mammalian species, is also involved in other species. Recently, Texada et al [47] have shown that autophagy plays a role in the mobilization of stored precursor cholesterol and its subsequent trafficking in relation to ecdysone production in Drosophila. It was found that autophagosomes gather and transport cholesterol substrate for steroidogenesis. Thus, the results from the study by Texada et al. [47] suggest that autophagy controls the steroidogenic process by rallying LD-derived cholesterol to supply the precursor substrate for steroidogenesis, indicating a link between new evidence related to autophagy and a well-established event involved in maintaining cholesterol homeostasis in steroidogenic cells. The interaction of the autophagosome-mediated cholesterol-trafficking with the endosome and lysosome system supports the idea of this role, since the endocytic trafficking of cholesterol is a delivery route for steroidogenesis [48]. Thus, the cell-intrinsic events in steroidogenic cells that converts

cholesterol and its intermediates into steroids might be a conserved mechanism, which requires further investigations.

1.7. Mitochondrial dynamics in steroidogenesis

The initiation of the biosynthesis of steroid hormones occur in the mitochondria, which are known to undergo dynamic changes called mitochondrial fission and fusion [49]. Therefore, it is likely that the cellular and molecular changes in mitochondria would influence steroidogenesis. Indeed, many studies have shown that mitochondrial dynamic changes are closely associated with the biosynthesis of steroid hormones in steroidogenic cells [50-52]. For example, cAMP-induced steroid hormone production has been reported to be accompanied by increased mitochondrial mass, specifically an increase in mitochondrial fusion, whereas a reduction in mitochondrial fission [49]. Among the mitochondrial-proteins that are involved in shaping the mitochondria, dynamin-related protein 1 (Drp1) level was found altered in response to cAMP stimulation. Particularly, an increase in the phosphorylation of Drp1 at Ser^{637} correlated with steroid hormone production in the primary adult rat Leydig cells and in a model murine cell line of Leydig cells. In addition, gonadotropin administration was found to alter the status of Drp1 phosphorylation in the Leydig cells isolated from immature rat testes [49]. Overall, mitochondrial dynamics at large were found to be directly linked to steroidogenesis, and Drp1 was found to play an important regulatory role during steroidogenesis [49]. Thus, cAMP-PKA pathway, which plays a central role in the Leydig cell steroidogenesis is also involved in the regulation of mitochondrial dynamics to facilitate steroidogenesis. Moreover, hypogonadism was found to affect mitofusin-1 (Mfn1) and mitofusin-2 (Mfn2) in the Leydig cells by reducing the transcription of Drp1, as well as Mfn1 and Mfn2, without changing protein optic atrophy 1

(Opa1) levels [49]. Collectively, this evidence suggests a relationship between trophic hormone-induced changes in mitochondrial dynamics and steroidogenesis.

1.8. Prohibitins

PHB1 is a hallmark protein of the IMM, which is reported to be involved in various aspects of mitochondrial biology, including mitochondrial dynamics [53]. PHB1 and its homologous protein PHB2 form large protein and lipid scaffolds in the IMM that are required for structural and functional integrity of the mitochondria [54]. Both PHBs belong to a group of proteins family, the PHB domain family, which are thought to function as lipid and protein scaffolds in the IMM that affect the lateral distribution of the membrane lipid and protein components [55,56]. PHBs form hetero-oligomeric mega complexes composed of multiple PHB1 and PHB2 subunits [57]. In mitochondria, the PHB complex interacts with the m-AAA and other proteases, which acts as a quality control enzyme with important regulatory functions in the IMM [58]. Moreover, the PHB family member protein SLP2 (stomatin-like protein-2) anchors a proteolytic hub in mitochondria containing PARL and the i-AAA protease YME1L [59,60], which are known to play a role in mitochondrial dynamics and autophagy/mitophagy. Thus, PHBs may affect mitochondrial activity in steroidogenic cells by modulating the turnover of a short-lived regulatory protein by the m-AAA pro-tease, such as the acute regulation of StAR during steroidogenesis. In addition, PHBs may play a role in the regulation of autophagy and lipophagy because both proteins contain LC3 binding motifs and interact with each other [61], and highly expressed in steroidogenic cells/tissues [62]. Because PHB1 has been implicated in lipid metabolism and homeostasis across species [63,64], including in mitochondrial phospholipids [56,65], and in autophagy/mitochondrial proteases [61], it is conceivable that PHB1 might be involved in steroid biosynthesis via lipid/cholesterol homeostasis across

steroidogenic cells/tissue types. Moreover, during autophagy, LC3-I is conjugated into phosphatidylethanolamine (PE) to form the LC3-PE conjugate, which is then tightly bound to the autophagosomal membranes [66]. Notably, PHB1 has relationship with both as it serves as a binding site for LC3 and is involved in PE synthesis [56,61].

1.8.1. The relationship between PHB family proteins and cholesterol

In addition to mitochondrial biology and autophagy, the PHB family member proteins Erlin-1 and Erlin-2 are shown to be highly enriched in the detergent-soluble, buoyant fraction of sucrose gradients in a cholesterol-dependent manner [67]. However, unlike other PHB family members (which localize to the mitochondria), these two proteins are localized to the ER membrane. In addition to membrane localization, a common feature reported on the PHB family of proteins is that they undergo post-translational modification by palmitoylation, which occur at a conserved cysteine residue in proximity of membrane targeting sequences [68,69]. For example, the lone cysteine in PHB1 at 69 position has been shown to undergo palmitoylation, which appears to be involved in its membrane anchoring and cell signaling function [69]. Moreover, in a separate study [70], it was found that PHB1 is a cholesterol-sensitive gene, and its expression levels increases when cholesterol levels are low. In addition, the authors showed that the PHB1 gene promoter contains regulatory elements that responds to cholesterol insufficiency [70].

Moreover, the PHB family member protein MEC-2 and podocin have both been found to bind cholesterol to regulate the activity of associated ion channels [68]. This binding requires the PHB domain, including a conserved palmitoylation site (a cysteine residue) within it and a part of the N-terminal hydrophobic domain that attaches the proteins to the cytosolic side of the plasma membrane [68]. By binding to MEC-2 and podocin, cholesterol associates with ion-

channel complexes to which these proteins bind [68]. Thus, MEC-2, podocin, and likely many other PHB-domain proteins regulate the formation and function of large protein–cholesterol super complexes in the plasma membrane by forming multimeric complex among themselves, cholesterol, and different target proteins. Moreover, in mitochondria, PHB1 is anchored to the IMM, and forms complexes with the group of proteases known as ATPases (m-AAA), which are associated with diverse cellular activities [58].

1.8.2. Prohibitins in steroidogenic tissues and steroidogenesis

PHB1 has been reported to play a role in granulosa cells [71]. However, the focus of these studies was on granulosa cell proliferation, differentiation, survival, and apoptosis rather than steroidogenesis [72-75], likely because of existing knowledge of PHB1's context-dependent role in cell proliferation, survival, and apoptosis in different cell types. For example, Choudhury et al [73] have reported that the administration of equine chorionic gonadotropin (eCG) increases PHB1 expression in the ovarian follicles and GC, but not in theca-interstitial cells within the pre-antral follicles. This increased expression of PHB1 corresponded with follicular growth, and decreased after the ovulatory luteinizing hormone (LH) surge and during follicular atresia. This finding would imply that the LH surge during the ovarian cycle may negatively regulate PHB1 expression. Moreover, a change in the phosphorylation levels of PHB1 and increased trafficking to the mitochondria was observed. Notably, the PHB1 phosphorylation sites under these culture conditions in response to FSH and testosterone were the Tyr^{249}, Thr^{258} and Tyr^{259} sites [73], which we have reported in relation to insulin signaling and lipid binding/metabolism [69,76-80]. Thus, a possibility exists that the phosphorylation of PHB1 may play a role in steroidogenic cells in response to hormones and growth factors (e.g. trophic hormones, insulin, IGF and EGF),

which are known to stimulate steroidogenesis. In summary, PHB1 possesses many features that may potentially contribute to steroidogenesis (Fig. 1.6).

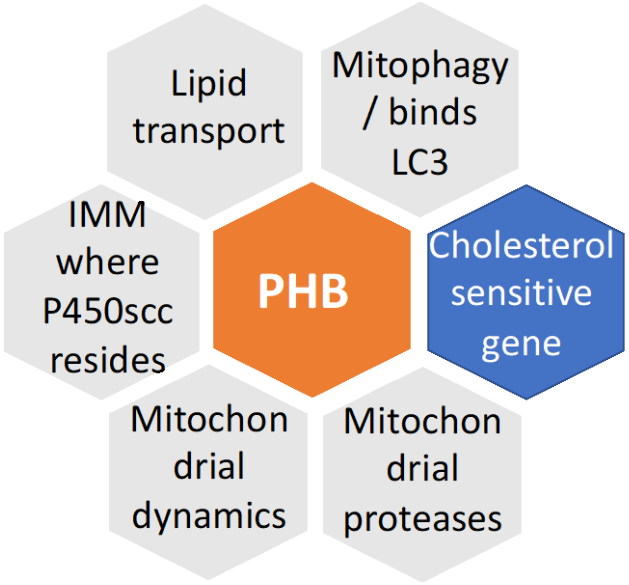


Figure 1.6. Schematic showing various attributes of PHB1 that may potentially contribute to steroidogenesis. Modified from Bassi et al. *Cells*, 2021 the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1.8.3. PHB1 — a novel player in steroidogenic mitochondria

Our laboratory has been interested in understanding the roles and regulation of PHB1, including its cell type-specific functions, such as adipocytes and pancreatic beta cells [79,80]. Recently, using a transgenic mouse model of PHB1 (by the fatty acid binding protein-4 gene promoter (which is primarily expressed in adipocytes and has been used successfully for gene targeting in adipocyte by others): *Fabp4*-PHB1-Tg) our laboratory has shown that PHB1 plays a crucial role in lipid homeostasis, which is mediated through its mitochondrial functions [64]. Subsequent studies revealed that the male PHB1 transgenic mice exhibit significantly higher testosterone levels. [81]. Further investigation revealed that it was due to overexpression of PHB1 in Leydig cells, which is consistent with a report that *Fabp4* expressed in Leydig cells [82]. The role of PHB1 in Leydig cell steroidogenesis is further confirmed using PHB1

manipulated cells [81]. Collectively, these evidences are suggestive of an important role of PHB1 in lipid processing, including steroidogenesis in the gonads. However, a potential role of PHB1 in corticosteroidogenesis is not explored, or at least have not been reported. Of note, a search of Human Protein Atlas [62] for PHB1 expression levels in different tissues informed that PHB1 is abundantly expressed in steroidogenic cells, with highest expression in adrenal cortex indicating a potential role in corticosteroidogenesis. However, virtually nothing is known about the role of PHB1 in adrenal steroidogenesis in the current literature. As the basic framework of pituitary-derived tropic hormones (i.e. ACTH, FSH and LH)-induced steroidogenesis in different steroidogenic tissue is very similar, including early cell signalling events leading to cholesterol transport to mitochondria, cholesterol side chain cleavage, and initiation of steroidogenic process, it is conceivable that PHB1 might play a role in ACTH-induced steroidogenesis in adrenocortical cells.

CHAPTER 2

Hypothesis and Objectives

2.1. Hypothesis

The PHB family of proteins interact with diverse group of lipid molecules (e.g., mitochondrial phospholipids, long chain fatty acids, and cholesterol) and have been implicated in various aspects of lipid biology, including lipid scaffolding, transport and homeostasis [83]. For example, overexpression of PHB1 in adipocytes leads to increase in adipose tissue mass due to mitochondrial biogenesis and adipogenesis [64] whereas its knockdown leads to decreased adipose tissue mass [84]. Subsequent studies in PHB1 transgenic mouse model revealed PHB1 as a target gene/protein for luteinizing hormone and led to the discovery of PHB1's role in cholesterol homeostasis and testosterone production by Leydig cells [81]. This discovery raised speculation about a potential role of PHB1 in steroidogenesis in adrenocortical cells leading to the present investigation into the role of PHB1 in ACTH-induced corticosteroidogenesis. In addition, growing evidence of cell-intrinsic events (e.g., autophagy/lipophagy, mitochondrial dynamics) in the regulation of cholesterol homeostasis in steroidogenic cells prompted me to explore their relationship with intracellular cholesterol pool and consequently in corticosteroidogenesis. The hypothesis of the thesis is that **PHB1 and the intracellular cholesterol pool in adrenocortical cells play a role in corticosteroidogenesis**.

2.2. Specific Aims

1) The first aim is to examine the role of PHB1 in ACTH-induced corticosteroidogenesis in adrenocortical cells.
2) The second aim is to explore the role of the intracellular cholesterol pool in the regulation of cellular events and factors involved in corticosteroidogenesis in adrenocortical cells.

CHAPTER 3

Materials and Methods

3.1. Materials

Table 3.1: Reagents used in the experiments and their sources.

Reagents	**Sources (Catalogue #)**
Y-1 cells	ATCC, Manassas, VA (CCL-79)
F12-K	Thermo Fischer Canada
Horse serum	Thermo Fischer Canada
Penicillin and streptomycin mix	Thermo Fischer Canada
Fetal bovine serum	Thermo Fischer Canada
HAC15 cells	ATCC, Manassas, VA (CRL-3301)
Insulin, human transferrin, and selenous acid (ITS) premix	Beckton Dickinson (354352)
F12-Ham (DMEM/F12)	Thermo Fischer Canada
ACTH	Sigma-Aldrich, Oakville, ON
Db-cAMP	Sigma-Aldrich, Oakville, ON
Lipoprotein depleted FBS	KALEN Biochemical, Germantown, MD
All antibodies (except SR-B1)	Cell Signaling Technology, Danvers, MA
SR-B1 antibody	Abcam, Cambridge, UK
pCMV6-XL5 vector	Origene Technology
shRNAs (shControl and shPHB1)	Dharmacon Inc.
X-tremeGENE HP DNA transfection reagent	Sigma-Aldrich, Oakville, ON
Amplex^{TM} Red cholesterol assay kit	Thermo Fischer Canada
Corticosterone DRG ELISA assay kit	Cedarlane, Burlington, ON
MTT(3-(4,5-dimethylthiazol-2-yl)-2,5 di-phenyltetrazolium bromide	Sigma-Aldrich, Oakville, ON

3.2. Methods

3.2.1. Cell culture – Human and murine adrenocortical cells

The murine adrenocortical Y-1 cells were cultured in ATCC recommended F12-K media supplemented with FBS to a final concentration of 2.5%, horse serum to a final concentration of 15%, and 1% penicillin and streptomycin mix at 37° C in 5% CO_2 atmosphere.

The human adrenocortical HAC15 cells were grown in ATCC recommended F12-Ham (DMEM/F12) culture media supplemented with 50 ml FBS and 5 mL ITS + Premix added in 500 ml media bottle and 1% penicillin and streptomycin mix at 37° C in 5% CO_2 atmosphere. Cells were plated overnight in 6-well plate at a density of 0.3 x 10^6/well. 6-8 hours before the treatment, growth media was replaced with serum free media. Cells were then treated with ACTH [15 nM] or dibutyryl cAMP (db-cAMP) [0.5 mM] for 2 hours or various time points, as indicated/applicable. The dose and time of treatment were determined from previous studies [85-87].

3.2.2. Cell transfection

The pCMV6-XL5 vector containing the human PHB1 clone was purchased from Origene Technologies and the cloning of the m-PHB1 cDNA construct has been described previously [77-79]. Glycerol stocks of StAR shRNA and scrambled control shRNA (shControl) were purchased from Dharmacon Inc. The bacterial transformation, culture, and plasmid preparation were performed as described previously pre(pre) or following the manufacturer's protocol. Cell transfection was performed using the X-tremeGENE HP DNA transfection reagent according to the manufacturer's protocol.

3.2.3. Depletion of intracellular cholesterol pool in Y-1 and HAC15 cells

For this, each cell type was washed 3-times with PBS and then cultured in cholesterol-depleted (CD) cell culture conditions (i.e., respective cell type-specific culture medium supplemented with lipoprotein depleted FBS containing only 0.04 mg/ml cholesterol (instead of normal FBS, which contains around 1.40 mg/ml cholesterol) [26]. An approximately 35-fold reduction in cholesterol content in lipoprotein depleted FBS compared with normal FBS. Cells were kept in CD culture condition for different time points (i.e., 6h, 12h and 24h). Cells in control experimental group in each case was continuously grown under normal condition (i.e. supplemented with normal FBS) [26].

3.2.4. MTT reduction assay

The effect of CD on cell viability was enumerated by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay [26]. For this, cells were seeded at a plating density of 3×10^3/well and cultured for 24h to allow them to adhere to the plate. The culture medium was then changed to CD condition and then cells were allowed to grow for another 24h (i.e. maximum time point used in CD experimental group). Cells in control group (NC condition) were continuously grown under normal culture condition (i.e. cell type-specific). At the end 10µg/100µl MTT was added and incubation continued for 3 h. Subsequently, the tetrazolium products were solubilized in acidic isopropanol and OD was read at 570 nm.

3.2.5. Cell lysate preparation and Western blotting

At each time point, cells were washed with ice-cold PBS and lysed in RIPA cell lysis buffer supplemented with proteinase and phosphatase inhibitor cocktails. After lysis, cell lysates

were kept at 4 °C for 30 min, vortexed intermittently every 5 min and then centrifuged at 4 °C for 10 min at 12000 x *g*. The supernatant was transferred into a new Eppendorf tube for subsequent use. The total protein concentration for all lysates was measured by Bradford protein assay method using bovine serum albumin as a standard [88]. Next, protein samples (20 µg each) were loaded for electrophoresis on 12% mini gel by standard SDS-PAGE procedures and electro transferred to polyvinylidene difluoride (PVDF) membranes by wet transfer method [26]. Then, the blots were blocked with 5% nonfat dry milk in TBS, 0.1% Tween-20 for 1h followed by incubation with respective primary antibodies overnight at 4 ºC with gentle shaking. The membranes were washed in TBST for 3 x 10 min, and incubated with respective secondary antibody conjugated with horseradish peroxidase for 1h at room temperature. Immunoreactivity of proteins were detected by chemiluminescence with Western blotting luminol reagent, and the images were captured with a ChemiDoc system (Bio-Rad Laboratories).

3.2.6. Transmission electron microscopy (TEM)

The TEM analysis of different steroidogenic cell lines cultured under different experimental conditions was performed using a Philips CM10 at 80 kV at the Histomorphology & Ultrastructural Imaging Platform, in the Department of Anatomy and Cell Sciences, at the Faculty of Health Sciences, University of Manitoba, as described previously [26,81]. Briefly, cells were trypsinised using 0.25% trypsin, followed by centrifugation at 1100 rpm for 5 min. Cell pellets were fixed with 3% glutaraldehyde in 0.1 M Sorensen's buffer for 3 hours. After fixation, the cells were resuspended in 5% sucrose in 0.1 M Sorensen's buffer and then embedded in EPON^{TM} resin. TEM analysis was performed on ultra-thin sections (100 nm), stained with uranyl acetate and counterstained with lead citrate [26,81].

3.2.7. Quantifications

Quantification of protein band densities in immunoblots, as well as lipid droplets and mitochondrial numbers in the transmission electron micrographs of adrenocortical cells were performed using ImageJ software (https://imagej.nih.gov/ij/) [26,89].

3.2.8. Amplex™ Red cholesterol assay

The cholesterol levels in HAC15 and Y-1 cells grown under normal and cholesterol depleted culture conditions were measured using an enzyme-coupled Amplex™ Red cholesterol assay kit as per the manufacturer's instructions [26]. This assay kit provides a simple fluorometric method for the sensitive quantitation of cholesterol using a fluorescence microplate reader or fluorimeter.

3.2.9. Measurement of corticosterone levels

Corticosterone levels in Y-1 total cells were measured using DRG ELISA kit (Catalogue # EIA-4164) with inter- and intra-assay co-efficient variation (%CV) of 5.5–6.3 and 2.4–4.0, respectively. The assay was performed according to the manufacturer's instructions.

3.2.10. Statistical analysis

GraphPad Prism 6.2 software was used for statistical analysis in all experiments. For comparisons between two groups, a two-tailed student's t test was performed. An analysis of variance (ANOVA) with Dunnett test was performed to compare every mean to a control mean whereas Tukey test was performed and for multiple comparisons [90]. A P value of < 0.05 was considered statistically significant in all cases. The graphs represent means, and error bars

indicate the standard error of the mean (SEM). All experiments were repeated for at least 3 times and p values are reported in the respective figure legends or indicated in figures.

CHAPTER 4

Results

Sections of this chapter have been published in *The Journal of Steroid Biochemistry & Molecular Biology* titled “The intracellular cholesterol pool in steroidogenic cells plays a role in basal steroidogenesis. 2022 Mar 24; 220: 106099.

In this article, I performed the majority of the experiments related to adrenocortical cells.

4.1. PHB1 is an ACTH-regulated protein in adrenocortical cells

Recently, our laboratory has reported that PHB1 is a LH regulated protein in testicular steroidogenic cells and plays a role in steroidogenesis [81]. This finding prompted me to investigate whether PHB1 is a target gene for ACTH in adrenocortical cells, as ACTH plays a central role in almost every aspect of corticosteroidogenesis [91], like LH in Leydig cells [92], and the basic framework of steroidogenesis is similar across steroidogenic cell types [16]. The stimulation of human HAC15 and mouse Y-1 adrenocortical cells with ACTH led to the upregulation of PHB1 protein levels in a time-dependent manner, especially an acute upregulation was observed in both cell lines (Fig. 4.1A, C). To further confirm acute regulation of PHB1 protein in adrenocortical cells, I repeated the experiment with dibutyryl cyclic-AMP (db-cAMP or cAMP), a cell permeable analogue of cAMP, which mimics ACTH action in corticosteroidogenesis [93]. A similar and sustained increase in PHB1 levels was observed in response to db-cAMP in both adrenocortical cell lines (Fig. 4.1B, D). Thus, in my subsequent experiments, I used only db-cAMP (when reasonable) for consistency and to avoid batch variation in full-length ACTH preparations. My finding of acute regulation of PHB1 during first few hours of ACTH or db-cAMP stimulation led me to investigate whether PHB1 is regulated at the translational level in adrenocortical cells. For this, I stimulated HAC15 and Y-1 cells with db-cAMP in the presence of cycloheximide (CHX), a protein synthesis inhibitor [94]. A reduction in the expression level of PHB1 was observed (Fig. 4.1E, F), confirming that PHB1 is regulated at the translational level in adrenocortical cells. Collectively, this data suggests that PHB1 is an ACTH-regulated protein in adrenocortical cells, which is acutely regulated in response to steroidogenic stimulation.

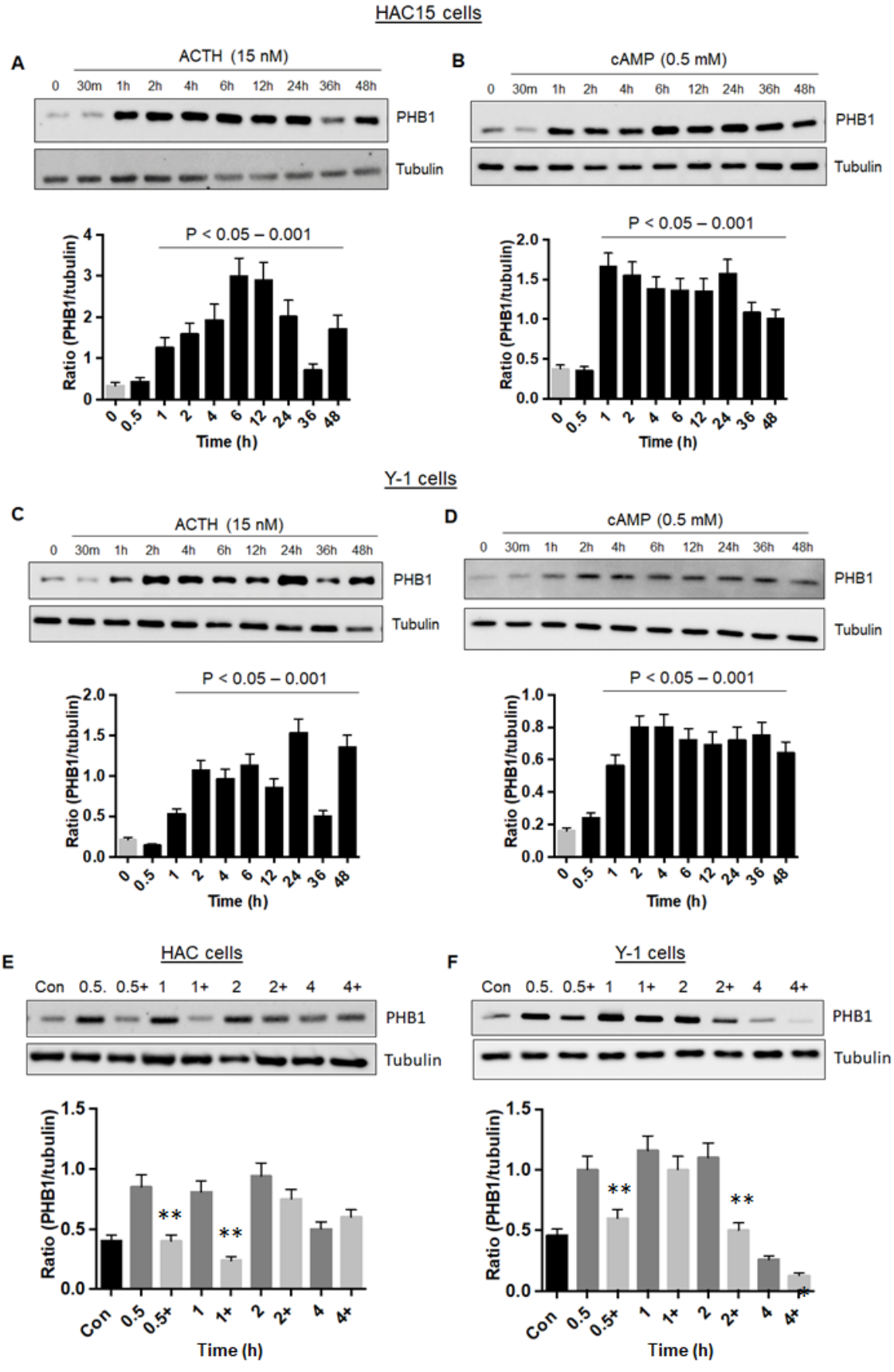

HAC15 cells
A
ACTH (15 nM)
0 30m 1h 2h 4h 6h 12h 24h 36h 48h
PHB1
Tubulin
P < 0.05 – 0.001
Ratio (PHB1/tubulin)
Time (h)
B
cAMP (0.5 mM)
0 30m 1h 2h 4h 6h 12h 24h 36h 48h
PHB1
Tubulin
P < 0.05 – 0.001
Ratio (PHB1/tubulin)
Time (h)
Y-1 cells
C
ACTH (15 nM)
0 30m 1h 2h 4h 6h 12h 24h 36h 48h
PHB1
Tubulin
P < 0.05 – 0.001
Ratio (PHB1/tubulin)
Time (h)
D
cAMP (0.5 mM)
0 30m 1h 2h 4h 6h 12h 24h 36h 48h
PHB1
Tubulin
P < 0.05 – 0.001
Ratio (PHB1/tubulin)
Time (h)
E
HAC cells
Con 0.5. 0.5+ 1 1+ 2 2+ 4 4+
PHB1
Tubulin
Ratio (PHB1/tubulin)
Time (h)
F
Y-1 cells
Con 0.5. 0.5+ 1 1+ 2 2+ 4 4+
PHB1
Tubulin
Ratio (PHB1/tubulin)
Time (h)

Figure 4.1. PHB1 is an ACTH-regulated protein in adrenocortical cells
(**A-D**) Representative immunoblots showing time-dependent effect of ACTH (**A** and **C**) or db-cAMP (**B** and **D**) on PHB1 expression levels in HAC15 and Y-1 adrenocortical cells. Tubulin blot is shown as a loading control. Quantification of band intensities are shown with histograms (lower panel).
Data are presented as mean ± SEM (n=3).
(**E** and **F**) Representative immunoblots showing the effect of cycloheximide (CHX) treatment on ACTH-induced changes in PHB1 levels in HAC15 and Y-1 adrenocortical cells.
*p <0.05, **p < 0.01 between ACTH and ACTH + CHX.

4.2. Manipulation of PHB1 levels in adrenocortical cells affects mitochondrial, lysosomes and lipid droplet characteristics

There is much evidence in previous literature suggesting that PHB1 plays a wide-ranging and interconnected role in mitochondrial biology and lipid homeostasis [53-61]. For example, our laboratory has shown that transgenic mice overexpressing PHB1 in adipocytes display increase in adipose tissue mass [64], whereas PHB1 knockdown in adipocytes was found to have opposite effect on adipose tissue mass [84]. Moreover, PHB1 has been reported to play a role in mitochondrial phospho-lipid homeostasis in different model organisms [83]. Furthermore, the PHB1 family member MEC-2 binds cholesterol in relation to membrane signaling and functions [68], whereas Erlin-1 and Erlin-2 are highly enriched in the detergent-soluble ER fraction in a cholesterol-dependent manner [67]. Moreover, our laboratory has recently found that PHB1 overexpressing Leydig cells display altered cholesterol handing and homeostasis [81]. Thus, a possibility exists that PHB1 may influence mitochondrial biology and cholesterol homeostasis within adrenocortical cells. Therefore, I analyzed adrenocortical cells after manipulating PHB1 levels using transmission electron microscopy (TEM) after validation of PHB1 overexpression and knockdown in adrenocortical cells (Fig. 4.2).

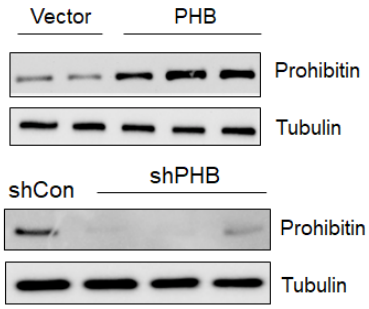


Figure 4.2. Representative immunoblots showing validation of PHB1 overexpression and knockdown in Y-1 adrenocortical cells. Tubulin blots are shown as loading control (n = 3).

An increase in mitochondrial numbers ($p < 0.05$) and a parallel decrease in lysosome numbers ($p < 0.01$) were observed in adrenocortical cells overexpressing PHB1 compared with vector transfected control cells (Fig. 4.3). Interestingly, shRNA-mediated PHB1 knockdown led to increase in lipid droplet number and size ($p < 0.01$), as well a reduction in mitochondrial number ($p < 0.05$) and structural abnormality (e.g., poor mitochondrial cristae organization) (Fig. 4.4). However, no difference was found in lysosome number and size (not shown). In addition, a sign of autophagy/lipophagy (e.g., autophagosome / lipophagosome) were apparent in adrenocortical cells after PHB1 knockdown (Fig. 4.3). Taken together, this data indicates a role of PHB1 in lipid/cholesterol homeostasis and processing, and potentially in the regulation mitochondrial biology in adrenocortical cells.

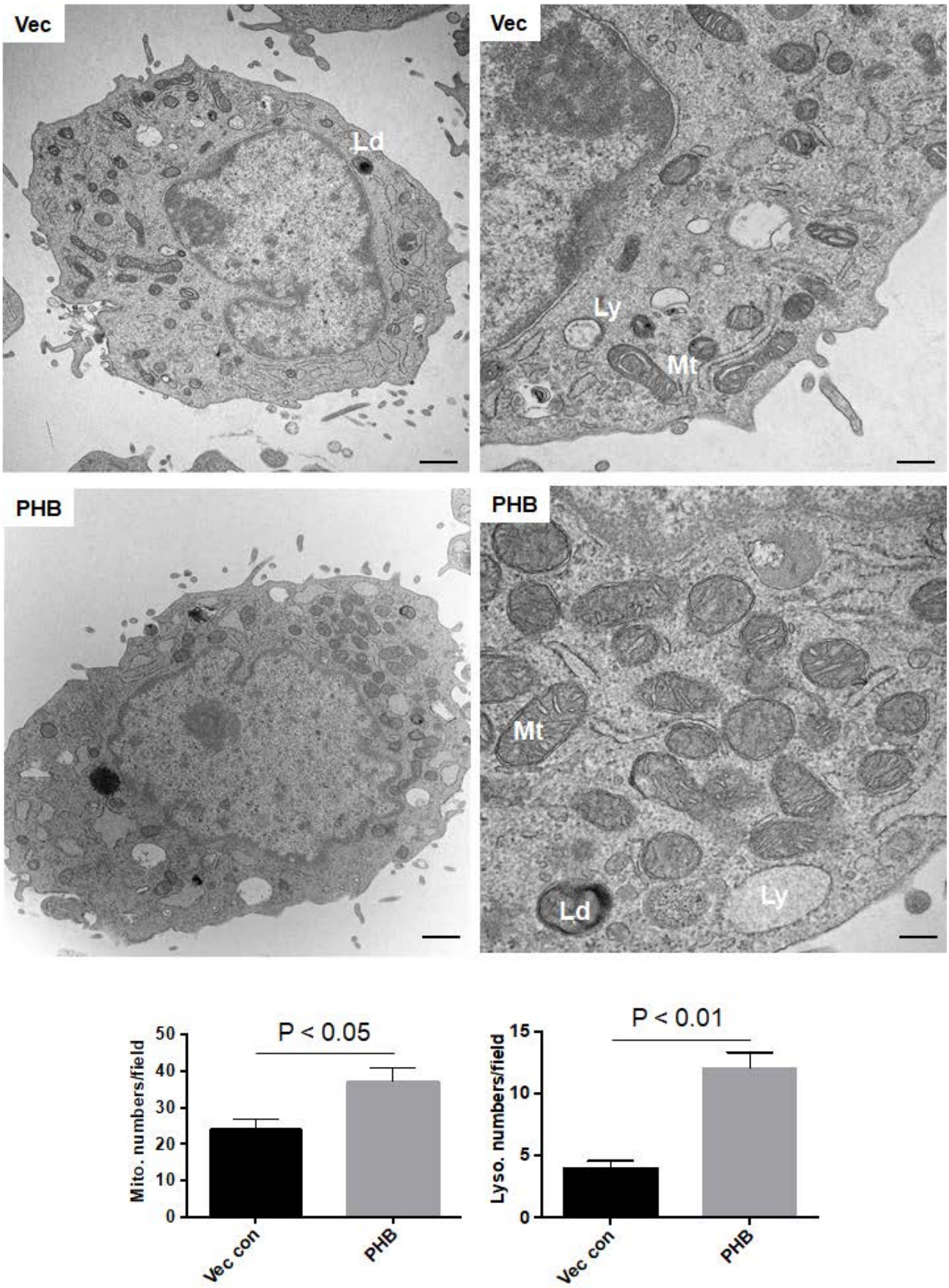
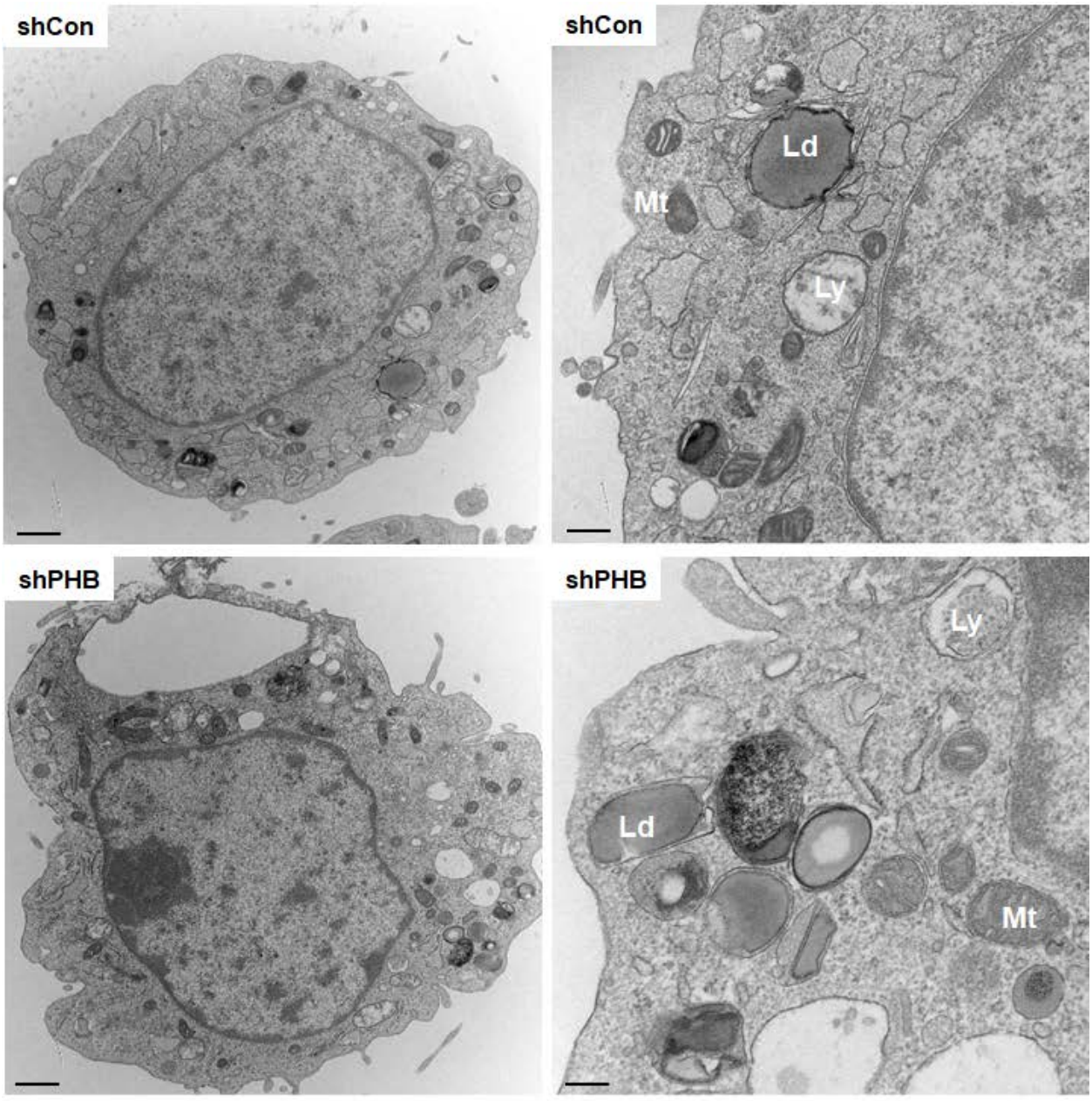
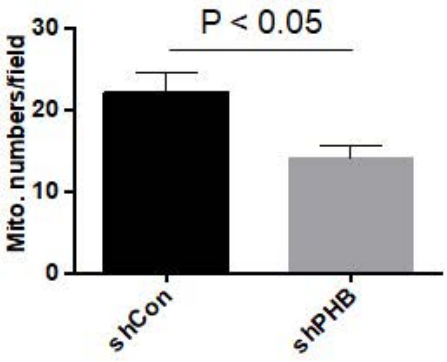

Vec
Ld
Vec
Ly
Mt
PHB
PHB
Mt
Ly
Ld
P < 0.05
Mito. numbers/field
50
40
30
20
10
0
Vec con
PHB
P < 0.01
Lyso. numbers/field
15
10
5
0
Vec con
PHB

Figure 4.3. Transmission electron micrographs showing the effect of PHB1 overexpression on mitochondrial and lysosomal characteristics in Y-1 adrenocortical cells (upper panel and middle panel). Histograms showing quantification of mitochondrial numbers and lysosome numbers (lower panel). Data are presented as mean ± SEM (7-10 cells were counted each group). Mt – mitochondria, Ld – lipid droplets and Ly – lysosomes.


shCon
shCon
Ld
Mt
Ly
shPHB
shPHB
Ly
Ld
Mt


P < 0.05
Mito. numbers/field
30
20
10
0
shCon
shPHB

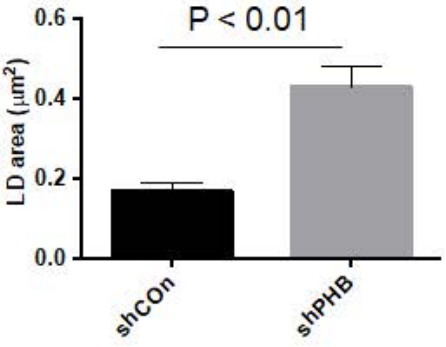

P < 0.01
LD area (μm²)
0.6
0.4
0.2
0.0
shCon
shPHB

Figure 4.4. Transmission electron micrographs showing the effect of shRNA-mediated PHB1 knockdown on mitochondrial and lipid droplet characteristics in Y-1 adrenocortical cells (upper panel and middle panel). Histograms showing quantification of mitochondrial numbers and lipid droplet area (lower panel). Data are presented as mean ± SEM (n =3). Mt – mitochondria, Ld – lipid droplets and Ly – lysosomes.

4.3. PHB1 modulates PKA and ERK signaling in adrenocortical cells

In addition to mitochondrial biology and lipid homeostasis, many studies have shown that PHB1 plays a role in the modulation of membrane signaling. For example, our laboratory has reported the phosphorylation of PHB1 in relation to insulin signaling [77-79], and have shown its importance in adipocyte differentiation, including a modulatory role in PI3K-Akt and MAPK-ERK signaling in a context-dependent manner [79,95]. Moreover, works by other laboratories have shown that PHB1 undergoes phosphorylation in many cell types in relation to growth factors (IGF, EGF) [77,78,95], hormones (FSH, LH) [73-75] and diverse immune signaling pathways [96,97]. Thus, I examined the activation level of cAMP-PKA and MAPK-ERK cell-signaling pathways in PHB1 manipulated adrenocortical cells because of their established roles in mediating the effects of trophic hormones in steroidogenic cells. As the cAMP-PKA signaling pathway plays a central role in mediating the ACTH response in adrenocortical cells [98], I first investigated the phospho-PKA (p-PKA) in adrenocortical cell lysates by immunoblotting using a phospho-specific antibody. An increase in p-PKA level was found in cells overexpressing PHB1 compared to control experimental group (Fig. 4.5), which was abrogated after PHB1 knockdown (Fig. 4.5). In addition, as PHB1 modulates MAPK-ERK signaling, we determined the phospho-ERK (p-ERK) levels in PHB1 manipulated adrenocortical cells using a phospho-specific antibody. The p-ERK level was also increased in PHB1 overexpressing cells, which was reversed by PHB1 knockdown (Fig. 4.5) indicating a potential connection between the two in mediating the role of PHB1 in adrenocortical cells.

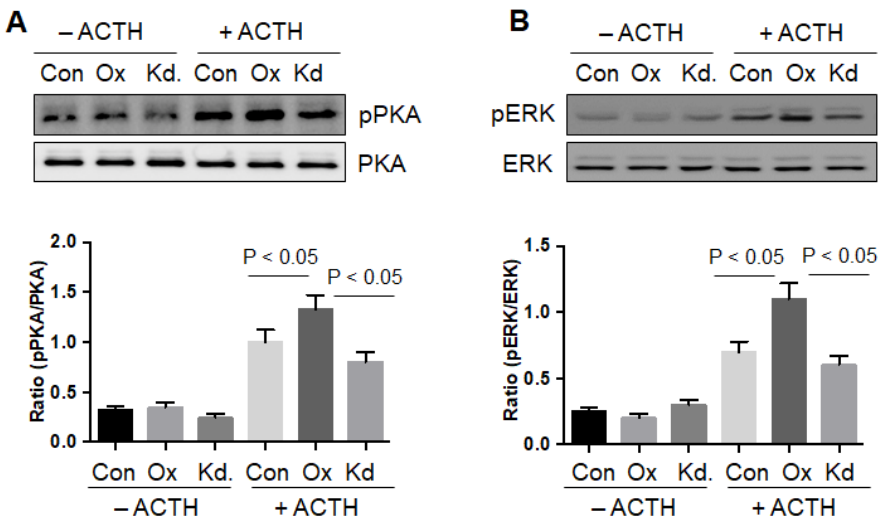


Figure 4.5. Immunoblots showing the effect of PHB1 overexpression on the phosphorylation of PKA (**A**) and ERK (**B**) with/without steroidogenic stimulation (upper panel) and their reversal by PHB knockdown. Quantification of band intensities are shown with histograms. Data are presented as mean ± SEM (n =3). Con – control, Ox – PHB1 overexpression and Kd – PHB1 knockdown.

4.4. PHB1 plays a role in steroid hormone production by adrenocortical cells

Finally, to determine the functional relevance of PHB1 in adrenocortical cells, I investigated the effect of PHB1 manipulation on corticoid production by adrenocortical cells. For this, I used a loss of function approach and examined the effect of shRNA-mediated PHB1 knockdown [79,80] on adrenocortical cells. A decrease in corticosterone production was observed in shPHB1 group when compared with scrambled shControl (scrambled shRNA) transfected group in response to db-cAMP stimulation ($p < 0.05$; Fig. 4.6). In addition, I used a gain of function approach and examined the effect of PHB1 overexpression on corticosterone production. An increase in corticosterone production was found in PHB1 overexpressing cells in comparison with vector only transfected control group ($p < 0.05$; Fig. 4.6). Together, this data suggests that PHB1 plays a role in ACTH-induced steroid production in adrenocortical cells.

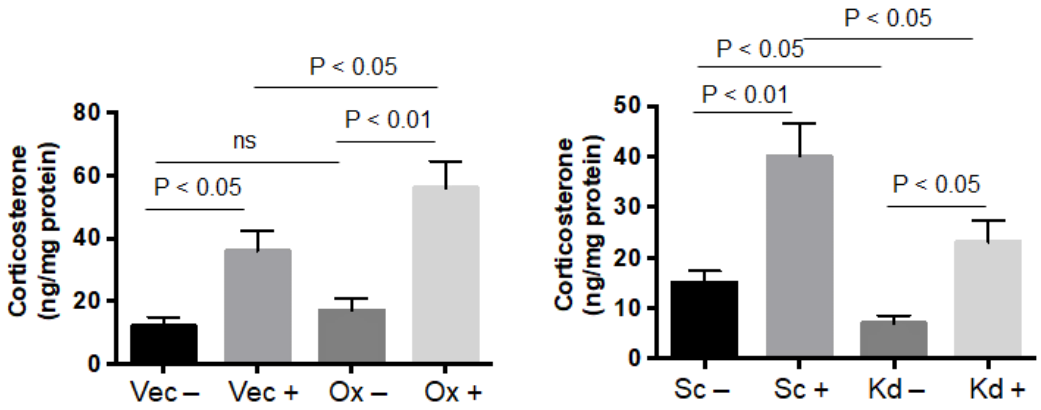


Figure 4.6. Histograms depicting the effect of PHB1 overexpression and knockdown on corticoid production from adrenocortical cells in response to db-cAMP. Data are presented as mean ± SEM (n =3). – and + indicates without or with db-cAMP stimulation, respectively. Vec – vector control, Ox – PHB1 overexpression, Kd – shRNA-mediated PHB1 knockdown, Sc – scrambled control shRNA.

4.5. Culturing adrenocortical cells in a lipoprotein-depleted medium leads to the depletion of the intracellular cholesterol pool and lipid droplets

As cholesterol is the precursor substrate for all steroid hormones, the steroidogenesis-related cellular events in steroidogenic cells must respond to intracellular cholesterol insufficiency to maintain basal hormone production, and to meet the physiological demands of different steroid hormones upon trophic hormone stimulation, which vary substantially in different steroidogenic cell types. In this context, the roles of different trophic hormones in the regulation of steroidogenic events and factors are well-established [99]. However, the potential role that cholesterol plays in these events especially under basal conditions is largely unexplored. As cholesterol uptake, autophagy, and mitochondrial dynamics are interlinked to maintain cholesterol homeostasis and steroidogenesis [100-102], I hypothesize that the depletion of the intracellular cholesterol pool must affect autophagy and mitochondrial dynamics in steroidogenic cells as a compensatory mechanism to maintain basal steroidogenesis. To explore this, I cultured Y-1 cells in cholesterol-depleted (CD) cell culture conditions (i.e., culture medium supplemented

with lipoprotein depleted FBS containing only 0.04 mg/ml cholesterol — an approximately 35-fold reduction compared with normal FBS, which contains around 1.40 mg/ml cholesterol) for different time points (i.e., 6h, 12h and 24h). To confirm intracellular cholesterol depletion under the experimental condition used, cells were harvested at each time point to determine their cholesterol contents.

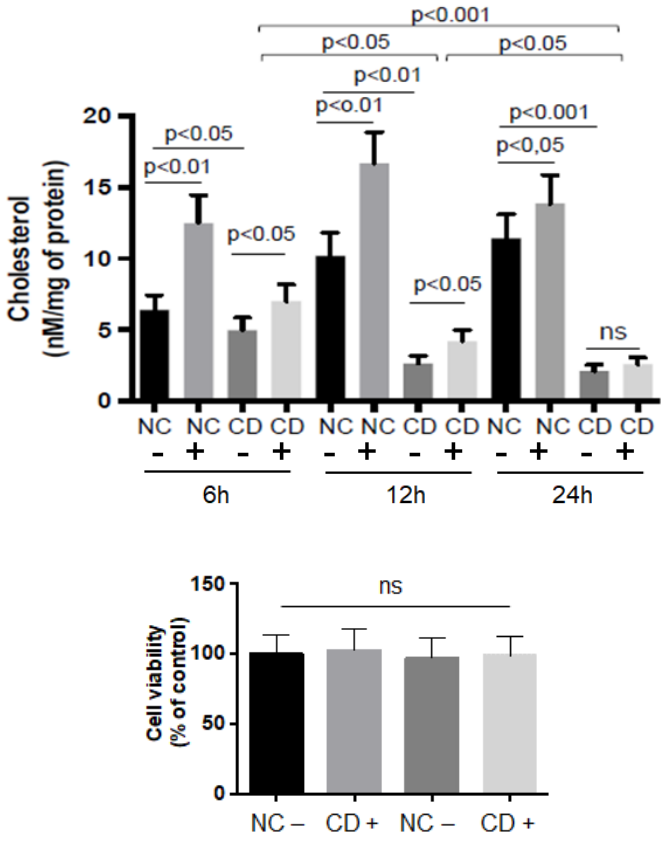


Figure 4.7. Culturing steroidogenic cells in lipoprotein-depleted medium leads to depletion of intracellular cholesterol pool.
Upper panel: Histograms depicting cholesterol levels as measured by Amplex™ Red Assay in Y-1 adrenocortical cells cultured under cholesterol depleted (CD) and normal culture (NC) conditions. Data are presented as mean ± SEM (n =3). Lower panel: Histograms showing Y-1 cell viability under NC and CD culture conditions (for 24h) as determined by MTT assay. Data are presented as percentage of control (mean ± SEM, n = 3). ns – not significant, – and + indicates without and with cAMP stimulation.

A gradual decrease in cholesterol content was observed in the cells grown in the CD medium, which were significantly lower than the cholesterol contents of cells cultured in the normal

culture (NC) condition (i.e., the culture medium supplemented with the normal FBS) (Fig. 4.7) at each time point. In addition, increase in cholesterol levels in response to db-cAMP was substantially higher in NC experimental group than CD experimental group at each time point. Moreover, no significant change in db-cAMP response was not observed at 24h in CD experimental group (Fig. 4.7). To determine whether CD culture condition affects cell viability, I performed MTT reduction assay. No significant difference in cell viability were observed between cells grown under NC and CD culture conditions during 24h time point used in this study (Fig. 4.7).

I also confirmed the depletion of intracellular lipid droplets and the cholesterol pool in cells cultured in the CD medium using transmission electron microscopy (TEM) (Fig. 4.8). Lipid droplets were apparent in Y-1 cells in the NC experimental group, which was significantly reduced in number ($p < 0.05$) and size ($p < 0.01$) in the CD experimental group (Fig. 4.8). In addition, a significant increase in lysosome numbers was observed in CD group ($p < 0.01$) compared to NC group. However, such difference in mitochondrial numbers was not observed between NC and CD experimental groups (Fig. 4.8). This data established that culturing the Y-1 steroidogenic cells in the CD medium leads to the depletion of intracellular cholesterol content without affecting cell viability under the culture conditions used.

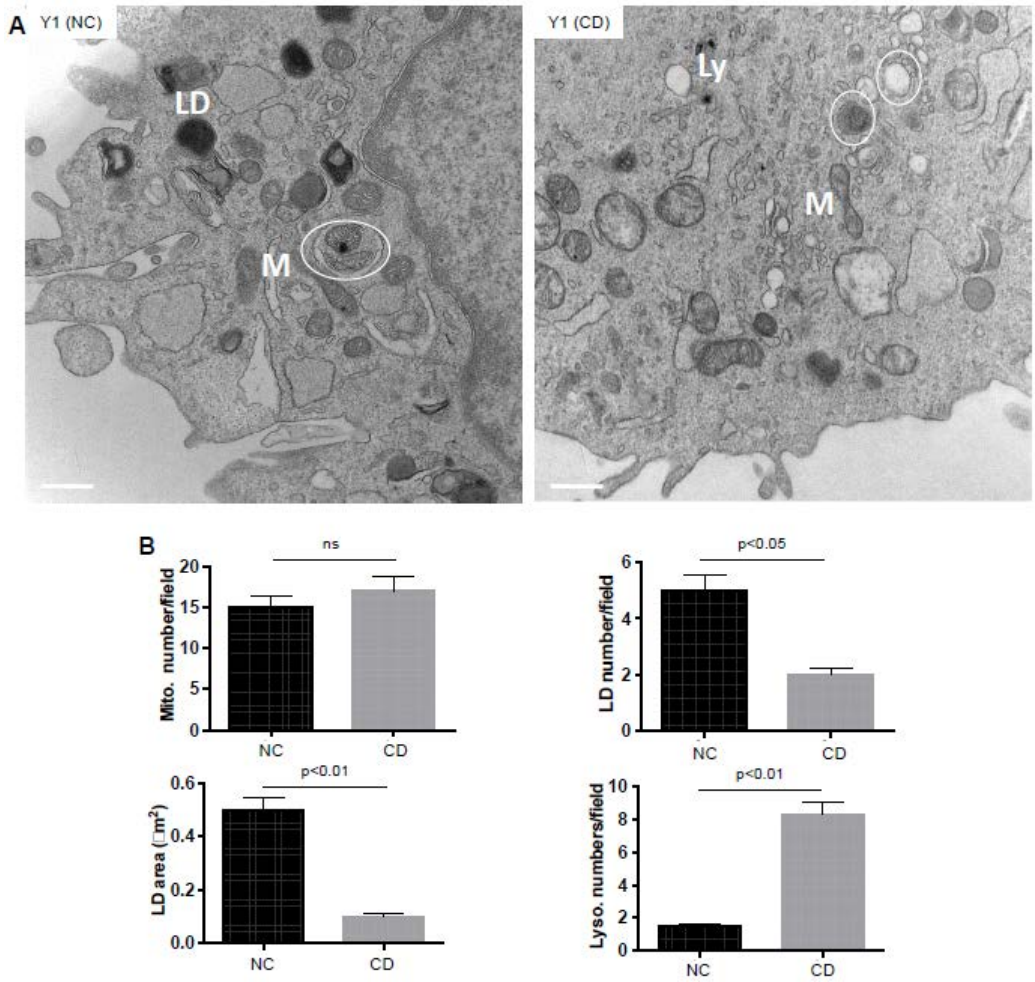


Figure 4.8. TEM analysis of Y-1 cells showing depletion of intracellular lipid droplets and associated ultrastructural changes under CD condition.
(**A**) Representative transmission electron micrographs (magnification: 50000x) of Y-1 cells cultured under cholesterol deficient and normal culture conditions. Scale bars = 500 nm. LD – lipid droplets, Ly – lysosomes, M – mitochondria. Circle – signs of autophagy/lipophagy, NC – normal culture condition, CD – cholesterol depleted culture condition. (**B**) Histograms showing quantification of LDs size, as well as lysosomal and mitochondrial numbers. Data are presented as mean ± SEM (n = 3).

4.6. Cholesterol deprivation induces steroidogenic events/factors in adrenocortical cells

Subsequently, I analyzed cell lysates prepared at each time point for the markers of autophagy, mitochondrial dynamics, and steroidogenesis, including proteins involved in cholesterol handling using immunoblotting.

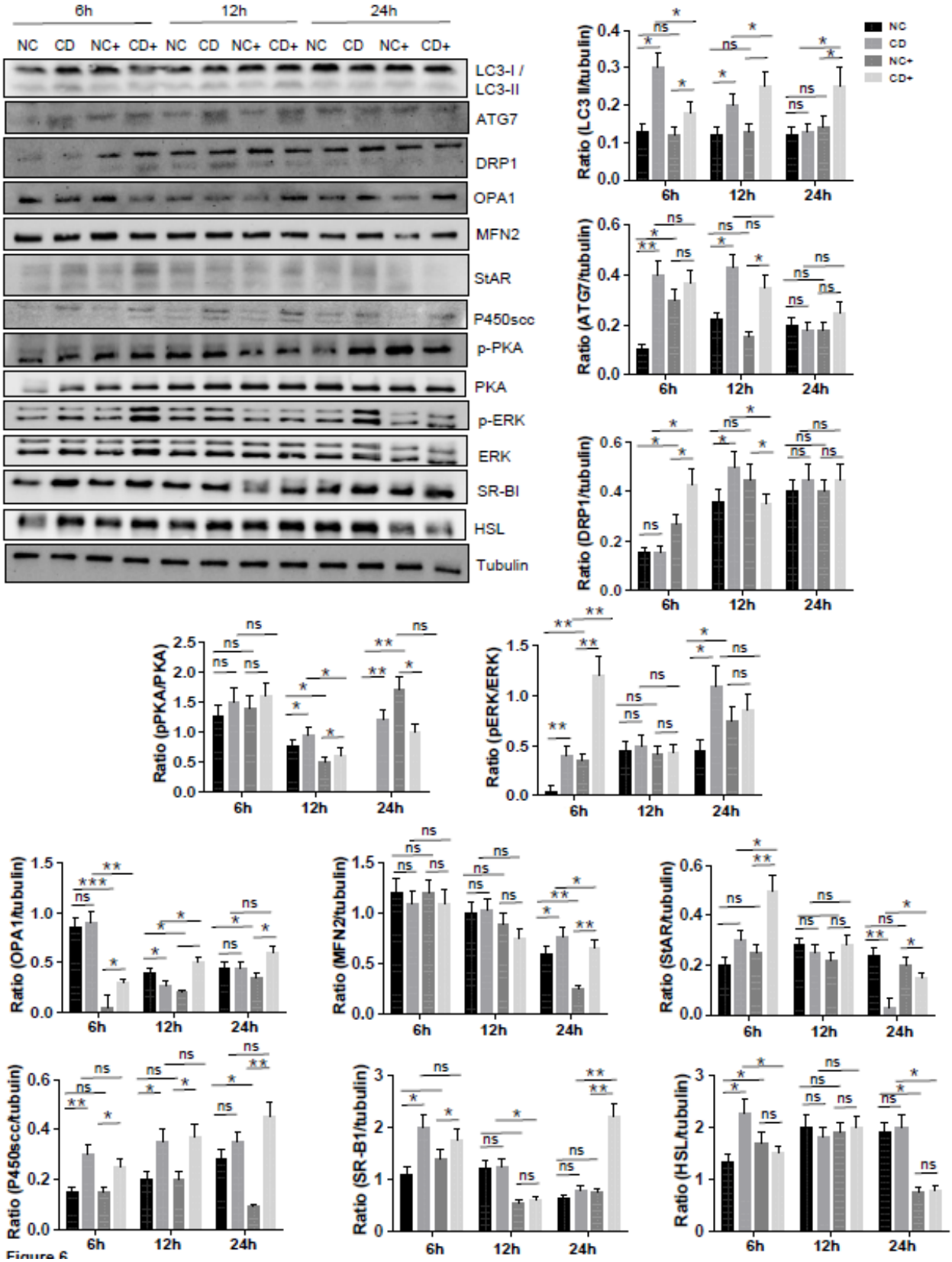

6h
12h
24h
NC CD NC+ CD+ NC CD NC+ CD+ NC CD NC+ CD+
LC3-I / LC3-II
ATG7
DRP1
OPA1
MFN2
StAR
P450scc
p-PKA
PKA
p-ERK
ERK
SR-BI
HSL
Tubulin
NC
CD
NC+
CD+
Ratio (LC3 II/tubulin)
Ratio (ATG7/tubulin)
Ratio (DRP1/tubulin)
Ratio (pPKA/PKA)
Ratio (pERK/ERK)
Ratio (OPA1/tubulin)
Ratio (MFN2/tubulin)
Ratio (StAR/tubulin)
Ratio (P450scc/tubuin)
Ratio (SR-B1/tubulin)
Ratio (HSL/tubulin)

Figure 6

Figure 4.9. Intracellular cholesterol depletion induces steroidogenic factors and events in Y-1 adrenocortical cells. Representative immunoblots showing protein levels of autophagy, mitochondrial dynamics, and steroidogenic markers in Y-1 cells cultured under normal and cholesterol depleted conations. Tubulin blot is shown as a loading control. + indicates respective hormonal stimulation (i.e., ACTH), NC – normal culture condition, CD – cholesterol depleted culture condition. All experiments were repeated at least for three times. Histograms showing quantification of protein band densities. Legend in LC3 II histograms is applicable to all histograms. Data are presented as mean ± SEM (n = 3). * $p<0.05$, ** $p<0.01$ *** $p<0.001$.

CD resulted in increased LC3-II levels at 6h and 12h (in unstimulated cells) when compared with NC group (Fig. 4.9). However, no difference in band intensity of LC3 (I and II) was observed between NC and CD groups at 24h. Notably, the effect of CD on ATG7 was relatively more apparent than LC3 II at 6h and 12h (Fig. 4.9). In addition, the effect of CD on LC3-II and TAG7 bands was tapered at 24h (Fig. 4.9). Drp1 (a marker for mitochondrial fission) levels remain unchanged between NC and CD experimental groups at 6h; however, an increase was observed in CD group at 12h (Fig. 4.9). In general, Opa1 (a marker for mitochondrial fusion) levels showed an opposite effect in relation to Drp1 during 6h-12h. However, a difference in Drp1 and Mfn2 was apparent between NC and CD groups at 24h, which were higher in CD group under both unstimulated and stimulated conditions (Fig. 4.9). Moreover, changes in StAR and P450scc levels (under NC and CD conditions) in Y-1 cells were consistently higher in CD experimental group (Fig. 4.9). In general, the higher molecular weight isoform of StAR was more apparent in Y-1 cells (Fig. 4.9). Notably, CD resulted in increase in p-PKA levels compared with NC group (Fig. 4.9) under unstimulated state; however, after hormonal stimulation in Y-1 cells, CD appears to have a negative effect on p-PKA suggesting a potential interplay between the two (Fig. 4.9). The p-ERK level in Y-1 cells was progressively increased in CD group compared with NC group under unstimulated state (Fig. 4.9). Whereas a varied effect on p-ERK level was observed in Y-1 cells under hormonal stimulation, ranging from synergistic effect with CD at 6h and an opposite effect at 24h (Fig. 4.9). In general, SR-B1

and HSL (proteins involved in intracellular cholesterol homeostasis) levels increased under CD and after hormone stimulation when compared with NC group and unstimulated group, respectively (Fig. 4.9). Collectively, this data suggests that steroidogenic stimulation appears to have a modulatory effect on CD-induced changes in steroidogenic events and factors, suggesting their cross-regulation (Fig. 4.9).

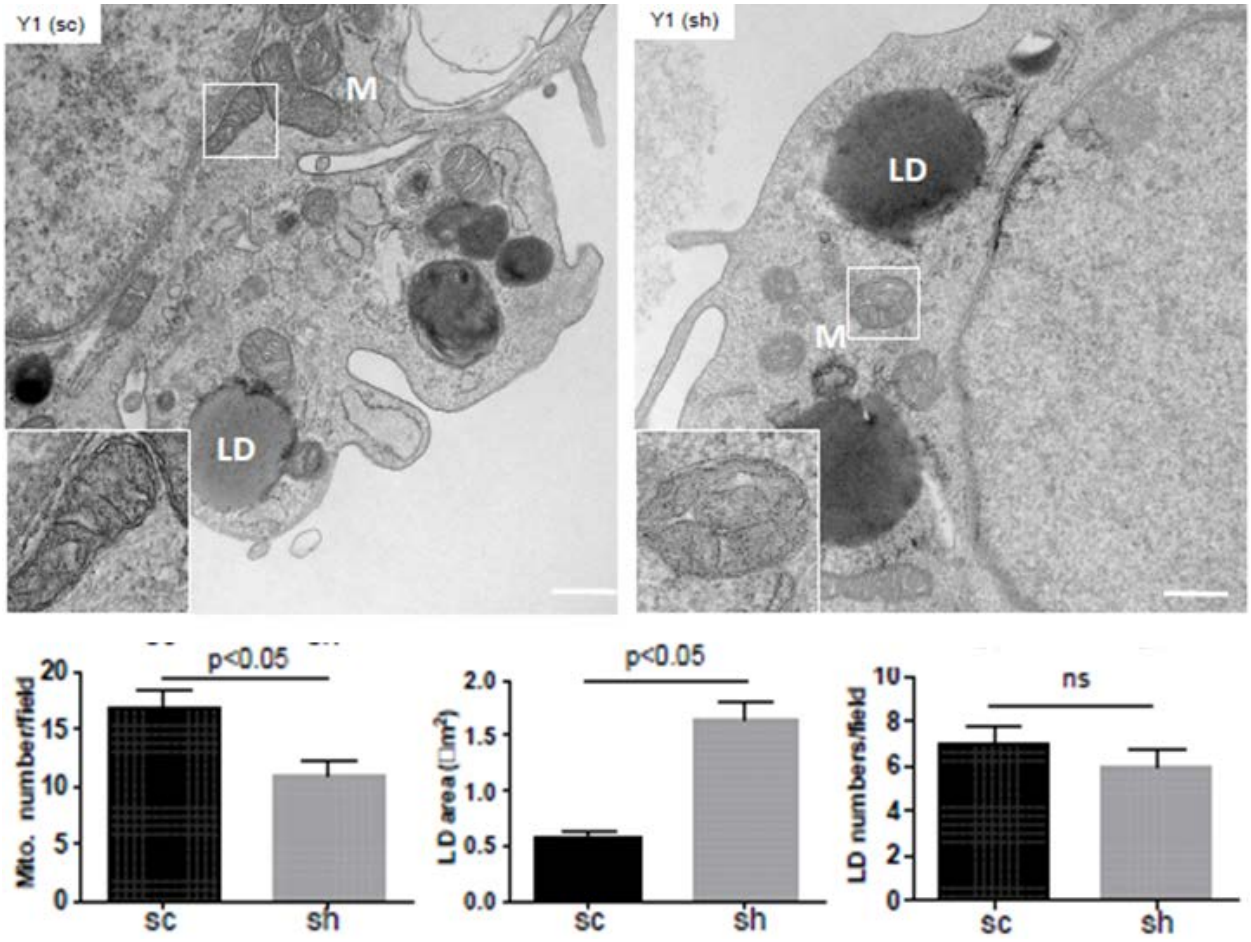


Figure 4.10. Ultrastructural analysis of Y-1 cells after augmenting intracellular cholesterol pool. (**A**) Upper panel: Representative transmission electron micrographs (magnification: 50000x) showing the effect of shRNA-mediated StAR knockdown in Y1 cells on intracellular lipid droplets and mitochondria. Scale bars = 500 nm. Sc – scramble control, sh –shStAR, M – mitochondria; LD – lipid droplets; Ly – lysosome. Asterisk indicates sign of autophagy/mitophagy. Lower panel: Histograms showing quantification of LDs and mitochondrial number and size. Data are presented as mean ± SEM.

4.7. Ultrastructural analysis of steroidogenic cells under increased intracellular cholesterol pool

In addition to depleting the intracellular cholesterol pool, I investigated the effect of an increased intracellular cholesterol pool on ultrastructural changes in the Y-1 cells. For this, I used a shRNA-mediated knockdown of *StAR* as a model, because in adrenocortical cells, the StAR protein is essential for cholesterol transport to the mitochondria for steroidogenesis [103,104], and loss of StAR function is known to cause lipid and cholesterol accumulation in these cell types [43]. As expected, the *StAR* knockdown in the Y-1 cells led to significant increase in the size of lipid droplets ($p < 0.05$) and a decrease in mitochondrial numbers ($p < 0.05$), as revealed by TEM analysis (Fig. 4.10). In addition, signs of mitochondrial damage, such as swelling and disorganization and fragmentation of mitochondrial cristae were observed (Fig. 4.10). Moreover, an early sign of lipophagy (e.g. pre-lipophagosomes) were noticeable, such as appearance of lipophagic membrane (Fig. 4.10). I also analyzed cells lysates for markers of autophagy. However, no difference in LC3-I and LC3-II band intensities were observed between different experimental groups, whereas ATG7 showed a modest increase in the shStAR cells, when compared with the scrambled shRNA transfected control cells (Fig. 4.11). Moreover, no difference was found in the expression level of mitochondrial dynamics markers between different experimental groups, particularly without steroidogenic stimulation (Fig. 4.11). This data, along with data obtained from the CD group, indicate that the role of cholesterol in autophagy and mitochondrial dynamics vary during basal and ACTH-stimulated conditions, as well as availability of steroidogenic cholesterol pool.

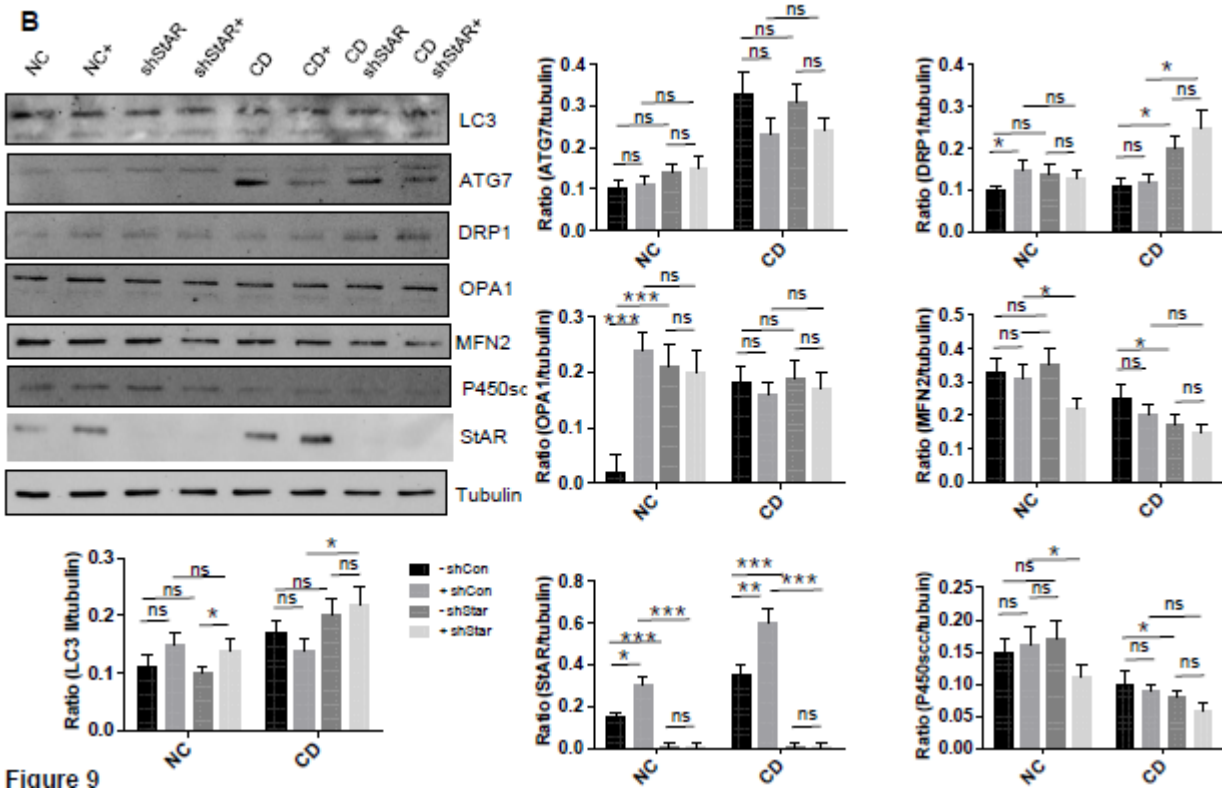


Figure 4.11. Immunoblots showing changes in protein levels of autophagy, mitochondrial dynamics and steroidogenic markers in response to StAR knockdown-mediated increased accumulation of lipid droplets/intracellular cholesterol pool in Y-1 cells. Quantification of protein band densities are shown with histograms (n = 3). Data are presented as mean ± SEM. Legend in LC3 II histograms is applicable to all histograms. NC – cells cultured under normal condition; CD – cell cultured under cholesterol depleted condition; + indicates hormonal stimulation; shStaR – StAR-specific shRNA; sc – scrambled control. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.8. CD-induced changes sustain basal steroidogenesis

Finally, I examined the functional consequences of CD-induced changes on steroidogenesis in Y-1 cells and measured pregnenolone levels in the presence and absence of hormone stimulation at 6h, 12h and 24h. CD increased steroid hormone production at all-time points, which was further increased in response to ACTH stimulation at 6h and 12h but not at 24h (Fig. 4.12). Moreover, a difference in the amplitude of pregnenolone production was observed under CD compared with the control groups (NC), which were higher during 6h-12h

and then decline at 24h (Fig. 4.12). In aggregate, our findings suggest a relationship between the intracellular cholesterol pool, the mitochondria, and steroidogenic capacity.

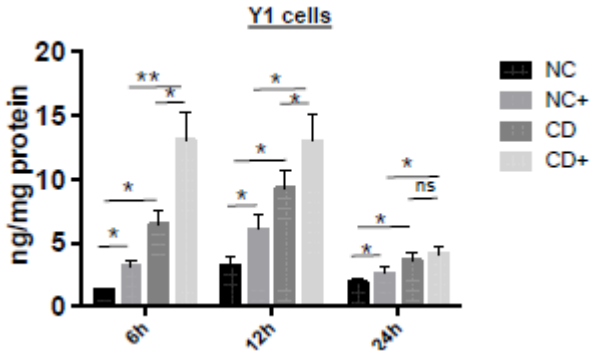


Figure 4.12. Cholesterol deprivation increase basal steroidogenesis in adrenocortical cells. Histograms showing pregnenolone production from Y-1 cells in response to cholesterol deprivation with/without ACTH stimulation. NC – normal culture condition, CD – cholesterol depleted culture condition. H – hormone treatment (ACTH dose), ns – not significant, Data are presented as mean ± SEM (n = 3). * $p<0.05$, ** $p<0.01$.

CHAPTER 5

Discussion

Sections of this chapter have been published as a research article in the *Journal of Steroid Biochemistry & Molecular Biology* titled “The intracellular cholesterol pool in steroidogenic cells plays a role in basal steroidogenesis”. 2022 Mar 24;220:106099.

For my thesis work, I investigated the role of PHB1 and intracellular cholesterol pool in corticosteroidogenesis in vitro in model adrenocortical cells. First, I investigated the effect of ACTH on PHB1 protein levels and the consequence of PHB1 manipulation in adrenocortical cells. Then, I undertook a simple and new approach to determine the effect of the depletion of intracellular cholesterol pool on steroidogenic factors and the markers of steroidogenic events in adrenocortical cells. I found that PHB1 is an important ACTH target protein in adrenocortical cells and plays a role in corticosteroidogenesis, including in cell signaling, cholesterol homeostasis, and mitochondrial biology involved in steroidogenesis. Thus, I found a previously unknown role of PHB1 in regulating interconnected steroidogenic events in different cellular compartments in adrenocortical cells. In addition, I found that the intracellular cholesterol pool in Y-1 adrenocortical cells plays a role the regulation of steroidogenic events and factors, especially under basal condition, which is consistent with our parallel findings in other steroidogenic cell types, including testicular MA-10 cells and placental BeWo cells [26].

The implications of my findings are broad as the fundamentals of steroidogenesis, such as cholesterol homeostasis, cholesterol transport to the mitochondria, and the initiation of steroidogenesis, are common among all steroidogenic tissues. For instance, cholesterol serves as an essential substrate for all steroid hormones and its trafficking and homeostasis in steroidogenic cells are tightly regulated [105.106]. Because of a highly hydrophobic chemical property, the cellular uptake and intracellular trafficking of cholesterol is mediated through different proteins [105]. Proteins that interact with cholesterol often contain cholesterol-binding domains or motifs [107,108]. For example, the StAR family members contain the START (StAR-related lipid-transfer) domain, which binds hydrophobic lipids [109], whereas P450scc contains cholesterol binding short linear motifs, known as CRAC and CARC [110]. Recently,

my laboratory has reported that PHB1 and its homologous protein PHB2 contains CRAC and CRAC motifs [81]. Moreover, previously our laboratory has shown that PHB1 contains lipid-binding motifs [78], and many PHB family members have been reported to bind cholesterol and be involved in lipid homeostasis [67-69], including mitochondrial phospholipid metabolism [56]. As lipid droplets in adrenocortical cells primarily contain cholesterol and cholesterol esters, an altered LDs characteristic in PHB1 manipulated Y-1 cells would mean that PHB1 plays a role in cholesterol homeostasis, which may include uptake, storage, transport, and their subsequent utilization for steroidogenesis. Of note, the ultrastructural features of adrenocortical cells (after PHB1 knockdown) were very similar to previous reports from *StAR* [43] and *Cyp11A1* knockout mice [44], such as increased lipid accumulation and signs of lipid droplet degradation. This would mean that dysregulation of lipid/cholesterol homeostasis in adrenocortical cells is likely due to adrenocortical cell-specific alterations independent of ACTH stimulation. It is possible that the manipulation of PB1 levels in adrenocortical cells leads to a change in adrenocortical cell-specific attributes involved in cholesterol handling, including uptake, storage, mobilization, and utilization. Of note, the cholesterol binding motifs in PHB1 and the recently identified LC3 binding motifs [61] overlap with each other. This raises the important question of whether these two features of PHB1 work in a mutually exclusive and context-dependent manner to maintain cholesterol homeostasis in adrenocortical cells for steroidogenesis in different conditions. For example, under cholesterol insufficiency, PHB1 may facilitate autophagy / mitophagy to recycle intracellular cholesterol (to maintain steroidogenesis), whereas cholesterol sufficiency and its binding to PHB1 may inhibit PHB1-mediated autophagy / mitophagy. Moreover, as PHB1 plays an important role in steroidogenesis, the loss of mitochondrial PHB1 due to autophagy / mitophagy may be compensated by upregulation of PHB1 under cholesterol deficiency because

PHB1 is a cholesterol sensitive gene and is upregulated under cholesterol deficiency [70]. The sign of lipophagy as observed in our TEM analysis in PHB1-manipulated adrenocortical cells would mean that PHB1's role in corticosterone production might in part be mediated through autophagy, mitophagy, or lipophagy-related cholesterol homeostasis. In addition, lipophagy may protect adrenocortical cells from the toxic effects of increased lipid accumulation on mitochondrial functions. Thus, PHB1 may function in multiple ways in adrenocortical cells in a context-dependent manner and may include steroidogenic events and factors. For instance, StAR protein levels are known to be acutely regulated in steroidogenic cells in response to trophic hormones and involved different isoforms as a result of proteolytic processing and post-translational modification by phosphorylation [111-114]. An altered processing of StAR in PHB1 manipulated adrenocortical cells indicate that PHB1 may be related to cellular mechanisms involved in the acute regulation of StAR and its function. In this context, it is important to note that PHB1 interacts with mitochondrial proteases, including m-AAA, YME1L and OMA1 [58, 59], which may be involved in this relationship.

My findings are consistent with the notion that the mitochondrial attributes of PHB1 contribute to the cell type-specific functions of PHB1 (in addition to its mitochondrial housekeeping functions) [64] and further supports my conclusion that PHB1 plays a multifaceted regulatory role in adrenocortical cells. In addition, my findings provide a tip-off on how a single protein may function at different levels in cell biology, from a cell-neutral mitochondrial housekeeping function to the cell type-specific functions of mitochondria. This would imply an additional means of creating diversity (due to cellular and functional compartmentalization) from a relatively limited number of genes and proteins, including but not limited to RNA splicing, post-translational modifications, and protein domains.

Furthermore, my findings suggest that the intracellular cholesterol pool in adrenocortical cells plays a role in the regulation of steroidogenic events and factors, and consequently, in steroid hormone production. The differences observed between different time points (i.e., 6h, 12h and 24h) under CD culture condition are likely due to temporal and dynamic changes in steroidogenic factors and events (e.g., activation levels of cell signaling intermediates, changes in their cholesterol pool, as well as expression levels and functions of different proteins involved in cholesterol uptake and handing for steroidogenic need). In addition, my study validated the feasibility of selectively probing the role of cholesterol in steroidogenic cell type-specific functions using a simple experimental approach without affecting the basic cellular need of cholesterol (as reflected in cell viability assay), which is challenging to achieve using chemical (e.g., methyl-β-cyclodextrin-mediated) or genetic approaches (e.g., perturbing de novo biosynthesis).

A modulatory effect on cell signaling intermediates (e.g., p-PKA and p-ERK) in Y-1 cells in response to cholesterol deprivation (independent of trophic hormone stimulation) would imply that cholesterol deficiency or insufficiency, in some way, leads to the activation of upstream signaling involved in steroidogenesis, which may be a compensatory response to maintain basal steroidogenesis. Moreover, the modulation of cholesterol deprivation-induced changes in Y-1 cells in response to steroidogenic stimulation indicate that a relationship exists between the intracellular cholesterol pool and the hormone ligand-induced activation of the G-protein coupled receptor (GPCR)-mediated steroidogenic signaling, which likely works in a context-dependent manner (e.g., the absence or presence of trophic hormones and the available steroidogenic cholesterol pool). Intriguingly, trophic hormone receptors are localized in the cholesterol-rich environment in the cell (i.e., the cell membranes) [115] whereas the

steroidogenic enzymes are localized in a cholesterol-poor environment (i.e., the IMM and smooth ER) [116]. It is conceivable that this unique set-up in steroidogenic cells, including cholesterol transport to the mitochondria as a committed step, makes cholesterol a critical player in the regulation of steroidogenesis (more than a precursor substrate for steroid hormones) across steroidogenic cell types, which will require further investigations to confirm. The physiological and pathophysiological implications of my findings are broad. For example, a similar mechanism might operate during the initiation of trophic hormone-independent adrenocortical and testicular steroidogenesis in the course of fetus development [117], as well as in steroid hormone-producing cancer cells [118], including recently reported role of cholesterol synthesis in cancer metastasis [119], which warrants further investigations.

Furthermore, as changes related to mitochondrial dynamics in Y-1 cells appeared in an overlapping manner alongside autophagy-related changes, it is possible that changes related to autophagy and mitochondrial dynamics are interlinked and may be a stepwise and integrated response to sustain steroidogenesis under varying cholesterol availability and steroidogenic needs (e.g., basal and hormone induced).

The precursor cholesterol for steroidogenesis is known to come from at least three different sources, including mobilization of cholesterol from the plasma membranes / intracellular LDs, the uptake of circulating cholesterol esters (in the form of lipoproteins), and the de novo synthesis of cholesterol [120,121]. In addition, autophagy-mediated cholesterol trafficking has been implicated in steroid production [47]. Among different sources, plasma lipoproteins are the major source of cholesterol for steroidogenesis. For example, low-density lipoprotein accounts for about 80% of cholesterol delivered to the steroidogenic gland for steroidogenesis [122]. Moreover, de novo synthesis of cholesterol from acetate does occur in

steroidogenic cells; however, its relative contribution to steroid hormones produced is less. It is conceivable that relative contribution by de novo cholesterol synthesis may substantially increase in the absence of lipoprotein uptake. A readily available pool of free cholesterol in the steroidogenic cells exist, which serve the basal and acute production of steroid hormones. In addition, steroidogenic stimulation increases hydrolysis of stored cholesterol esters to free cholesterol, increase uptake from plasma lipoproteins, and increased cholesterol biosynthesis within the gland [123]. The acute response to a steroidogenic stimulation is mediated by the StAR protein, which facilitate cholesterol transport across the OMM. However, our understanding of the relative contributions of various sources of cholesterol to the different stages of steroidogenesis (i.e., basal, acute, and chronic) in major steroidogenic cells remains limited. It is likely that these processes work in a coordinated manner to maintain the physiological needs of different steroid hormones, which vary substantially under basal and stimulated conditions. The mobilization of cholesterol from LDs may play a major role in the acute response to trophic hormones, whereas the de novo synthesis of cholesterol may be primarily involved in basal and chronic steroidogenesis, whereas the cellular uptake of cholesterol in replenishing the depleted, readily available pool due to the acute response, and in the maintenance of a chronic response. By the same token, the instantaneous contribution of the de novo synthesis of cholesterol for acute steroid production in response to trophic hormones is expected to be minimal. My findings provide a proof of principle that cholesterol is more than a substrate for steroid hormones and plays a role in the regulation of steroidogenic factors and events (Fig. 5.1). Future studies warranted to better understand the multifaceted relevance of cholesterol-centric set-up of steroidogenesis.

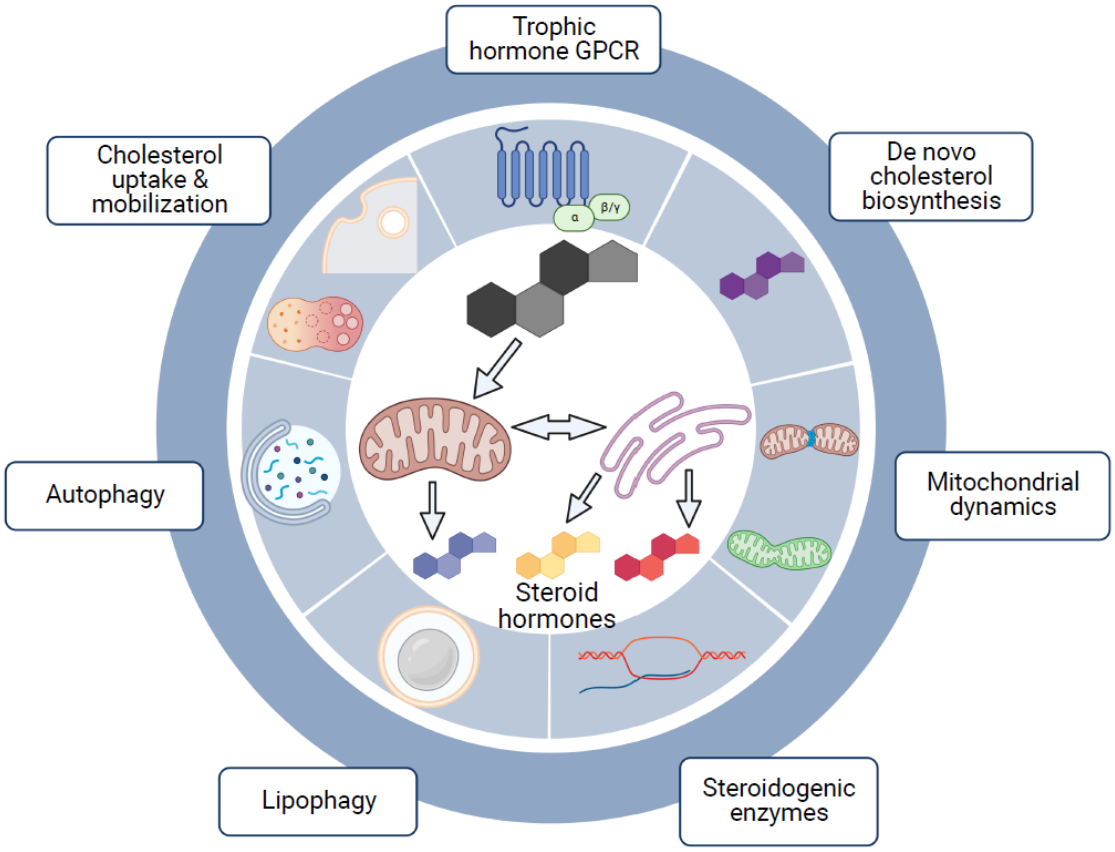


Figure 5.1. Cholesterol – more than a substrate for steroid hormones.
Reproduced from Bassi G, Sidhu SK, Mishra S. The intracellular cholesterol pool in steroidogenic cells plays a role in basal steroidogenesis. *J Steroid Biochem Mol Biol*. 2022 Mar 24;220:106099.

In summary, data presented in this thesis suggests that PHB1 plays a role in ACTH-induced steroidogenesis and the intracellular cholesterol pool in basal corticosteroidogenesis. Altered cholesterol homeostasis and reduced corticoid production from adrenocortical cells in response to PHB1 knockdown support a role of PHB1 in corticosteroidogenesis. Furthermore, my data suggests that cholesterol plays a role in the maintenance of basal steroidogenesis whereas cholesterol depletion leads to a compensatory response pertaining to steroidogenesis. It is anticipated that a better understanding of the relationship between cholesterol and PHB1 in

steroidogenesis will shed new light on the relationship between extrinsic and intrinsic factors and events pertaining to steroidogenesis.

5.1. Limitations

This study is not without limitations. For example, in this study, I have only used in vitro cell culture models. Further investigations using adrenocortical cell-specific transgenic mouse models of PHB1 and cholesterol targeting (e.g. adrenocortical cell-specific targeting of SR-B1) are necessary to confirm the physiological and pathological significance of my findings related to the role of PHB1 and cholesterol in adrenocortical cells. Moreover, depletion of exogenous supply of cholesterol to steroidogenic cells expected to affect different endogenous sources of cholesterol (e.g., plasma membranes, LDs and de novo synthesis) for steroidogenesis differently, which is not examined in this study. For example, it would be interesting to know whether depleting extracellular source of cholesterol and consequently readily available pool leads to upregulation of de novo cholesterol biosynthesis in steroidogenic cells.

5.2. Conclusion and Future Directions

In addition to a cell neutral role in the maintenance of structural and functional integrity of mitochondria, PHB1 plays a role in cell type-specific function in in adrenocortical cells. This finding is in consistent with the role of PHB1 in testosterone production from Leydig cells [81], lipogenesis in adipocytes [79], and insulin secretion from pancreatic beta cells [80]. The mechanism by which cholesterol is transported from outer to inner mitochondrial membrane is not clear. Recently, PHB1 has been reported to facilitate lipid transport across plasma membrane [84]. Our findings of an inner mitochondrial protein PHB1 steroidogenesis in two different species and in two different steroidogenic cell types is a step forward in this direction. However,

the molecular mechanisms involved in the interplay between PHB1 and cholesterol in steroidogenesis, including cell compartment specific functions remain to be determined. Future studies focusing on these issues will enhance our understanding of the relationship between PHB1, mitochondria, and cholesterol in steroidogenesis, which is a fundamental aspect of whole-body physiology in mammals.

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