

Very low-density lipoprotein (VLDL)-induced signals mediating aldosterone production

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Abstract

Aldosterone, secreted by the adrenal zona glomerulosa, enhances sodium retention, thus increasing blood volume and pressure. Excessive production of aldosterone results in high blood pressure and contributes to cardiovascular and renal disease, stroke and visual loss. Hypertension is also associated with obesity, which is correlated with other serious health risks as well. Although weight gain is associated with increased blood pressure, the mechanism by which excess fat deposits increase blood pressure remains unclear. Several studies have suggested that aldosterone levels are elevated with obesity and may represent a link between obesity and hypertension. In addition to hypertension, obese patients typically have dyslipidemia, including elevated serum levels of very low-density lipoprotein (VLDL). VLDL, which functions to transport triglycerides from the liver to peripheral tissues, has been demonstrated to stimulate aldosterone production. Recent studies suggest that the signaling pathways activated by VLDL are similar to those utilized by AngII. Thus, VLDL increases cytosolic calcium levels and stimulates phospholipase D (PLD) activity to result in the induction of steroidogenic acute regulatory (StAR) protein and aldosterone synthase (CYP11B2) expression. These effects seem to be mediated by the ability of VLDL to increase the phosphorylation (activation) of their regulatory transcription factors, such as the cAMP response element-binding (CREB) protein family of transcription factors. Thus, research into the pathways by which VLDL stimulates aldosterone production may identify novel targets for the development of therapies for the treatment of hypertension, particularly those associated with obesity, and other aldosterone-modulated pathologies.

Key Words

- ▶ adrenal cortex
- ▶ obesity
- ▶ hypertension
- ▶ phosphorylation
- ▶ intracellular signalling

Journal of Endocrinology
(2017) **232**, R115–R129

Introduction

Aldosterone is the primary mineralocorticoid hormone involved in maintaining fluid and electrolyte balance through its control of sodium and potassium homeostasis, thereby regulating blood volume and pressure under physiological conditions. In addition to its role in helping to regulate blood pressure, accumulating evidence points

to the idea that excessive production/secretion of this hormone not only results in hypertension but also likely contributes to cardiac fibrosis and congestive heart failure and exacerbates the morbidity and mortality associated with these disorders. Hypertension, which can be induced by dysregulated aldosterone secretion among other causes,

is prevalent in the United States, with over 80 million diagnosed with this disease (Mozaffarian *et al.* 2016). Importantly, a significant proportion of individuals with essential hypertension in the absence of hypokalemia have been diagnosed with hyperaldosteronism (reviewed in Brown *et al.* 1996, Rossi *et al.* 2008). In fact, primary aldosteronism has been estimated to occur in approximately 10% of all hypertensives, especially in those with resistant hypertension (on 3 or more medications to control blood pressure) (reviewed in Brown 2011). In addition, results from the Framingham Offspring Study suggest that higher aldosterone levels, even if these values are within the normal range, are associated with an enhanced risk of developing hypertension (Garrison *et al.* 1987).

Hypertension, in turn, has been associated with cognitive impairment (Kilander *et al.* 1998 and reviewed in Knopman & Roberts 2010) and is a contributing factor in renal disease, stroke, visual loss and congestive heart failure. It has also been postulated that there are direct effects of aldosterone in renal disease, independent of its effects on blood pressure (Hostetter *et al.* 2001) and indeed, recent clinical trials support this idea. Thus, studies using eplerenone in individuals with chronic kidney disease have demonstrated an involvement of the mineralocorticoid receptor (MR) activated by aldosterone in renal damage (reviewed in Kawarazaki & Fujita 2016). In addition to effects on blood pressure that promote heart dysfunction, aldosterone also appears to exhibit direct actions on cardiomyocytes, contributing to cardiac fibrosis and congestive heart failure (reviewed in Brown 2011). Aldosterone can also induce vascular damage by stimulating the generation of reactive oxygen species and activating pro-inflammatory and pro-fibrotic pathways in endothelial cells, leading to chronic vascular dysfunction (Brown 2005). Furthermore, some studies suggest that the interactions between aldosterone and AngII can enhance inflammation, fibrosis and cell proliferation. Indeed, in two clinical studies, the Randomized Aldactone Evaluation Study (RALES) and the Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS), antagonists of the mineralocorticoid receptor (MR) activated by aldosterone improve the morbidity and mortality observed in congestive heart failure patients (Pitt *et al.* 1999, O'Keefe *et al.* 2007), when added on top of standard treatments. The results from these studies suggest the importance of aldosterone in cardiac pathologies. In addition, several reports have suggested that aldosterone may be one of the causal links between obesity and hypertension (Briet & Schiffrin 2011,

Kawarazaki & Fujita 2016, Xie & Bollag 2016), although the mechanism(s) underlying this association among obesity, aldosterone and high blood pressure are unclear. Nevertheless, because of the profound effects that aldosterone can exert on the cardiovascular system, understanding the regulation of its biosynthesis is important, particularly as aldosterone may exert functions independently of the MR, such that MR antagonists may not completely inhibit all of aldosterone's adverse effects (e.g., Hofmann *et al.* 2016).

On the other hand, it is thought that perhaps not all effects of MR are the result of its activation by aldosterone. Cortisol is known to bind to the MR with approximately equal affinity as aldosterone. In certain tissues such as the kidney, cortisol is prevented from activating MR by co-expressed 11-beta-hydroxysteroid dehydrogenase 2 (11 β -HSD2), which converts active cortisol to inactive cortisone. However, some tissues such as cardiac muscle express minimal 11 β -HSD2, suggesting the possibility that in these cells cortisol may activate MR and contribute to its fibrotic effects, particularly as serum levels of cortisol are one hundred to one thousand times higher than those of aldosterone (Brown 2013). However, in vascular smooth muscle 11-beta-hydroxysteroid dehydrogenase 1 (11 β -HSD1), which usually catalyzes conversion of inactive cortisone to active cortisol, showed unusual oxidase activity, that is, an 11 β -HSD2 activity (Young *et al.* 2003). In this report, an inhibitor of 11 β -HSD2 activity increased blood pressure, cardiac and kidney weights and inflammatory marker levels, mimicking the effects of a glucocorticoid (deoxycorticosterone), in an experimental rodent model of cardiac hypertrophy and fibrosis (Young *et al.* 2003). These results suggest that endogenous glucocorticoid levels can induce cardiac pathology upon inhibition of 11 β -HSD2 activity. As 11 β -HSD2 activity has been shown to be decreased in some hypertensive patients (van Uum *et al.* 1998), a role for glucocorticoids in MR activation and hypertension remains uncertain (Brown 2013).

Aldosterone biosynthesis

Aldosterone is synthesized by four enzymes: the cholesterol side-chain cleavage complex (CYP11A1), type II 3 beta-hydroxysteroid dehydrogenase (HSD3B2), 21-hydroxylase (CYP21) and aldosterone synthase (CYP11B2). CYP11A1 and CYP11B2 are localized to the inner side of the inner mitochondrial membrane. The three CYP enzymes (CYP11A1, CYP21 and CYP11B2)

belong to the cytochrome P450 family, which can accept electrons from NADPH and use molecular oxygen to perform hydroxylation or oxidative conversion reactions. HSD3B2 belongs to the short-chain dehydrogenase family and is localized to the endoplasmic reticulum along with CYP21. The first reaction in aldosterone biosynthesis (Fig. 1) is the conversion of cholesterol to pregnenolone by CYP11A1 within mitochondria. However, in order for cholesterol to access this enzyme on the inner mitochondrial membrane, the cholesterol must first be transported by the steroidogenic acute regulatory (StAR) protein from the outer membrane of the mitochondria to the inner membrane where CYP11A1 is located (Capponi 2004). This step is the initial rate-limiting reaction. Pregnenolone is more water-soluble than cholesterol and

can then move by passive diffusion to the endoplasmic reticulum where it is converted to progesterone by HSD3B2. Progesterone is then hydroxylated to deoxycorticosterone by CYP21 and is converted to aldosterone by three oxidation reactions: an 11 beta- and 18-hydroxylation, followed by an 18-oxidation, which are catalyzed by aldosterone synthase CYP11B2 (in humans). The expression of CYP11B2 occurs only in the zona glomerulosa, which prevents aldosterone production in the other adrenocortical zones, the zona fasciculata and the zona reticularis (Domalik *et al.* 1991, Ogishima *et al.* 1992, LeHoux *et al.* 1995, Pascoe *et al.* 1995). Thus, aldosterone production involves two key steps: the first (acute) rate-limiting step requires the expression and activity of the steroidogenic acute regulatory (StAR)

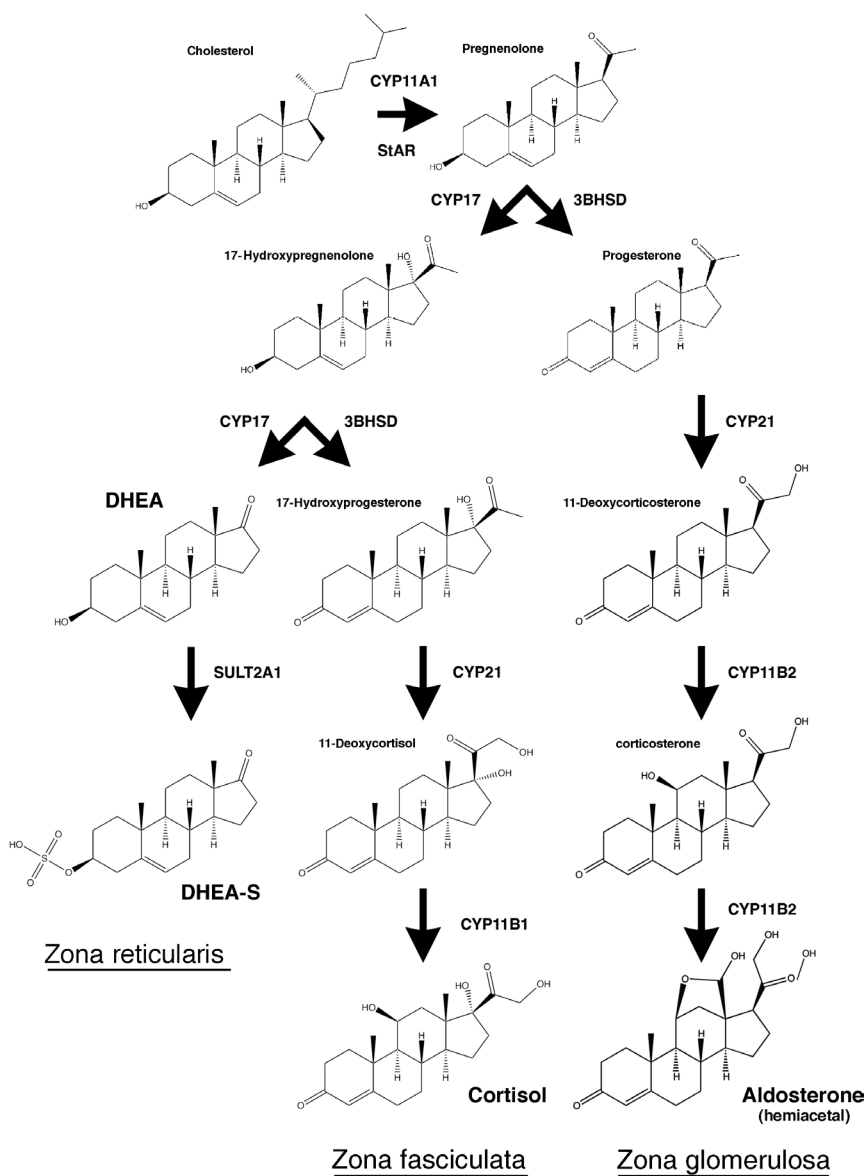


Figure 1

Steroidogenesis in the adrenal cortex. Shown are the pathways by which the adrenal cortex synthesizes aldosterone in the zona glomerulosa, cortisol in the zona fasciculata and dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEA-S) in the zona reticularis. All steroid hormones are synthesized from cholesterol, which is transported by the protein steroidogenic acute regulatory (StAR) protein from the outer to the inner mitochondrial membrane, where the initial step of side-chain cleavage is catalyzed by CYP11A1 to yield pregnenolone. CYP17 is an enzyme with two activities, hydroxylation and lyase, which allows the synthesis of cortisol and DHEA. CYP11B2 is expressed in the zona glomerulosa where the protein encoded by this gene, aldosterone synthase, is required for aldosterone production, catalyzing the last three reactions in aldosterone biosynthesis, 11-hydroxylation followed by 18-hydroxylation and subsequent oxidation to yield an aldehyde. CYP11B1, on the other hand, produces cortisol in the zona fasciculata.

protein, necessary for mitochondrial delivery of precursor cholesterol to the initial synthetic enzyme located in the inner mitochondrial membrane. A second (chronic) phase involves the regulation of the expression of aldosterone synthase (CYP11B2), the enzyme that catalyzes the final reactions in aldosterone biosynthesis.

Various studies suggest that StAR activity is regulated at the transcriptional, translational and post-translational levels (reviewed in Miller & Bose 2011). In addition, StAR is co-translationally phosphorylated in response to elevations in cAMP, thus converting StAR to the active form of the protein (phospho-StAR) (Moore *et al.* 1990). StAR moves cholesterol from the outer to inner mitochondrial membrane, but appears to act on the outer membrane (Stocco 2001, Soccio *et al.* 2002). However, the mechanism by which StAR acts on the outer mitochondrial membrane to stimulate the flow of cholesterol to the inner membrane remains unclear. One hypothesis is that when StAR interacts with protonated phospholipid head groups on the outer mitochondrial membrane, it changes its conformation, opening and closing its cholesterol-binding pocket; this conformational change is presumably required for cholesterol binding and translocation (Tsujiyama & Hurley 2000).

The importance of StAR to steroidogenesis is demonstrated by the finding that StAR mutation leads to a severe form of congenital lipoid adrenal hyperplasia, a group of autosomal recessive disorders resulting from deficiency of enzymes required for the synthesis of steroid hormones in the adrenal gland (Tee *et al.* 1995, Arakane *et al.* 1996, Bose *et al.* 1997). This disease is characterized by reduced or absent steroid hormone production and enlarged adrenal glands. Transcription factors, such as cAMP response element-binding protein (CREB), steroidogenic factor 1 (SF-1) and adrenal hypoplasia critical region on chromosome X gene 1 (DAX-1 or NROB1), may regulate StAR expression by acting directly or indirectly on the promoter region (Sandhoff & McLean 1999, Manna *et al.* 2002, Osman *et al.* 2002, Kim *et al.* 2004, Ragazzon *et al.* 2006, Okuhara *et al.* 2008, Olala *et al.* 2014). On the other hand, the transcription factors for CYP11B2 have been demonstrated to include members of the activating transcription factor (ATF)/CREB family of transcription factors, such as ATF-1, ATF-2, CREB and cAMP-responsive element modulator (CREM), as well as nerve growth factor-induced clone B (NGFIB) (Bassett *et al.* 2004).

The cholesterol required as a precursor for aldosterone synthesis can be synthesized *de novo*

or derived from lipoproteins. *De novo* cholesterol biosynthesis from acyl-coenzyme A occurs through the pathway involving the mevalonate pathway and hydroxymethylglutaryl-CoA reductase (HMG-CoAR), the rate-limiting step in cholesterol biosynthesis (Ribas *et al.* 2016). This enzyme is regulated by exogenous cholesterol, that is, cholesterol absorbed from the diet and/or transported by lipoproteins, by an elegant feedback mechanism, as defined by Brown and Goldstein (reviewed in Brown & Goldstein 2009). Lipoproteins are macromolecules that contain protein and lipid and function mainly to transport lipids and cholesterol throughout the body. The outside portion of lipoproteins is composed of phospholipids, cholesterol and apoproteins possessing hydrophilic groups, and the inside portion of these particles is formed of triglycerides and cholesterol esters (Fig. 2). There are five major groups of lipoproteins, which are divided by their densities: (1) chylomicrons, which carry triglyceride absorbed in the gastrointestinal tract from the intestine to the adipose tissue, liver and skeletal muscle and have the lowest density; (2) very low-density lipoproteins (VLDL) that carry triglyceride from the liver to adipose and other tissues; (3) intermediate-density lipoproteins (IDL), which are formed upon the metabolism of VLDL and can carry cholesterol from the liver throughout the body; IDLs are the intermediate between VLDL and LDL and usually are not detectable in the blood; (4) low-density lipoproteins (LDL), which also carry cholesterol from the liver to other tissues of the body; and (5) high-density lipoproteins (HDL), which collect cholesterol from the tissues of the body and return this lipid to the liver.

Studies suggest that both LDL and HDL are important sources, depending on the organism, of the cholesterol used to produce aldosterone (Capponi 2002, 2004) in response to the classical secretagogues angiotensin II (AngII), elevated extracellular potassium levels or adrenocorticotrophic hormone (ACTH) (Fig. 3). LDL is suggested to provide the bulk of cholesterol to glomerulosa cells (Rone *et al.* 2009), but HDL may also be an important source of cholesterol for aldosterone biosynthesis (Capponi 2002, Rone *et al.* 2009). Indeed, some studies have shown that HDL and LDL have equivalent stimulatory effects on AngII-induced steroidogenesis (measured as pregnenolone production) in bovine adrenal glomerulosa and human adrenocortical carcinoma cells (Cherradi *et al.* 2001). However, whether or not LDL stimulates aldosterone production *in vitro* is at present controversial

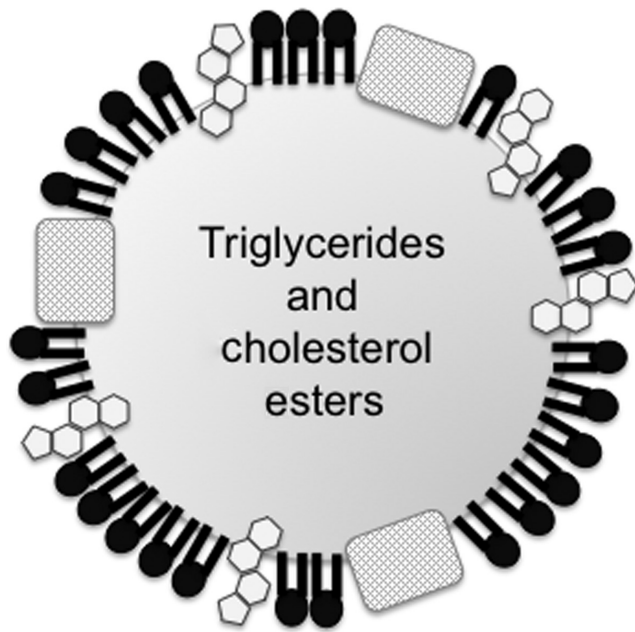


Figure 2

Schematic structure of lipoproteins. Lipoproteins are composed of cholesterol, phospholipids and apolipoproteins surrounding a hydrophobic core of triglycerides and cholesteryl esters. VLDL has a greater diameter (with a very low density) and contains a large amount of triglyceride and small amounts of cholesteryl ester in its core. VLDL is metabolized to IDL and then to LDL, which has a smaller diameter (with a greater density than VLDL or IDL) and a large proportion of cholesteryl esters with small amounts of triglycerides (reviewed in Feingold & Grunfeld 2015).

(e.g., Simpson *et al.* 1989, Saha *et al.* 2012c, reviewed in Capponi 2002, and see below). LDL binds to the LDL receptor (LDLR), with this LDL/LDLR complex internalized by endocytosis (Miller & Bose 2011) to enter the zona glomerulosa cell. LDLR levels can be regulated by the cAMP signaling system but not by AngII (Cherradi *et al.* 2001). On the other hand, AngII can increase the levels of the scavenger receptor, class B, type 1 (SRB1), required for uptake of cholesterol from HDL, and this protein is also expressed in adrenal glomerulosa cells (Cherradi *et al.* 2001). In several studies, HDL has also been found to robustly stimulate aldosterone production in zona glomerulosa cell models (Cherradi *et al.* 2001, Xing *et al.* 2011, Saha *et al.* 2012c).

Obesity, aldosterone and hypertension

Globally, overweight and obesity have reached epidemic proportions. The World Health Organization reports that there are currently nearly 2 billion adults that are overweight and 600,000 million of these are obese; within the United States the trend is similar. Obesity

poses serious health risks that include type 2 diabetes, hypertension, vascular disease, stroke and some forms of cancer (reviewed in Kaidar-Person *et al.* 2011). In addition, although the military places a high regard on physical health and enforces body weight standards, once removed from the military, United States veterans are also at risk for weight issues. Indeed, a recent large-scale study of almost 2 million veterans indicates that approximately 73% of male and 68% of female veterans are overweight and about 33% of males and 37% of females can be categorized as obese (Das *et al.* 2005). Although excess fat deposits have been determined to contribute to increased blood pressure in patients with essential hypertension, and weight gain is associated with increased blood pressure (Juhaeri *et al.* 2002), it is unclear as to how excess weight results in higher blood pressure. Possible mechanisms that have been proposed include an activation of the sympathetic nervous system and/or the renin-angiotensin II-aldosterone system (RAAS) by the extra adipose tissue. In addition, over-secretion of adipose-derived cytokines (known as adipokines) and/or pro-inflammatory cytokines and physical compression of the kidneys, especially with increased visceral adiposity, may also be involved (Bogaert & Linas 2009, da Silva *et al.* 2009, Dall'Asta *et al.* 2009) and reviewed in Canale *et al.* (2013).

On the other hand, several reports have suggested that aldosterone levels are a major link between obesity and hypertension (Egan *et al.* 1994, Kidambi *et al.* 2009, Nagase & Fujita 2009, Kawarazaki & Fujita 2016). Thus, aldosterone/renin ratios are elevated in obese patients (Hiramatsu *et al.* 1981, Rocchini *et al.* 1986), with this association becoming more obvious in obese individuals receiving a high-salt diet, in which renin activity is suppressed (Goodfriend *et al.* 1998). In addition, visceral fat has been suggested to increase aldosterone production (Aneja *et al.* 2004, Krug *et al.* 2007, Fujita 2008, Ronconi *et al.* 2008). This increase in aldosterone may not be related to activation of the RAAS as aldosterone levels in obese patients are higher than what would be predicted by renin concentrations. These observations raise the possibility that in obese patients, there is an alternative aldosterone regulatory system in addition to the classical agonists AngII, serum potassium levels and ACTH.

In addition to an elevated risk of hypertension and cardiovascular disease, obese patients typically have increases in lipoprotein levels, or dyslipidemia, characterized by increased circulating plasma triglycerides, VLDL and low-density lipoprotein (LDL)

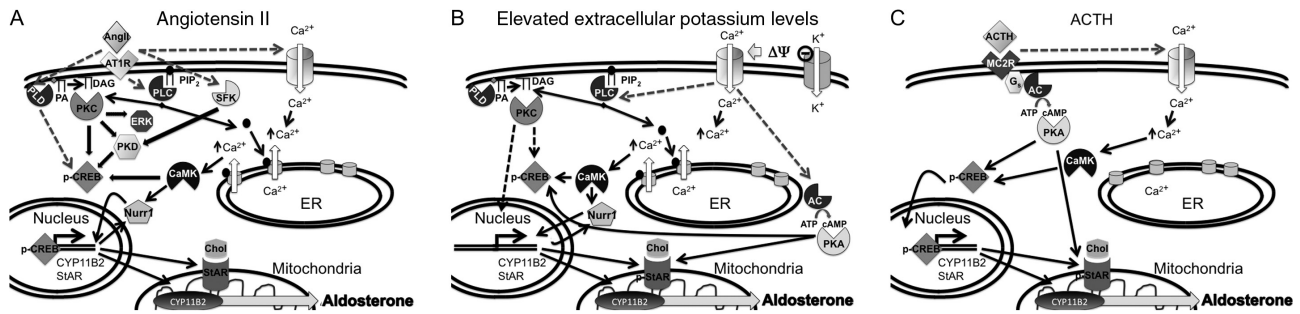


Figure 3

Traditional regulators of aldosterone production and their signaling pathways. The traditional aldosterone secretagogues are angiotensin II (AngII), elevated extracellular potassium (K^+) levels and adrenocorticotropic hormone (ACTH), which function through different signal transduction pathways. (A) AngII binds to angiotensin II type 1 receptors (AT1R) to activate phosphoinositide-specific phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ binds to IP₃ receptors (IP₃R) on the endoplasmic reticulum (ER) to release ER calcium (Ca^{2+}) ions and increase cytosolic Ca^{2+} levels. The increase in intracellular Ca^{2+} concentration activates Ca^{2+} /calmodulin-dependent protein kinases (CaMK) that can phosphorylate and activate various members of the cAMP response element-binding protein (CREB)/activating transcription factor (ATF) family of transcription factors (represented as CREB). This family, as well as Nurr1, the levels of which are also elevated, can induce the expression of genes encoding steroidogenic proteins, such as steroidogenic acute regulatory protein (StAR) and CYP11B2 (coding for aldosterone synthase). AngII also increases Ca^{2+} influx through voltage-dependent Ca^{2+} channels and store-operated Ca^{2+} channels. The other second messenger produced by PLC activity, DAG, activates the protein kinase C (PKC) family of isoenzymes, some of which phosphorylate and activate protein kinase D (PKD); PKD is also known to phosphorylate and activate members of the CREB family of transcription factors. PKC can also activate extracellular signal-regulated kinase-1/2 (ERK), which is able to phosphorylate and activate cholesterol ester hydrolase, to release cholesterol from its storage as cholesteryl ester in lipid droplets, and likely also StAR. DAG can also be generated from the phosphatidic acid (PA) produced by phospholipase D (PLD), which is also activated by AngII and underlies steroidogenesis, although PA can itself serve as a second messenger to mediate the activation of various enzymes (reviewed in Bollag 2016). In addition, AngII working through the AT1R activates Src family kinases (SFK), which can also stimulate PKD activity and appear to underlie aldosterone production. Finally, AngII can also activate JAK2 (not shown). (B) An elevated extracellular K^+ level uses many of the same signal transduction pathways as AngII. In this case, an increased K^+ concentration depolarizes the glomerulosa cell to open voltage-dependent Ca^{2+} channels, increase intracellular Ca^{2+} levels and activate CaMK, with its downstream targets. There is evidence that K^+ , like AngII, may also induce phosphoinositide hydrolysis, through an unknown mechanism, although controversy remains concerning this point. Elevated K^+ also seems to activate PKC and PLD (Betancourt-Calle *et al.* 2001), which likely play similar roles as in AngII's effects. Another signal that may or may not be used by elevated K^+ levels to mediate steroidogenesis is the adenylate cyclase (AC)/cAMP/cAMP-dependent protein kinase (also known as protein kinase A or PKA) pathway. PKA is also known to phosphorylate and activate CREB family transcription factors as well as StAR. (C) ACTH stimulates aldosterone production by binding to its receptor, the melanocortin 2 receptor (MC2R), to activate AC that converts ATP to cAMP. cAMP then stimulates the activity of PKA, thereby activating CREB family transcription factors and StAR. ACTH also increases Ca^{2+} influx through an unclear mechanism, and the resulting increased Ca^{2+} cytosolic levels can activate CaMK, with its downstream effectors. These pathways have been recently reviewed in Bollag (2014).

(Repas 2011); this dyslipidemia is thought to play an important role in the damaging effects of being obese. Importantly, triglyceride levels (a surrogate measure of VLDL levels) are positively correlated with serum aldosterone levels in obese subjects (Goodfriend *et al.* 1995), and eplerenone, an MR antagonist, decreases blood pressure and serum triglyceride levels in hypertensive individuals with or without metabolic syndrome (Sato & Fukuda 2010). In addition to providing cholesterol for steroid hormone biosynthesis as described previously, recent results suggest that lipoproteins can increase aldosterone levels by initiating signaling pathways that regulate steroidogenesis. For example, HDL induces the expression of CYP11B2 through calcium-activated signaling events, such that a voltage-dependent calcium channel blocker and a calcium/calmodulin-dependent protein kinase inhibitor decrease the HDL-induced effects (Xing *et al.* 2011).

In addition, accumulating evidence supports an ability of VLDL to induce various signal transduction events. VLDL, with a triglyceride content of approximately 50%, is synthesized by the liver and is responsible for transporting fatty acids and triglyceride from the liver to peripheral tissues. Upon entering the circulation, nascent VLDL picks up ApoC-II and ApoE proteins from HDL to convert to its mature form. As VLDL circulates through the body, triglyceride is removed by lipoprotein lipase for storage or energy production, forming first an intermediate-density lipoprotein (IDL) and then a low-density lipoprotein (LDL). Although the receptor for VLDL is present in the adrenal gland (Iijima *et al.* 1998), until recently, the function of VLDL in adrenal steroidogenesis had not been thoroughly examined. However, studies in other tissues have established that VLDL is able to initiate various signaling pathways, suggesting roles for

VLDL beyond that of merely a lipid transport particle (see below).

Previous studies have shown that VLDL can regulate signaling pathways in different tissue types. For example, HepG2 cells incubated with VLDL showed an increase in radiolabeled inositol 1,4,5-trisphosphate (IP_3) levels (suggesting a stimulation of a phosphoinositide-specific phospholipase C), arachidonate release and protein kinase C and extracellular signal-regulated kinase-1 and -2 (ERK1/2) activities (Banfi *et al.* 1999). Furthermore, pharmacologic inhibition of phospholipase C, endoplasmic reticulum calcium release or ERK1/2 activation resulted in a decrease in the VLDL-induced secretion of plasminogen activator inhibitor type 1 (PAI-1) (Banfi *et al.* 1999), which promotes platelet aggregation and clot formation (reviewed in Olufadi & Byrne 2006). In addition, in vascular smooth muscle cells treated with VLDL, Src-dependent assembly of fibronectin and type I collagen is inhibited (Frontini *et al.* 2009), and in PC-3 prostate cancer cells, the lipoprotein can stimulate the proliferation and activation of the ERK1/2 and Akt signaling pathways (Sekine *et al.* 2007). Incubation of macrophage-derived cell lines with VLDL also stimulates ERK1/2 activity, in a protein kinase C (PKC)-dependent manner, leading to increased expression of the VLDL receptor (Liu *et al.* 2009). Additionally, VLDL appears to be a negative regulator of the Wnt pathway in endothelial cells, in which selective knockdown of its receptor resulted in the upregulation of low-density lipoprotein (LDL) receptor-related protein 5 and 6 (LRP5/6) expression and activation of beta-catenin (Chen *et al.* 2007). Treatment of endothelial cells with VLDL or oxidized VLDL upregulates the expression of certain genes and decreases that of others (Norata *et al.* 2003). The expression of some genes is altered similarly by both VLDL and oxidized VLDL, whereas the intact vs modified lipoprotein also modulates the expression of distinct sets of genes (Norata *et al.* 2003). Thus, VLDL activates different signaling pathways in various cell types.

VLDL and aldosterone production

Based on the fact that VLDL levels are elevated in obesity (Repas 2011), we sought to determine whether this lipoprotein might link obesity and hypertension by investigating whether VLDL can stimulate aldosterone synthesis in cell models of the zona glomerulosa. In collaboration, we showed that VLDL stimulates aldosterone production in multiple zona glomerulosa

cell models, including primary cultures of bovine adrenal glomerulosa cells and human adrenocortical cells as well as the human adrenocortical carcinoma cell line, H295R cells (Xing *et al.* 2012). In subsequent studies, we also showed a similar effect in the HAC15 cell line (Tsai *et al.* 2014), a clone of H295R, which shows good responses to AngII and ACTH (Wang & Rainey 2012, Wang *et al.* 2012). The ability of VLDL to enhance CYP11B2 expression is not additive with a near-maximal concentration of angiotensin II (10 nM) but VLDL enhances the stimulatory effect of the cAMP pathway agonist forskolin, even at a high dose (10 μ M) that causes a large increase in cAMP levels and aldosterone production in H295R cells (Fassnacht *et al.* 1998). In addition, VLDL's stimulation of steroidogenesis was not unique to aldosterone production. Thus, VLDL also enhanced the production of dehydroepiandrosterone (DHEA) in H295R cells, although less robustly than its stimulatory effect on aldosterone and without affecting CYP17 expression (Xing *et al.* 2012). On the other hand, VLDL did not significantly alter cortisol formation, although it did significantly increase CYP11B1 mRNA levels; VLDL did not affect the expression of CYP11A1 or CYP21 after a 24-h exposure (Xing *et al.* 2012). Similar effects have been observed in H295R cells exposed to HDL, with this lipoprotein inducing CYP11B2 expression without affecting mRNA levels of CYP11A1, CYP21 or HSD3B2 (Xing *et al.* 2011).

The steroidogenic effect of VLDL is mediated by its ability to increase both StAR and CYP11B2 expression likely through a calcium-initiated signaling cascade. Thus, VLDL increases cytosolic calcium levels in a manner similar to AngII, and inhibitors of calcium/calmodulin-dependent protein kinases (CaMK) reduce VLDL-induced steroidogenic events (Xing *et al.* 2012). In addition, voltage-dependent calcium channel antagonists also inhibit VLDL-induced aldosterone production, suggesting the possibility that VLDL treatment initiates calcium signaling through effects on calcium channels (Xing *et al.* 2012). Nevertheless, calcium channel blockers can also inhibit AngII-elicited aldosterone secretion (e.g., Kojima *et al.* 1985a,b and reviewed in Bollag 2014, Spat & Hunyady 2004), despite the fact that changes in cytosolic calcium concentrations in the case of this agonist result from both IP_3 -induced release from intracellular calcium stores and calcium influx through calcium channels (reviewed in Bollag 2014). Therefore, in additional studies, we demonstrated that xestospongine C, an IP_3 receptor blocker (Gafni *et al.* 1997), reduces aldosterone production in response to VLDL without affecting adrenocortical cell viability (Tsai *et al.* 2016).

A similar ability to inhibit VLDL-elicited aldosterone production is observed with the phosphoinositide-specific phospholipase C (PI-PLC) inhibitor U73122; U73122 also blocks a VLDL-induced increase in radiolabeled levels of diacylglycerol, the other product (in addition to IP₃) of PI-PLC-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (Tsai *et al.* 2016). Thus, VLDL appears to utilize signaling pathways similar to those of AngII, an idea supported by our result that sub-maximal concentrations of AngII and VLDL act in an additive manner to stimulate aldosterone production (Tsai *et al.* 2016). On the other hand, HDL does not enhance AngII- (or elevated potassium-) induced aldosterone production but does increase that elicited by forskolin (Xing *et al.* 2011). Nevertheless, aldosterone production stimulated by HDL, like that induced by VLDL, is also decreased by inhibitors of calcium signaling (Xing *et al.* 2011), as well as of scavenger receptor, class B, type 1 (SRB1) (Xing *et al.* 2011, Saha *et al.* 2012b), extracellular signal-regulated kinase-1 and -2, Janus kinase 2 and protein kinase C (Saha *et al.* 2012b). Thus, HDL also uses many of the same pathways as does AngII (reviewed in Bollag 2014), despite showing more lot-to-lot variability and lower efficacy.

It should be noted that the *in vitro* results observed are corroborated with studies performed *in vivo*, in which rats were fed a chow diet supplemented with liquid sucrose; this manipulation is known to raise triglyceride levels. Indeed, serum triglyceride levels were elevated in these rats in comparison with the control group that received a chow diet alone (Xing *et al.* 2012). As serum triglycerides were measured in a post-absorptive state, these values serve as a surrogate for VLDL levels. These studies also demonstrated increased CYP11B2 expression in the adrenal glands of the liquid sucrose-fed animals, and in fact, serum triglyceride and CYP11B2 mRNA levels show a strong correlation (Xing *et al.* 2012). This result suggests that VLDL may have similar effects *in vivo* as it does *in vitro*.

In further studies, a key role of phospholipase D (PLD) in the VLDL response was also demonstrated (Tsai *et al.* 2014), similar to the involvement of this lipid-metabolizing enzyme in AngII- and sphingosine 1-phosphate-induced aldosterone secretion (e.g., Brizuela *et al.* 2006, Qin *et al.* 2010 and reviewed in Bollag 2016). Thus, PLD inhibitors reduce VLDL-induced CYP11B2 expression and aldosterone secretion, as does overexpression of lipase-inactive mutants of either PLD1 or PLD2 (Tsai *et al.* 2014). Inhibition of PLD by either method also results in decreased VLDL-stimulated StAR levels, presumably by reducing the phosphorylation

and activation of cAMP response element modulator (CREM) (Tsai *et al.* 2014), a member of the cAMP response element-binding (CREB) protein family of transcription factors. Members of this group have been shown to bind to and activate StAR promoter activity (Clem *et al.* 2005, Jo *et al.* 2005, Olala *et al.* 2014); the CYP11B2 promoter also possesses cAMP response elements to which this transcription factor family binds (Clyne *et al.* 1997 and reviewed in Bollag 2014).

Similar results concerning the ability of VLDL to stimulate aldosterone production were also obtained by Saha and coworkers (Saha *et al.* 2012a). These authors demonstrated that VLDL and *in vitro*-modified oxidized and glycoxidized VLDL increase aldosterone production in H295R cells by inducing CYP11B2 expression. Both the intact and modified lipoproteins function through ERK1/2, cAMP-dependent protein kinase (protein kinase A or PKA) and Janus kinase-2, as inhibitors of these pathways reduce lipoprotein-stimulated steroidogenesis (Saha *et al.* 2012a). Results with a scavenger receptor class B type 1 (SR-B1) inhibitor also suggested that these VLDLs exert their actions through SR-B1, rather than other known receptors for VLDL, such as the VLDL receptor, the LDL receptor (Chappell *et al.* 1993) or CD36 (Calvo *et al.* 1998), as well as through activation of ERK1/2 (Saha *et al.* 2012a) (Table 1). Subsequent studies by this group also showed that *in vivo* modified VLDL lipoproteins also induce similar effects, such that VLDL from individuals with impaired glucose tolerance promotes greater aldosterone secretion than that isolated from subjects with normal glucose tolerance (Saha *et al.* 2013). It should be noted that others have demonstrated that high-density lipoprotein (HDL) can also stimulate aldosterone production (Cherradi *et al.* 2001, Saha *et al.* 2012a). We have determined that VLDL is more efficacious and less variable from lot-to-lot than HDL (Xing *et al.* 2012). Thus, in primary cultures of both bovine adrenal glomerulosa and human adrenocortical cells, VLDL is more effective than HDL at stimulating aldosterone production (unpublished data), as was also observed by Saha and coworkers (Saha *et al.* 2012a). These authors also observed an ability of native LDL, as well as modified LDL, to stimulate aldosterone production in H295R human adrenocortical carcinoma cells, although to a lesser extent than VLDL (Saha *et al.* 2012a). However, in our hands, LDL has no such steroidogenic effect (unpublished data). Together, these results support an interesting role for VLDL as an aldosterone secretagogue and suggest that this agent acts through activation of various signal transduction pathways known to induce steroidogenesis in the zona glomerulosa (Fig. 4).

Table 1 Signaling pathways used by VLDL* to mediate aldosterone production.

Signal	Evidence	References
VLDL binds to SR-B1 VLDL induces phosphoinositide hydrolysis	Cell response [†] reduced by the SR-B1 inhibitor, BLT-1 VLDL increases cytosolic Ca ²⁺ and DAG levels; cell response inhibited by U73122, a PLC inhibitor (as is the increase in DAG) and xestospongine, an IP ₃ receptor inhibitor; the increase in cytosolic calcium levels is inhibited by BAPTA-AM, an intracellular calcium chelator	Saha <i>et al.</i> (2012a,c) Xing <i>et al.</i> (2012), Tsai <i>et al.</i> (2016)
VLDL activates PKC	Cell response (and StAR protein levels) decreased by PKC inhibitors (Ro31-8220 and Gö6983); CREM and ERK phosphorylation also reduced by PKC inhibitors	Tsai <i>et al.</i> (2016)
VLDL activates CaMK VLDL increases Ca ²⁺ influx through voltage-dependent Ca ²⁺ channels VLDL activates PLD	Cell response inhibited by KN93, a CaMK inhibitor Cell response inhibited by nifedipine, a voltage-dependent Ca ²⁺ channel antagonist VLDL increases a marker of PLD activity (PET); cell response (as well as CREM phosphorylation and StAR protein levels) decreased by PLD inhibitors (CAY10593, CAY10594 and FIPI) and overexpression of lipase-dead PLD mutants	Xing <i>et al.</i> (2012) Xing <i>et al.</i> (2012) Tsai <i>et al.</i> (2014)
VLDL activates ERK1/2	VLDL increases the phosphorylation (activation) of ERK1/2; cell response reduced by U0126, a MEK inhibitor that prevents ERK activation	Saha <i>et al.</i> (2012a), Tsai <i>et al.</i> (2016)
VLDL activates JAK2	VLDL increases the phosphorylation of STAT3, a JAK2 substrate; cell response decreased by AG490, a JAK2 inhibitor	Saha <i>et al.</i> (2012a)
VLDL activates PKA VLDL increases StAR expression	Cell response reduced by H89, a PKA inhibitor VLDL increases StAR mRNA and protein levels	Saha <i>et al.</i> (2012a) Saha <i>et al.</i> (2012a), Xing <i>et al.</i> (2012)
VLDL increases Nurr1 expression VLDL activates CREB/ATF transcription factors	VLDL increases Nurr1 mRNA and protein levels VLDL increases phosphorylation of ATF2 and CREM	Xing <i>et al.</i> (2012) Xing <i>et al.</i> (2012), Tsai <i>et al.</i> (2014, 2016)

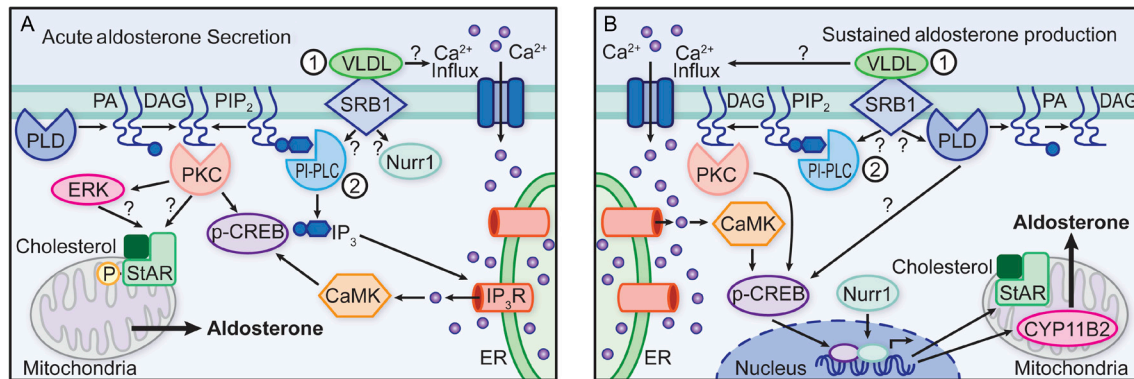
*ATF, activating transcription factor; CaMK, calcium/calmodulin-dependent kinase; CREB, cAMP response element-binding protein; CREM, cAMP responsive element modulator; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; IP₃, inositol 1,4,5-trisphosphate; JAK2, Janus kinase 2; PET, phosphatidyl ethanol; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; SR-B1, scavenger receptor, class B, type 1; StAR, steroidogenic acute regulatory protein; STAT3, signal transducer and activator of transcription 3; VLDL, very low-density lipoprotein.

[†]Cell response monitored: aldosterone production and/or CYP11B2 expression.

Some of these signals are activated acutely, within minutes to an hour of exposure of glomerulosa cells to secretagogues such as VLDL. These include binding of the agonist to its receptor, which in the case of VLDL appears to be SR-B1, and initiation of phosphoinositide hydrolysis upon activation of PI-PLC. Within minutes, the resultant generation of diacylglycerol and IP₃ activates PKC that stimulates PLD activity and releases intracellular calcium stores to raise cytosolic calcium levels, respectively. Other signals are activated in a more delayed fashion to maintain and sustain steroidogenesis. These sustained signals are generated over several hours and include elevations in protein levels of transcription factors, such as Nurr1, and increases in CYP11B2 transcription. For additional details about acute and sustained effects of VLDL, please see Fig. 4 and its legend.

It should be noted that in all of the previously mentioned studies, concentrations of VLDL of

30–100 µg protein/mL were used. Based on the calculation that protein represents approximately 10% and triglyceride levels roughly 55% of the VLDL particle (Kuchinskiene & Carlson 1982), 100 µg protein/mL VLDL translates to a concentration of about 55 mg/dL triglyceride, well below the 150 mg/dL threshold for normal triglyceride levels. Therefore, it seems that serum VLDL levels likely contribute to physiological aldosterone levels, particularly in view of the fact that lower concentrations of VLDL and AngII act additively to promote aldosterone production (Tsai *et al.* 2016). Thus, the supraphysiological AngII levels required to stimulate significant aldosterone production *in vitro* may relate to the lack of *in vivo* contributing factors such as VLDL in the culture environment. In addition, as VLDL production in the liver is enhanced when lipid substrate supplies are increased (Sundaram & Yao 2010), for example, after ingestion of an animal-based diet, this ability of VLDL to stimulate aldosterone production might have been an evolutionary physiological mechanism to

**Figure 4**

The VLDL-activated pathway to aldosterone production in the zona glomerulosa. (A) Shown are the early effects of VLDL that promote acute aldosterone secretion. (1) Very low-density lipoprotein (VLDL) apparently binds to the scavenger receptor, class B, type 1 (SR-B1) and (2) activates, through an unknown mechanism, a phosphoinositide-specific phospholipase C (PI-PLC) that cleaves phosphatidylinositol 4,5-bisphosphate (PIP₂) to yield inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). Various signaling cascades are then initiated simultaneously. For example, IP₃ binds to IP₃ receptors (IP₃R) on the endoplasmic reticulum to release the stored calcium and increase cytosolic calcium levels (occurring within a few minutes of VLDL exposure); the increased cytosolic calcium levels activate calcium/calmodulin-dependent protein kinase (CaMK). At the same time, the other signal formed within minutes is DAG; DAG activates protein kinase C (PKC) isoforms to stimulate the activity of extracellular signal-regulated kinase (ERK), again within minutes of VLDL treatment. VLDL-stimulated ERK, like the angiotensin II (AngII)-activated enzyme, may, in turn, phosphorylate and activate cholesterol ester hydrolase (Cherradi *et al.* 2003), also known as hormone-sensitive lipase, to release cholesterol esters stored in lipid droplets. ERK also likely phosphorylates StAR, thereby enhancing its cholesterol transport activity (Poderoso *et al.* 2008). In addition, within minutes of VLDL exposure, activated PKC induces phosphorylation and activation of members of the activating transcription factor/cAMP response element binding protein family of transcription factors (ATF/CREB) to increase after a few hours the levels of steroidogenic acute regulatory protein (StAR). StAR allows transport of cholesterol into the inner mitochondrial membrane where it can be acted upon by the cholesterol side-chain cleavage complex (CYP11A1) located there to initiate steroidogenesis. (B) The initial (1) binding of VLDL to SRB1 and (2) activation of PI-PLC also activates phospholipase D (PLD) (within an hour, the earliest time tested) via an unknown mechanism. PLD catalyzes phosphatidylcholine hydrolysis to yield phosphatidic acid (PA), which can be dephosphorylated to DAG (by lipins) to sustain PKC activation and aldosterone production. PA may also have its own effector enzymes, such as Raf-1, a protein kinase upstream of ERK, as reviewed in Bollag (2014). PKC-elicited phosphorylation of ATF/CREB also induces the expression not only of StAR but also of aldosterone synthase, encoded by the gene CYP11B2, after 6–12 h. (By analogy to angiotensin II, CaMK also may induce the expression of members of this family (Felizola *et al.* 2014). CYP11B2 transcription is also promoted by the VLDL-induced increase in the mRNA and protein expression of the transcription factor Nurr1 (the protein levels of which are elevated after several hours of VLDL treatment). CYP11B2 in the mitochondria catalyzes the final steps in the production of aldosterone.

promote the retention of dietary sodium when access to this mineral was uncertain. Based on our preliminary results showing a dose-dependent increase in CYP11B2 promoter activity and aldosterone production in the range of 0–300 µg protein/mL VLDL (data not shown), we presume that pathologically high concentrations of VLDL, as seen for example in obesity (Repas 2011), would play an even more important role in elevating serum aldosterone concentrations *in vivo*.

On the other hand, serum triglyceride levels can be decreased by certain medications. For example, statins decrease not only serum cholesterol but also triglyceride levels; in addition, data in the literature suggest that statins can reduce blood pressure while also improving dyslipidemia (e.g., Myerson *et al.* 2005 and reviewed in Drapala *et al.* 2014). Several lines of evidence support the idea that statins can regulate various components of the renin-angiotensin II-aldosterone system (e.g., Long *et al.* 2015 and reviewed in Chiong & Miller 2002, Drapala *et al.* 2014). Indeed, a recent well-controlled study demonstrated that statin use in hypertensive and

diabetic subjects with statins, in particular lipophilic statins, reduces basal serum aldosterone levels as well as the aldosterone response to angiotensin II and low-sodium diet (Baudrand *et al.* 2015). Although these authors also showed that statins inhibit aldosterone production *in vitro* (Baudrand *et al.* 2015), it is tempting to speculate that at least some of the antihypertensive effect of the statins may be the result of their ability to decrease VLDL, with the lower levels of this lipoprotein thereby also resulting in reduced aldosterone production. Such an idea is consistent with data in hereditary hypertriglyceridemic rats, in which both a statin and the mineralocorticoid antagonist spironolactone are able to inhibit elevated blood pressure values (Torok *et al.* 2007). Fibrates reduce serum triglyceride levels as well; these agents also appear to decrease blood pressure (Jonkers *et al.* 2001, Gilbert *et al.* 2013). Thus, although the exact mechanism(s) by which the fibrates and statins improve hypertension is/are not yet known, clearly these agents appear to be beneficial, and further research is needed.

Conclusion

Many medications used to treat hypertension antagonize some aspect of the aldosterone pathway. Thus, inhibitors of the synthesis or action of angiotensin II (AngII), the primary physiological regulator of aldosterone production, all function by interfering with the secretion or action of aldosterone. These include angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs), mineralocorticoid receptor blockers (spironolactone and eplerenone) and antagonists of aldosterone action (amiloride and triamterene). Although these medications are important therapies in the armamentarium for treating various cardiovascular conditions, not only can these therapies have serious side effects but also their control of blood pressure is often sub-optimal, suggesting the necessity of identifying additional selective agents. In addition, the likely presence of adipose-derived releasing factors, such as 12,13-epoxy-9-keto-10(trans)-octadecenoic (EKODE) (Goodfriend *et al.* 2004, reviewed in Kawarazaki & Fujita 2016), stimulating aldosterone production in obesity-associated hypertension suggests that the ARBs and ACE inhibitors at least will be relatively ineffective at controlling high blood pressure resulting from excess weight. Moreover, the involvement of VLDL in increasing aldosterone secretion suggests that, particularly in cases of obesity-associated hypertension, this molecule and/or its downstream signaling pathway may represent reasonable targets for the development of drug therapies to treat high blood pressure. Nevertheless, other factors derived from adipocytes may also contribute to obesity-related hypertension. For example, a recent study demonstrated a role for adipocyte-released leptin, the serum levels of which are proportional to fat mass, in regulating aldosterone levels both *in vitro* and *in vivo* (Huby *et al.* 2015 and reviewed in Xie & Bollag 2016). Other studies have suggested effects of adipocyte-released cytokines, or adipokines, such as tumor necrosis factor- α , interleukin-6 and complement-C1q TNF-related protein 1 (CTRP1) (Jeon *et al.* 2008), on aldosterone levels as well (Briet & Schiffrin 2011, Xie & Bollag 2016). Therefore, it is clear that further studies are needed to define not only the complete pathway through which VLDL promotes aldosterone production in the adrenal zona glomerulosa but also the possible involvement of other adipokines and adipocyte-derived factors.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding

Dr Bollag was supported by a VA Research Career Scientist Award. The contents of this article do not represent the views of the Department of Veterans Affairs or the United States Government.

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Received in final form 21 November 2016

Accepted 2 December 2016

Accepted Preprint published online 2 December 2016