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VLDL-activated cell signaling pathways that stimulate adrenal cell aldosterone production

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Abstract

Aldosterone plays an important role in regulating ion and fluid homeostasis and thus blood pressure, and hyperaldosteronism results in hypertension. Hypertension is also observed with obesity, which is associated with additional health risks, including cardiovascular disease. Obese individuals have high serum levels of very low-density lipoprotein (VLDL), which has been shown to stimulate aldosterone production; however, the mechanisms underlying VLDL-induced aldosterone production are still unclear. Here we demonstrate in human adrenocortical carcinoma (HAC15) cells that submaximal concentrations of angiotensin II and VLDL stimulate aldosterone production in an additive fashion, suggesting the possibility of common mechanisms of action. We show using inhibitors that VLDL-induced aldosterone production is mediated by the PLC/IP₃/PKC signaling pathway. Our results suggest that PKC is upstream of the extracellular signal-regulated kinase (ERK) activation previously observed with VLDL. An understanding of the mechanisms mediating VLDL-induced aldosterone production may provide insights into therapies to treat obesity-associated hypertension.

Keywords

adrenal cortex; aldosterone; obesity; phospholipase C; protein kinase C; VLDL; zona glomerulosa

1. Introduction

The mineralocorticoid hormone aldosterone is responsible for sodium retention that directly impacts body fluid levels and blood pressure. Excessive aldosterone production causes about

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8% of the diagnosed cases of hypertension [1], a disorder that is associated with an increased risk of cardiovascular disease, stroke, visual loss and renal disease [2,3]. In addition, hyperaldosteronism contributes to congestive heart failure and cardiac fibrosis [4], which increases patient morbidity and mortality. Aldosterone is synthesized from cholesterol stores in adrenal zona glomerulosa cells and is mainly regulated by the traditional secretagogues angiotensin II (AngII) and serum potassium levels. (ACTH can also potentially increase aldosterone production *in vitro* but its effect *in vivo* is transient.) The two key rate-limiting steps in aldosterone biosynthesis are steroidogenic acute regulatory (StAR) protein, which is involved in transporting cholesterol to the inner mitochondrial membrane to initiate steroid hormone synthesis, and aldosterone synthase (CYP11B2), which catalyzes the final reactions in aldosterone production.

Globally, overweight and obesity are reaching epidemic proportions. According to reports from the World Health Organization, currently 2 billion adults are overweight and 600,000 million of these people are obese [5]. Obesity is linked to serious health risks, for example, hypertension, type II diabetes, vascular disease, stroke and some forms of cancer [6]. In obese patients plasma levels of lipoproteins, such as triglycerides, low-density lipoprotein and very-low-density lipoprotein (VLDL) are typically elevated (dyslipidemia) in association with the increased risk of cardiovascular disease and hypertension. Previous studies have indicated a positive correlation between triglyceride levels and serum aldosterone in both normal and metabolic-syndrome subjects [7]. Indeed, several studies have indicated that aldosterone levels are a major link between obesity and hypertension [8–11]. Thus, aldosterone levels are typically elevated in obese individuals and drop upon weight loss [11], with a correlation between decreases in serum aldosterone levels and in blood pressure with the reduction in weight [12]. Greater amounts of visceral fat are also suggested to result in increased aldosterone production [13]; adipose tissue can secrete a variety of adipokines, such as leptin, tumor necrosis factor- α , interleukin-6, complement-C1q, TNF-related protein 1 and fatty acids. In obese patients these adipokines have been suggested to be factors that stimulate aldosterone production via an alternative aldosterone regulatory system [14–16].

Very-low-density lipoprotein (VLDL) is synthesized in the liver as a light-density lipoprotein with a composition of approximately 50% triglyceride (TG) and 10% protein [17]. In addition to its well-known function of transporting fatty acids and triglyceride from the liver to peripheral tissues, VLDL has been suggested to activate several signaling cascades in different tissues, including adrenocortical cells. Indeed, we have previously shown that VLDL stimulates aldosterone secretion in a variety of zona glomerulosa cell models, including primary cultures of human adrenocortical and bovine glomerulosa cells [18] and in the human adrenocortical cell line H295R and its clone [19], HAC15 [20]. This effect of VLDL to increase aldosterone biosynthesis is related to its ability to increase the expression of CYP11B2 (the gene encoding aldosterone synthase) and steroidogenic acute regulatory (StAR) protein levels. In addition, VLDL stimulates CYP11B1 expression; however, it has no effect on cortisol production either in the H295R or in primary human adrenocortical cells although it increases DHEA production [18]. The expression of other steroidogenic genes, such as 17 α -hydroxylase (CYP17), is not altered by VLDL [18]. Saha et al. [21,22] also demonstrated an ability of VLDL, both native and modified *in vitro*, to

stimulate CYP11B2 expression and aldosterone production. VLDL acted, at least in part, through scavenger receptor, class B, type 1 (SR-B1), cAMP-dependent protein kinase (protein kinase A or PKA), extracellular signal-regulated kinase-1 and -2 (ERK1/2) and Janus kinase-2 (JAK2) [21]. In other cases, VLDL appears to be altered in the circulation *in vivo* (e.g., in patients with diabetes) and this modified VLDL can also stimulate cell signaling events [23]. In addition to VLDL, high-density lipoprotein (HDL) has also been found to induce aldosterone production [24], although VLDL is more effective than HDL [18]. Others have reported that LDL can also stimulate aldosterone biosynthesis [21], although this effect has been somewhat controversial (reviewed in [25]).

AngII regulates aldosterone production by activating, via the AngII receptor, type 1 (AT1R), a phosphoinositide-specific phospholipase C (PLC), which hydrolyzes the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate to generate two second messengers, inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) ([26–28][29]). IP₃ then releases calcium from the endoplasmic reticulum (ER) and activates calcium/calmodulin-dependent protein kinase. On the other hand, DAG stimulates PKC activity, which has been suggested to sustain AngII-elicited aldosterone production in bovine adrenal glomerulosa cells [26,28,30,31]. Sustained calcium influx is required to maintain aldosterone production in response to AngII, such that inhibition of voltage-dependent calcium channels decreases hormone-induced steroidogenesis (reviewed in [29]).

In the present study we examined the role of the PLC/PKC signaling pathway in VLDL-regulated aldosterone production. Our findings demonstrate that VLDL increased DAG levels; in addition, a PLC inhibitor, as well as two PKC inhibitors and an IP₃ receptor inhibitor, reduced aldosterone production and CYP11B2 expression stimulated by VLDL. Taken together, our results suggest that VLDL induces PLC-mediated phosphoinositide hydrolysis and the resultant production of IP₃ and DAG to mediate aldosterone production. Finally, our data suggest that PKC downstream of PLC and phosphoinositide hydrolysis mediates VLDL-induced activation of ERK, a protein kinase that has also been reported to be involved in steroidogenesis [32].

2. Materials and Methods

2.1. Cell culture

A clone of the H295R cell line, human adrenocortical (HAC15) cells [33] were cultured in DMEM/F12 (Gibco) with 10% Cosmic calf serum (Thermo Scientific), 0.1% gentamicin (Invitrogen), 1% penicillin/streptomycin (Life Technologies), and 1% ITS (insulin-transferrin-selenous acid with linoleic acid and bovine serum albumin) (Becton Dickinson Labware). Cells were plated in 6- or 12-well plates and cultured at 37°C for 2 days. Before experimentation, cells were incubated with low-serum experimental medium (0.1% Cosmic calf serum) for 24 hours. For inhibitor studies, cells were preincubated with either the PLC inhibitor U73122 (Enzo Life Sciences), the IP₃ receptor inhibitor xestospongine C (Cayman Chemicals) or one of two PKC inhibitors, Ro31-8220 or Gö6983 (Calbiochem) for 30 minutes before treatment with or without VLDL (Kalen Biomedical, LLC) or AngII (Sigma-Aldrich) for the appropriate time periods. None of the inhibitors used in this study exhibited

toxicity towards HAC15 cells (Supplemental Figure 1), determined using MTT assays as described below.

2.2. Cell toxicity assay

Cell toxicity was determined using an MTT cell proliferation assay kit (Vybrant). Briefly, HAC15 cells were plated in 96-well microplates with growth medium for 48 hours; after 24 hours in low-serum medium the cells were incubated with fresh low-serum medium with or without inhibitors for 30 minutes prior to stimulation with or without AngII for 24 hours in low-serum medium. Cells were then incubated with the MTT solution provided in the kit at 37°C for 4 hours. After labeling, SDS-HCl solution was added to solubilize the cells, which were incubated at 37°C for another 4–18 hours. The absorbance at 570 nm was measured in the SDS lysate using a PolarSTAR plate reader.

2.3. Aldosterone production

After a 24-hour treatment of the HAC15 cells with or without VLDL or AngII (after a 30-minute pretreatment with or without PLC, IP₃ receptor or PKC inhibitors), the supernatants were collected and assayed with a solid-phase radioimmunoassay kit (Siemens Products, Los Angeles, CA) according to the supplier's directions.

2.4. RNA Extraction, cDNA Synthesis, and Real-Time RT-PCR

RNA was extracted from cells using an RNeasy kit (5 PRIME, Gaithersburg, MD) following the protocols of the manufacturer. RNA concentration was determined using a Nanodrop instrument (NanoDrop Technologies, Wilmington, DE). Total RNA was reverse transcribed with a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA) following the manufacturer's recommendations. Primer/probe sets for the amplification of target sequences were purchased from Applied Biosystems, and PCR amplifications were performed using the ABI Step One Fast Real-Time PCR System using the reaction parameters recommended by the manufacturer: 20 µl total volume consisting of primer/probe mix and Fast Reagent Master Mix (Applied Biosystems), with 18S or cyclophilin (PPIA; Applied Biosystems) used as an endogenous normalization control gene. Water served as the negative control. The relative gene expression was calculated by the $\Delta\Delta C_t$ method and the resultant values normalized to a calibrator. In every experiment the calibrator was the control (untreated or vehicle-treated) sample. Final results were expressed as n-fold difference in gene expression relative to the housekeeping gene and calibrator and were analyzed and plotted as the means $\pm$ SEM of these fold values.

2.5. Western analysis

After treatment with or without inhibitors for 30 minutes, followed by stimulation with VLDL for 30 minutes, 3 hours or 24 hours, human adrenocortical cells (HAC15) were harvested with warm lysis buffer [0.1875M Tris-HCl (pH 8.5), 3% SDS, 1.5 mM EGTA]. Sample buffer containing β -mercaptoethanol, glycerol and bromophenol blue was added to constitute Laemmli buffer, and equal volumes of boiled sample were loaded on to a 10% SDS gel. Resolved samples were then transferred to FL transfer membrane (Immobilon-FL). After blocking with LI-COR blocking buffer (1:1 diluted with PBS) for 1 hour, the

membranes were incubated with anti-CREM or -pCREM, (1:1000, Abcam), anti-StAR (1:10000, Abcam) or anti-total ERK or -pERK (1:1000, Cell Signaling) antibodies overnight at 4°C, followed by the appropriate secondary antibody (1:3000, LI-COR) for 45 minutes. Signal development and quantitation were performed using an Odyssey imaging system (LICOR, Biosciences, Lincoln, NE).

2.6. Measurement of DAG levels

HAC15 cells were labeled in serum-free medium containing 5 $\mu\text{Ci/ml}$ [^3H]oleic acid for 20 hours. The prelabeled cells were then equilibrated for 30 minutes in serum-free medium before preincubation with or without the PLC inhibitor U73122. After a 30-minute preincubation with U73122, the cells were stimulated with 100 $\mu\text{g/ml}$ VLDL with/without inhibitor for 30 minutes. Reactions were terminated by adding 0.2% SDS containing 5 mM EDTA, and then phospholipids were extracted into chloroform/methanol containing acetic acid (1:2:0.04 vol/vol/vol). After drying under nitrogen gas, the samples were resuspended in chloroform/methanol (2:1) and spotted onto heat-activated silica gel 60 thin-layer chromatography plates (0.25mm thickness aluminum backed with concentrating zone). Phospholipids were separated using a mobile phase consisting of benzene/ethyl acetate (7:3 vol/vol) and visualized with autoradiography using En 3 Hance (PerkinElmer, Waltham, MA). Spots corresponding to 1,2-DAG, identified by comigration with authentic standards visualized with iodine vapor, were excised, placed in liquid scintillation fluid and counted.

2.7. Statistical Analysis

Experimental fold values from a minimum of 3 experiments (and performed at least in duplicate) were graphed as the means $\pm$ SEM of the mean values from each experiment and analyzed by ANOVA using the program Prism (GraphPad Software, San Diego, CA) with a Newman-Keuls post-hoc test. For Figure 1, a three VLDL (0, 30, 100 $\mu\text{g/ml}$) by four AngII (0, 0.1, 1, 10 nM) ANOVA was performed on the Ct values, i.e., the Ct value for CYP11B2 minus that for the housekeeping gene (18S or PPIA), or the log of the raw aldosterone values. An interaction was explored and experiment was included to account for variation. No interactions were determined for either parameter, but there were significant additive effects for both VLDL and AngII. Significant differences between pairs of means within a treatment were determined using a Tukey's multiple comparison test. SAS© 9.3 (SAS Institute, Inc., Cary, NC) was used for these analyses.

3. Results

3.1. VLDL and angiotensin II act in an additive manner to induce CYP11B2 expression and aldosterone production

Both VLDL and angiotensin II (AngII) can induce aldosterone production in HAC15 cells, and we wished to determine whether the two agonists can act together to induce steroidogenesis in an additive or synergistic manner. We treated HAC15 cells with different doses of AngII (0, 0.1, 1 and 10 nM) with various concentrations of VLDL (0, 30 and 100 $\mu\text{g/ml}$) for 24 hours in low-serum medium and isolated RNA for qRT-PCR analysis and collected the medium for aldosterone assay. Our data (Figure 1A and B) showed that VLDL and AngII act in an additive fashion to stimulate CYP11B2 expression ($p < 0.001$ and

p=0.0495, respectively) and aldosterone production (p<0.001 for both). Thus, at the highest concentrations of VLDL and AngII, VLDL induced an approximate 70-fold increase, AngII elicited a 6-fold rise and the combination produced an about 100-fold increase in CYP11B2, whereas the values for aldosterone production were 4-fold, 9-fold and 13-fold, respectively. This result suggested that VLDL and AngII induce aldosterone production via similar pathways.

3.2. The PLC inhibitor U73122 reduced VLDL-induced CYP11B2 mRNA expression and aldosterone production in HAC15 cells

To determine whether PLC is involved in VLDL-induced aldosterone production, we used the PLC inhibitor U73122. After pretreatment and incubation with or without VLDL in the presence or absence of U73122 for 24 hours, we found that U73122 significantly inhibited VLDL-induced CYP11B2 mRNA expression (Figure 2A), reducing the VLDL-induced increase from more than 100-fold to approximately 70-fold over control, and aldosterone production (Figure 2B), to a value of approximately 4-fold over control from a VLDL-elicited increase of about 10-fold.

3.3. VLDL induced an increase in radiolabeled DAG levels, and the PLC inhibitor U73122 blocked this increase in HAC15 cells

It is known that AngII binds to AT1R and activates PLC in glomerulosa cells (reviewed in [29]). PLC then increases the levels of DAG, which promotes PKC association with the cell membrane and activation [34]. Here we showed that after a 30-minute stimulation of HAC15 cells with VLDL, DAG levels were elevated by approximately 30% whereas the PLC inhibitor U73122 blocked this VLDL-induced increase (Figure 3). This result suggests that VLDL stimulated aldosterone production, at least in part, by regulating PLC and DAG signaling.

3.4. The IP₃ receptor inhibitor xestospongin C decreased VLDL-induced CYP11B2 mRNA expression and aldosterone production in HAC15 cells

In addition to DAG, PLC activity also generates IP₃, which binds to IP₃ receptors in the ER and lead to the release of calcium efflux from this store as well as increased cytosolic calcium levels. To determine whether VLDL-induced IP₃ production and binding to its receptor could underlie aldosterone biosynthesis, HAC15 cells were pretreated with or without the IP₃ receptor inhibitor xestospongin C [35] prior to incubation in the presence or absence of VLDL. Consistent with results showing increased DAG levels (Figure 3) and increased cytosolic calcium levels [18], xestospongin C significantly decreased VLDL-induced CYP11B2 mRNA expression (Figure 4A) and aldosterone production (Figure 4B), with VLDL producing an increase of almost 40-fold for CYP11B2 expression and about 4-fold for aldosterone and xestospongin C decreasing these values to approximately 12-fold and 2-fold.

3.5. The PKC inhibitors Ro31-8220 and Gö6983 reduced VLDL-induced CYP11B2 mRNA expression and aldosterone production in HAC15 cells

It has previously been shown that inhibition of the effector enzyme for calcium, CaMK, decreases VLDL-stimulated CYP11B2 expression [18]. On the other hand, a downstream effector of DAG is the PKC family of serine/threonine protein kinases. Therefore, we used two PKC inhibitors: Ro31-8220 and Gö6983 to determine whether PKC also plays a role in VLDL-induced aldosterone production. As with the PLC inhibitor, we showed that the two PKC inhibitors significantly decreased VLDL-induced CYP11B2 mRNA expression (Figure 5A) and aldosterone production (Figure 5B). Thus, VLDL induces CYP11B2 expression to about 60-fold over control, and Ro31-8220 and Gö6983 reduce this increase to 20- and 40-fold, respectively. Similar, VLDL stimulated aldosterone production about 3-fold and Ro31-8220 and Gö6983 inhibited this increase by about 50 and 75%, respectively.

3.6. PKC mediated VLDL-induced steroidogenic acute regulatory protein levels and transcription factor phosphorylation (activation)

A previous study [18] suggested that VLDL increases the levels of steroidogenic acute regulatory protein (StAR), which is involved in transporting cholesterol from the outer to the inner mitochondrial membrane, the early rate-limiting step during steroid hormone synthesis [29]. We further explored the role of PKC in this VLDL-induced increase in StAR levels as well as the activity of the transcription factor, cAMP responsive element modulator (CREM), which can regulate StAR expression [36]. After pretreatment with the PKC inhibitors Ro31-8220 and Gö6983 for 30 minutes, cells were treated with VLDL for either 30 minutes (Figure 6A) or 3 hours (Figure 6B); the phosphorylation (activation) of the transcription factor CREM and the levels of StAR protein were then monitored. VLDL induced a more than 2-fold increase in CREM phosphorylation and the PKC inhibitors returned this phosphorylation to control levels without affecting basal phosphorylation. Similarly, StAR protein levels were also significantly enhanced approximately 2-fold by VLDL, and this increase was essentially completely inhibited by the inhibitors. These results suggest that PKC activity may be involved in VLDL-induced CREM phosphorylation (activation) and StAR protein expression.

3.7. PKC inhibitors reduced VLDL-Induced ERK activation in HAC15 cells

Previous studies indicated that LDL and VLDL induce the phosphorylation (activation) of ERK1/2 as a downstream signaling molecule to transmit signals for regulating the expression of different genes involved in aldosterone production [21,37]. Although the mechanism of ERK-1/2 activation was not determined in this prior work, in the case of VLDL-induced activation of ERK-1/2 in HepG2 cells, a likely involvement of PKC was demonstrated [38]. Therefore, to determine the role of PKC in this VLDL-induced ERK activation, we pretreated cells with the PKC inhibitors Ro31-8220 and Gö6983 for 30 minutes and with VLDL for an additional 30 minutes. Our results showed that VLDL induced an approximate 50% increase in ERK-1/2 phosphorylation and that the inhibitors completely inhibited this VLDL-induced phosphorylation (activation) of ERK-1/2 (Figure 7).

4. Discussion

The plasma levels of VLDL are typically high in obese individuals; previous studies have shown that VLDL induces aldosterone biosynthesis in several glomerulosa cell models [18,20,21], suggesting a possible mechanism linking obesity, hypertension and aldosterone levels [5]. In these studies VLDL was found to stimulate steroidogenic acute regulatory (StAR) protein and CYP11B2 and transcription factor expression [18]. VLDL has also been reported to enhance aldosterone production and CYP11B2 expression in part by recruiting PKA, ERK1/2 and JAK2 in the H295R human adrenocortical carcinoma cell line [21]. We have also shown a role for phospholipase D signaling in VLDL-induced aldosterone production in HAC15 cells, a clonal derivative of the H295R cell line [20]. In addition, VLDL increases intracellular calcium levels in H295R cells [18] thereby inducing CYP11B2 expression. Nevertheless, it is not clear whether the calcium involved is released from intracellular stores or enters the cell from the extracellular fluid (or both), although data demonstrating that VLDL-induced CYP11B2 expression can be blocked by a voltage-dependent calcium channel inhibitor [18] suggest a key role for calcium influx. However, a calcium channel antagonist also inhibits chronic aldosterone production in response to AngII despite the importance of ER calcium release in steroidogenesis stimulated by this agonist (reviewed in [29]). Our current data suggest that VLDL also releases calcium from the ER, since interfering with stimulation of the IP₃ receptor inhibits CYP11B2 and aldosterone production in response to VLDL (Figure 4). VLDL-induced CYP11B2 expression is also reduced by an inhibitor of calcium/calmodulin-dependent protein kinase (CaMK) [18], indicating that VLDL likely induces aldosterone production via similar pathways to those utilized by AngII. Our data using the PLC inhibitor U73122 also supports this idea since this inhibitor reduced VLDL-induced aldosterone production and CYP11B2 mRNA expression (Figure 2), indicating that VLDL activates PLC. Such an idea is also consistent with our results that VLDL and AngII induce aldosterone production in an additive manner (Figure 1). A previous study in our laboratory [20] showed that VLDL does not directly interact with the AT1R to increase aldosterone production. Saha et al. [22] have reported that VLDL exerts its effects, at least in part, through SR-B1, although there may be other receptors as well (e.g., VLDL receptor, the LDL receptor [39] and CD36 [40]). Thus, although they do not share the same receptor, our results suggest that VLDL and AngII, at some level, share similar signaling pathways leading to aldosterone production.

To confirm an ability of VLDL to activate PLC suggested by our results with U73122, we next focused on the second messengers generated by the PLC signaling pathway, DAG and IP₃. Our results showed that VLDL significantly increased DAG levels, and this increase was inhibited by the PLC inhibitor (Figure 3). In addition, the ability of the IP₃ receptor inhibitor to decrease aldosterone production and CYP11B2 expression (Figure 4) argues for an important signaling role for IP₃ in mediating VLDL's action. Next we determined whether PKC, which is a downstream effector activated by DAG, is involved in VLDL-induced aldosterone production. Our results demonstrated that upon treatment with either of the two PKC inhibitors, Ro31-8220 (a pan-PKC inhibitor) and Gö6983 (a classical PKC inhibitor), aldosterone production and CYP11B2 expression were inhibited (Figure 5), indicating that PKC is involved in VLDL-induced aldosterone production. In AngII

signaling PKC lies upstream of the serine-threonine protein kinase, protein kinase D (PKD), which has been shown to be activated by AngII and to mediate aldosterone production in AngII-treated H295R human adrenocortical carcinoma cells and primary cultures of bovine adrenal glomerulosa cells (e.g., [41,42]). PKD possesses two cysteine-rich domains that allow its direct activation by DAG and additionally the enzyme can be transphosphorylated and activated by novel PKC isoenzymes [43]. Therefore, given the parallels between the signaling pathways activated by VLDL and AngII, as well as the fact that PKD is a downstream mediator of PKC action [44–46], it seems possible that VLDL might also activate PKD. Experiments are in progress to investigate this possibility.

In addition to the late rate-limiting enzyme CYP11B2, we also showed that VLDL increased the levels of the early rate-limiting protein StAR [18]. Based on previous studies indicating the involvement of the activating transcription factor/cAMP response element binding protein (CREB) family of transcription factors in AngII-induced StAR mRNA and protein expression [28], we postulated the involvement of one of these factors. Our findings demonstrated that the transcription factor CREM was phosphorylated (activated) via PKC signaling, as the two PKC inhibitors inhibited this event (Figure 6). These results, together with data in bovine adrenal glomerulosa cells indicating that activated CREB can associate with the StAR promoter [47], suggest that VLDL increases StAR expression and thus protein levels by activating CREM.

ERK1/2 is reported to activate cholesterol ester hydrolase, also known as hormone-sensitive lipase [32]. Previous studies have indicated that ERK1/2 regulates aldosterone production and can be activated by both VLDL [18] and LDL [37]. Here we showed that PKC inhibitors reduced VLDL-induced ERK1/2 phosphorylation (activation), suggesting that PKC may control ERK1/2 activity thereby regulating aldosterone biosynthesis (Figure 7). This result is consistent with findings in macrophages that PKC is upstream of ERK1/2 in a VLDL-induced signaling cascade [48]. Nevertheless, controversy remains as to whether ERK1/2 mediates aldosterone production, as some investigators have found no effect of ERK pathway inhibitors on agonist-induced steroidogenesis [49–52]. It is known that ERK1/2 can phosphorylate StAR on serine 232 and stimulate its cholesterol-transporting activity ([53] and reviewed in [54,55]), suggesting that this signaling pathway likely does promote aldosterone production, although additional studies are necessary to resolve this controversy. In addition, membrane polarization maintained by various potassium channels and calcium influx through voltage-dependent and store-operated calcium channels is also very important in regulating aldosterone production in response to a variety of agonists (reviewed in [29]).

In conclusion, the present study expands our understanding of the signaling pathways through which VLDL induces aldosterone production. Figure 8 summarizes the findings of the present study: the pathways activated by VLDL include calcium, PLC, DAG, PKC and ERK signaling to increase StAR and CYP11B2 expression through phosphorylation (activation) of members of the CREB family of transcription factors. Supplemental Figure 2 collates information from the current study as well as previous work examining the regulation of aldosterone secretion by VLDL, inferring information also from the signaling pathways utilized by angiotensin II. In summary, our results indicate that VLDL stimulates aldosterone production by similar mechanisms to those used by AngII. Although it is

difficult to directly extrapolate *in vitro* results to the organismal level, our data demonstrating a correlation between triglyceride levels (a surrogate for VLDL levels) and adrenal CYP11B2 expression in rats fed normal diets and high-fat, high-sucrose diets [18] suggests that this effect may also occur *in vivo*. Indeed, it should be noted that serum aldosterone levels exhibit a positive correlation with the triglyceride levels, which in fasted individuals are a surrogate measure of VLDL levels, in obese subjects [56]. Finally, treatment of hypertensive patients with or without metabolic syndrome with the mineralocorticoid receptor antagonist, epleronone, results in reductions in serum triglyceride levels concomitantly with decreases in blood pressure upon treatment [57]. Thus, future studies into the role of VLDL in regulating aldosterone production are clearly warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AngII	angiotensin II
CREB	cAMP response element binding protein
CREM	cAMP responsive element modulator
DAG	diacylglycerol
ERK	extracellular signal-regulated kinase
IP₃	inositol 1,4,5-trisphosphate
PLC	phospholipase C
PKC	protein kinase C
StAR	steroidogenic acute regulatory protein
VLDL	very low-density lipoprotein

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Highlights

- In glomerulosa cells VLDL increases aldosterone secretion and diacylglycerol levels
- VLDL induces similar signal transduction mechanisms to those used by angiotensin II
- Inhibitor studies show that VLDL induces aldosterone synthesis via phospholipase C
- Protein kinase C inhibitors also reduce VLDL-induced steroidogenesis
- VLDL-stimulated protein kinase C induces transcription factor activity

Figure 1. VLDL and angiotensin II acted in a largely additive manner to induce CYP11B2 expression and aldosterone production

HAC15 cells were treated with VLDL (0, 30 or 100 µg/mL) and/or AngII (0, 0.1, 1 or 10 nM) for 24 hours. (A) CYP11B2 expression was measured using qRT-PCR. (B) Aldosterone levels in the media were measured using a radioimmunoassay. Results represent the means $\pm$ SEM of 3 separate experiments. Statistical analyses were performed as described in the Methods section. There was no significant interaction observed between VLDL and AngII; however, there were significant additive effects for both VLDL and AngII across the dose range for aldosterone levels ($p < 0.001$ for both) and CYP11B2 expression ($p < 0.001$ and $p = 0.0495$, respectively). Means for each concentration of VLDL were significantly different from each other across the dose range of AngII for both aldosterone and CYP11B2 values; for AngII 1 and 10 nM values for aldosterone production were significantly greater than 0, 0.1 nM was significantly less than 10, and 1 and 10 nM were not significantly different across all concentrations of VLDL. For CYP11B2 expression 10 nM AngII was statistically greater than 0 and 0.1 and 1 nM were intermediate and were not significantly different across all concentrations of VLDL. The symbols (*, # and †) and the letters (a, b and c), as indicated on the graphs, show pairs of means, with different letters representing significantly different ($p < 0.05$) values (and similar letters indicating no difference) within a treatment by a Tukey's multiple comparison test.

Figure 2. A PLC inhibitor decreased VLDL-induced aldosterone production and CYP11B2 expression

HAC15 cells were pretreated for 30 minutes with or without the PLC inhibitor U73122 (10 μ M) or the DMSO vehicle, prior to incubation in the presence or absence of 100 μ g/mL VLDL, or 10 nM AngII as a positive control, for 24 hours. (A) CYP11B2 expression was measured with qRT-PCR. (B) Aldosterone levels in the supernatant were measured using a 20 radioimmunoassay. Results represent the means $\pm$ SEM of 3 separate experiments; * p <0.05, *** p <0.001 versus the control, † p <0.05 versus AngII alone, †† p <0.01 versus VLDL alone.

Figure 3. The PLC inhibitor U73122 inhibited the VLDL-induced increase in DAG levels in HAC15 cells

HAC15 cells were pre-treated with or without 10 μ M U73122 for 30 minutes and then incubated in the presence or absence of 100 μ g/mL VLDL. Cell lipids were extracted with chloroform-methanol and DAG levels analyzed by thin-layer chromatography. Results represent the means $\pm$ SEM of data from 3 independent experiments. * p <0.05 versus the control, † p <0.05 versus VLDL alone.

Figure 4. The IP₃ receptor inhibitor xestospongine C decreased VLDL-induced aldosterone production and CYP11B2 expression

HAC15 cells were pretreated for 30 minutes with or without the IP₃ receptor inhibitor xestospongine C (XC) (10 μ M) or the DMSO vehicle, prior to incubation in the presence or absence of 100 μ g/mL VLDL for 24 hours. (A) CYP11B2 expression was measured with qRT-PCR. (B) Aldosterone levels in the supernatant were measured using a radioimmunoassay. Results represent the means $\pm$ SEM of 3 separate experiments;

***p<0.001 versus the control, †p<0.05 and †††p<0.001 versus VLDL alone.

Figure 5. PKC inhibitors reduced VLDL-induced aldosterone production and CYP11B2 expression

HAC15 cells were pretreated for 30 minutes with or without the pan-PKC inhibitor Ro31-8220 (5 μ M), the classical PKC Inhibitor Gö6983 (5 μ M) or the DMSO vehicle, prior to incubation in the presence or absence of 100 μ g/mL VLDL for 24 hours. (A) CYP11B2 expression was measured with qRT-PCR. (B) Aldosterone levels in the supernatant were measured using a radioimmunoassay. Results represent the means $\pm$ SEM of 3 separate experiments; * p <0.05, *** p <0.001 versus the control, † p <0.05 versus AngII alone, †† p <0.01 versus VLDL alone.

Figure 6. PKC inhibitors reduced VLDL-stimulated phosphorylation of the transcription factor CREM as well as StAR protein levels

HAC15 cells were pretreated for 30 minutes with or without the PKC inhibitors Ro31-8220 (5 μ M), Gö6983 (5 μ M) or the DMSO vehicle prior to incubation in the presence or absence of 100 μ g/mL VLDL. (A) After 30 minutes cells were harvested to examine phosphorylation (activation) of CREM. (B) After 3 hours cells were harvested to monitor StAR protein levels. Shown is a representative blot as well as cumulative results of 3 separate experiments presented as means $\pm$ SEM of levels normalized to CREM (panel A) or β -actin (panel B); ** p <0.01, *** p <0.001 versus the control, † p <0.05, †† p <0.001, ††† p <0.001 versus VLDL alone.

Figure 7. PKC inhibitors decreased VLDL-induced ERK phosphorylation (activation) in HAC15 cells

HAC15 cells were pre-treated with the PKC inhibitors Ro31-8220, Gö6983 (5 μ M) or DMSO vehicle for 30 minutes prior to treatment with or without VLDL (100 μ g/mL) for 1 hour. Cells were then harvested for analysis of ERK phosphorylation. Shown is a representative blot as well as cumulative results of 3 independent experiments presented as means $\pm$ SEM of levels normalized to ERK; ** p <0.05 versus the control, †† p <0.001 versus VLDL alone.

Figure 8.

Summary of the signaling pathways activated by VLDL to induce aldosterone production in glomerulosa cells demonstrated in the current report. Data presented here indicate that VLDL activates a phosphoinositide-specific phospholipase C (PLC) to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate the two second messengers, inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃, in turn, binds to IP₃ receptors on the endoplasmic reticulum (ER) to release calcium (Ca²⁺) from this intracellular storage site. Ca²⁺ then stimulates the expression of aldosterone synthase, encoded by CYP11B2, to increase aldosterone production. The other signal, DAG activates protein kinase C (PKC), which can result in the phosphorylation and activation of cAMP response element modulator (CREM) and 22 extracellular signal-regulated kinase-1/2 (ERK), as well as the induction of CYP11B2 expression and aldosterone biosynthesis.