Aldosterone Production in Human Adrenocortical Cells Is Stimulated by High-Density Lipoprotein 2 (HDL2) through Increased Expression of Aldosterone Synthase (CYP11B2)

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Adrenal aldosterone production is regulated by physiological agonists at the level of early and late rate-limiting steps. Numerous studies have focused on the role of lipoproteins including high-density lipoprotein (HDL) as cholesterol providers in this process; however, recent research suggests that HDL can also act as a signaling molecule. Herein, we used the human H295R adrenocortical cell model to study the effects of HDL on adrenal aldosterone production and *CYP11B2* expression. HDL, especially HDL2, stimulated aldosterone synthesis by increasing expression of *CYP11B2*. HDL treatment increased *CYP11B2* mRNA in both a concentration- and time-dependent manner, with a maximal 19-fold increase (24 h, 250 μg/ml of HDL). Effects of HDL on *CYP11B2* were not additive with natural agonists including angiotensin II or K^+. HDL effects were likely mediated by a calcium signaling cascade, because a calcium channel blocker and a calmodulin kinase inhibitor abolished the *CYP11B2*-stimulating effects. Of the two subfractions of HDL, HDL2 was more potent than HDL3 in stimulating aldosterone and *CYP11B2*. Further studies are needed to identify the active components of HDL, which regulate aldosterone production. **(*Endocrinology* 152: 751–763, 2011)**

The adrenal cortex is the only source of the mineralocorticoid aldosterone. Aldosterone secretion is normally under the tight control of the renin/angiotensin II (AngII)/aldosterone system (RAAS). Considerable evidence supports the role of two key regulatory steps in the control of adrenal cell aldosterone synthesis. The first has been termed the early rate-limiting step. AngII and K^+ control this step through regulation of both protein expression and activities of the steroidogenic acute regulatory (StAR) protein, which facilitates cholesterol transport from the outer to inner mitochondrial membrane where conversion to pregnenolone occurs (1, 2). The second limiting reaction has been termed the late rate-limiting step, which represents the conversion of deoxycorticosterone to aldosterone by the mitochondrial enzyme aldosterone synthase (CYP11B2) (3, 4).

CYP11B2 is expressed almost solely within the zona glomerulosa of the adrenal cortex (5, 6). As the final enzyme needed for aldosterone synthesis, CYP11B2 is regulated by multiple physiological agonists, including AngII and K^+. Both agonists increase intracellular calcium levels and activate calmodulin and calmodulin kinases (7, 8), which leads to increased CYP11B2 transcription (9–13). The expression of CYP11B2 influences directly the capacity of zona glomerulosa cells to produce aldosterone, and its adrenal expression varies *in vivo* depending on dietary sodium through regulation of the RAAS.

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Abbreviations: AngII, angiotensin II; FPLC, fast protein liquid chromatography; HDL, high-density lipoprotein; 12-HETE, 12-hydroxyeicosatetraenoic acid; LDL, low-density lipoprotein; qPCR, quantitative real-time RT-PCR; RAAS, renin/AngII/aldosterone system; siRNA, small interfering RNA; S1P, sphingosine 1 phosphate; SR-BI, scavenger receptor, class B type I; StAR, steroidogenic acute regulatory.

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Circulating lipoproteins are important sources of cholesterol for adrenal steroidogenesis. Earlier studies have shown that in both fetal and adult human adrenals, low-density lipoprotein (LDL) increases ligand-stimulated steroid production by providing needed cholesterol for steroid biosynthesis (14–22). However, the function of high-density lipoprotein (HDL) in human adrenal steroidogenesis and particularly aldosterone synthesis remains poorly defined. Recent reports in vascular model systems suggest that, in addition to the traditional role in cholesterol transport, HDL can also act as a signaling molecule by activating the ERK1/2 pathway (23–26). The observation that HDL receptors [scavenger receptor, class B type I (SR-BI)] are highly expressed in both normal adrenal glands and aldosterone-producing adenomas (27, 28) supports a potential role for HDL in adrenal function and particularly aldosterone biosynthesis. Capponi and co-workers (29) have reported that HDL can stimulate aldosterone production in both bovine glomerulosa and H295R cells, but the mechanism was assumed to be through provision of cholesterol as a substrate for steroid synthesis.

In the current study, we demonstrate for the first time that HDL, especially HDL2, is able to stimulate adrenal cell aldosterone synthesis and steroidogenic enzyme expression. This indicates a potential new physiological mechanism for the regulation of aldosterone production by diseases or drugs that raise serum HDL2 levels.

Materials and Methods

Cell culture and reagents

The human adrenocortical cell line H295R was routinely cultured in DME/Ham F12 medium (Life Technologies, Inc., Carlsbad, CA) supplemented with 10% Cosmic calf serum (Hyclone, Logan, UT) and antibiotics including 1% penicillin/streptomycin (Life Technologies) and 0.1% gentamicin (Sigma-Aldrich, St. Louis, MO). One day before the experiment, cells were changed to low-serum experimental medium overnight (DME/F12 medium supplemented with 0.1% Cosmic calf serum and antibiotics). On the next morning, cells were treated with the indicated reagents in the low-serum experimental medium for the indicated time. For inhibitor studies, cells were preincubated with inhibitors for 30 min before any ligands were added. BLT-1 was purchased from Chembridge Corp. (San Diego, CA) and stored in dimethylsulfoxide. Other reagents, unless otherwise noted, were ordered from Sigma-Aldrich.

Lipoproteins

Native human HDLs were purchased commercially from Calbiochem (Darmstadt, Germany) (three lots tested), AbD Serotec (Raleigh, NC) (one lot tested), and Millipore (Billerica, MA) (two lots tested) or prepared by our collaborators using previously described methods (30). All the figures represent results obtained using more than one source.

HDL2 and HDL3 (three sets) were provided and prepared as previously described (30). Briefly, HDL2 (1.066 g/ml $\leq$ d $\leq$ 1.125 g/ml) and HDL3 (1.125 g/ml $\leq$ d $\leq$ 1.210 g/ml) were isolated by sequential ultracentrifugation. Before use, HDL was dialyzed extensively against 0.9% NaCl, 10 mM HEPES (pH 7.4) and sterilized by filtration through a 0.45-μm Millipore filter. Concentrations used for HDL represent the protein content in the lipoprotein particle.

RNA isolation and quantitative real-time RT-PCR (qPCR)

Total RNA was extracted from cells using RNeasy mini kit (QIAGEN, Valencia, CA) following the manufacturer recommendations. Purity and integrity of the RNA was checked spectroscopically using a NanoDrop instrument (NanoDrop Technologies, Wilmington, DE). Total RNA (2 μg) treated with deoxyribonuclease I (Ambion Inc., Austin, TX) was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) following the manufacturer recommendations and stored at −80 C for further application.

The primer and probe sets for human *StAR*, side-chain cleavage (*CYP11A1*), 3β-hydroxysteroid dehydrogenase-2 (*HSD3B2*), 21-hydroxylase (*CYP21*), *CYP11B1*, and *CYP11B2* were designed using Primer Express 3.0 (Applied Biosystems) and purchased from Integrated DNA Technologies (Coralville, IA) as published previously (12). *SR-BI* and *LDLR* gene expression assays were obtained from Applied Biosystems.

qPCR were performed in the ABI Prism 7500 sequence detection system (Applied Biosystems) in a total reaction mixture volume of 20 μl following the reaction parameters recommended by the manufacturer. The TaqMan 2× Master Mix (Applied Biosystems), 900 nM of each primer, 250 nM of probe, and 5 μl of each first-strand cDNA sample were combined in each reaction vessel for gene detection. Negative controls contained water instead of first-strand cDNA. Relative quantification of mRNA levels between different tissues were determined using the comparative cycle threshold value as described previously (31), and *18S* rRNA was used as an internal control. The quantification of the *18S* rRNA in each sample was performed using a TaqMan ribosomal RNA reagent kit (Applied Biosystems) following the manufacturer recommendations.

Protein extraction and protein assay

Cells were lysed in 100 μl 1× mammalian protein extraction reagent (Pierce Chemical Co., Rockford, IL). The protein content of samples was then determined by the bicinchoninic acid protein assay using the micro bicinchoninic acid protocol (Pierce).

Steroid assay

Cell medium was collected after treatment and stored at −20 C. The aldosterone content of the medium was analyzed using an RIA kit from Siemens Healthcare Diagnostics (Los Angeles, CA), and radioactivity was read by multicrystal γ-counter (Berthold Technologies, Bad Wildbad, Germany). Assays were conducted following the manufacturer's recommendation except that standard curves were prepared in the experimental cell culture medium. The results were normalized to protein amount and shown as the fold change over basal condition.

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Small interfering RNA (siRNA) silencing

Cell electronucleation was performed using an AMAXA nucleofector kit following the manufacturer's recommendation (Lonza Group Ltd., Basel, Switzerland). Briefly, H295R cells were cultured until 80% confluence in normal growth medium before use. Cells were trypsinized and pelleted at a speed of 1000 rpm for 5 min. Supernatants were discarded, and cell pellets were resuspended in supplemented buffer R (Lonza) at a ratio of 3×10^6 cells/100 μl. For siRNA silencing experiments, Mission predesigned siRNA was ordered from Sigma-Aldrich and dissolved in nuclease-free water at a stock concentration of 50 μM, and 2 μl of siRNA was used per 1×10^6 cells and electronucleation was performed using program T-20 in the AMAXA system. Cells were allowed to recover in growth medium for 10 min before plating, and treatments were performed 48 h later.

Western analysis

Immunoblotting was performed using XCell SureLock system (Invitrogen) following the manufacturer's recommendation. Briefly, samples were lysed with lysis buffer (2% sodium dodecyl sulfate, 62.5 μM Tris, 0.04% bromophenol blue, 0.5 M dithiothreitol) and heated at 95 C for 5 min. Proteins were loaded in equal amounts to 10% Bis-Tris gel and allowed to separate for 1.5 h before transferring to polyvinylidene difluoride membranes. After blocking with 5% BSA for 1 h, the membrane was incubated with either SR-BI antibody (rabbit antihuman, 1:5000 in BSA; Novus Biologicals, Littleton, CO) or Nurr-1 antibody (rabbit antihuman, 1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA), and second antibody (goat antirabbit, 1:7000; Invitrogen).

Transfection and luciferase assay

The 5′ flanking DNA from the human *CYP11B2* (−1521/+2) was inserted upstream of the firefly luciferase gene in the reporter vector pGL3 Basic (Promega, Madison, WI). Transient transfection assays were performed using Transfast (Promega) reagents in a ratio of 4 μl/μg plasmid DNA. Luciferase activity was tested using the Luciferase assay system (Promega), and values are expressed as arbitrary light units normalized to the β-galactosidase activity of each sample and compared with basal condition for the fold change.

Intracellular calcium assay

Calcium concentration was measured using Fluo-4 NW calcium assay kit (Invitrogen) following the manufacturer's recommendation. Briefly, H295R cells were cultured to 80–90% confluence in 96-well culture plates and preloaded with fluo-4 dye for 30 min at 37 C and measured at the same temperature with FLUOstar OPTIMA system (BMG LABTECH, Offenburg, Germany) using an excitation filter of 494 nm and emission of 516 nm. Basal levels were measured for 20 sec before injection, followed by 2 sec shaking and 100 sec of measurement. Inhibitors (losartan and BAPTA-AM) were added to loading dye buffer and incubated for 30 min before experiments.

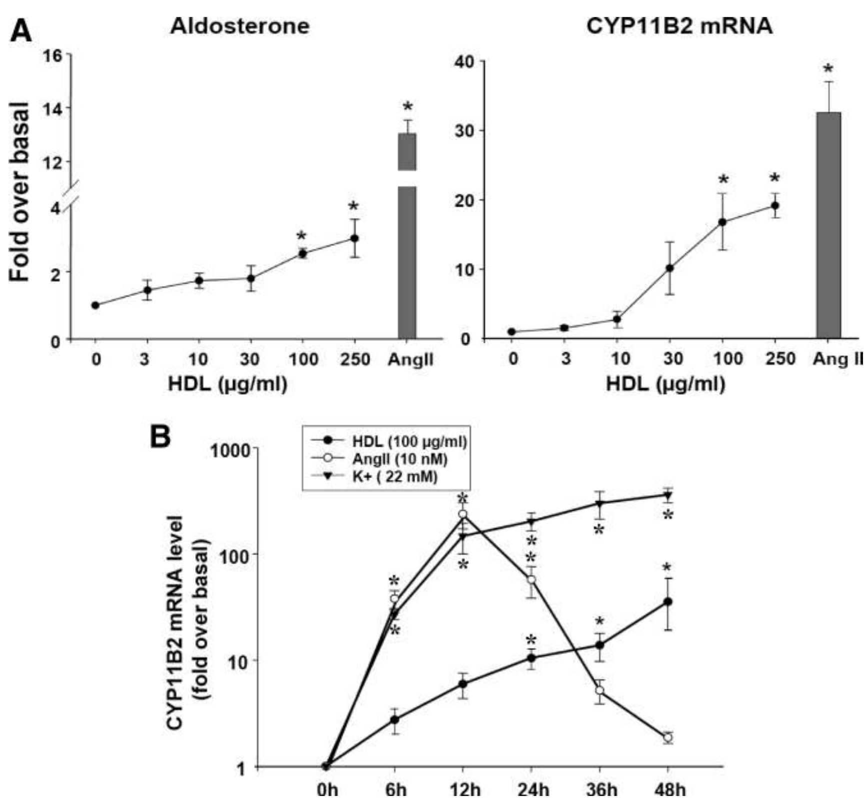

FIG. 1. Effects of HDL on aldosterone production and *CYP11B2* expression in human H295R adrenal cells. A, HDL concentration-dependent effects on aldosterone production (*left*) and aldosterone synthase expression (*right*). Cells were treated with the indicated concentrations of HDL for 24 h. Media content of aldosterone was measured by immunoassay, and qPCR was used to quantify cellular *CYP11B2* mRNA. B, HDL time-dependent effects on *CYP11B2* expression. Cells were treated with HDL (100 μg/ml), AngII (10 nM), or K^+ (22 mM) for the indicated times, and the expression of *CYP11B2* mRNA was quantified by qPCR. qPCR data were normalized to *18S* rRNA. Data are shown as the fold change compared with basal. Results represent the means ± SEM from at least three independent experiments. Statistical significances were determined using one-way ANOVA followed by a Dunnett test. *, $P < 0.05$.

Gel filtration chromatography [fast protein liquid chromatography (FPLC)]

FPLC was performed as previously described (32). Briefly, the lipoprotein samples were loaded (1 ml) onto a Superdex 200 column (60 × 1.6 cm) using an Akta FPLC system and eluted with 10 mM Tris buffer at a flow rate of 1 ml/min (120 ml total column volume). After each run, the Superdex 200 column was washed with one column volume each of water and elution buffer.

Statistical analysis

Results are provided as means ± SEM. Individual experiments were repeated at least three times. Statistical analysis was performed using one-way ANOVA (including one-way ANOVA on rank) and paired *t* tests with a confidence interval of 95% using GraphPad Prism version 3.0 (GraphPad Software, Inc., San Diego, CA).

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Results

HDL stimulates aldosterone production and CYP11B2 mRNA expression

Incubation of H295R cells with native human HDL for 24 h increased both aldosterone production and *CYP11B2* mRNA expression in a concentration-dependent manner with maximal responses detected at a concentration of 250 μg/ml HDL (3-fold for aldosterone and 19-fold for *CYP11B2*) (Fig. 1A). Compared with AngII (10 nM), HDL stimulated aldosterone production only moderately (3-fold *vs.* 13-fold). This may be explained by the difference in StAR regulation by the two reagents. StAR protein Western analysis indicated that HDL did not significantly change basal StAR levels, whereas AngII stimulated its expression by 6-fold after a 6-h treatment (data not shown).

The time-dependent effects of HDL on *CYP11B2* mRNA expression were also examined in H295R (Fig. 1B). Although AngII caused a significant time-dependent increase in *CYP11B2* mRNA that peaked at 12 h, this increase was short-lived, and *CYP11B2* transcript levels almost returned to basal by 48 h. On the other hand, K^+-stimulated *CYP11B2* expression plateaued after 12 h. Like K^+ treatment, HDL (100 μg/ml) increased the expression of *CYP11B2* transcript continuously for up to 2 d, suggesting that HDL is not working through the AngII signaling pathway. This was supported by the ability of the type 1 AngII receptor antagonist losartan to block AngII but not HDL stimulation of *CYP11B2* transcript levels (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Effects of HDL alone and with aldosterone-stimulating agonists on CYP11B2 expression

Cells were treated with HDL (100 μg/ml) alone or together with AngII (10 nM), forskolin (10 μM), or K^+ (22 mM) for 24 h. Although HDL alone stimulated *CYP11B2* transcript levels by 15-fold, this effect was not additive with either AngII or K^+ in H295R cells. However, when combined with the cAMP pathway agonist forskolin, HDL enhanced forskolin stimulation of *CYP11B2* from 15-fold to more than 80-fold (Fig. 2A). This result strongly suggests that HDL used a signaling pathway other than cAMP, but a pathway that was shared by AngII and K^+.

HDL effects on steroidogenic enzyme expression

To further characterize the effects of HDL on aldosterone stimulation, the mRNA encoding the enzymes/proteins involved in aldosterone biosynthesis were examined and compared with AngII and K^+. As reported previously, AngII significantly increased expression of *HSD3B2*, *CYP21*, and *CYP11B2* (33–36). However, *CYP11B2* was the only transcript that was significantly up-regulated by HDL treatment (Fig. 3). This is similar to the effects of K^+ on steroidogenic enzyme transcript expression. Transcripts related to cortisol and dehydroepiandrosterone production were also examined, and there was no difference in *CYP17* expression level, whereas *CYP11B1* was moderately increased (4.3-fold with 100 μg/ml HDL treatment, data not shown). The expression of this transcript can also be increased by Ang II and K^+ (37, 38).

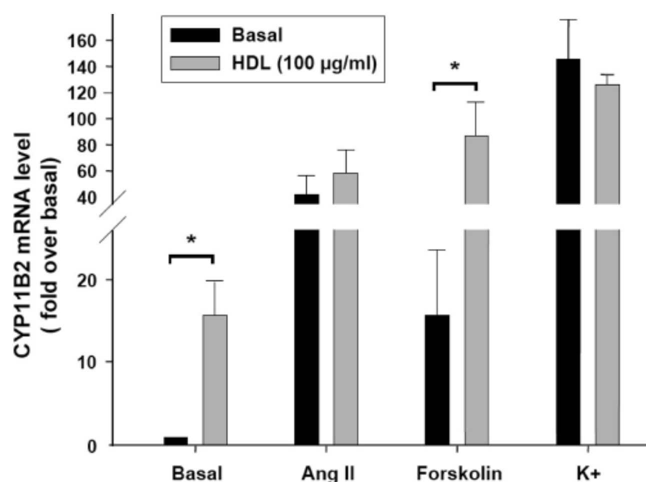

FIG. 2. Effects of HDL alone and with aldosterone-stimulating agonists on *CYP11B2* expression. H295R adrenal cells were treated with AngII (10 nM), forskolin (10 μM), or K^+ (22 mM) with or without HDL for 24 h. RNA was isolated and used for qPCR measurement of *CYP11B2* with data normalized to *18S* rRNA and shown as the fold change compared with basal. Results represent the means ± SEM from at least three independent experiments. Statistical significances were determined using one-way ANOVA followed by a Dunnett test. *, $P < 0.05$.

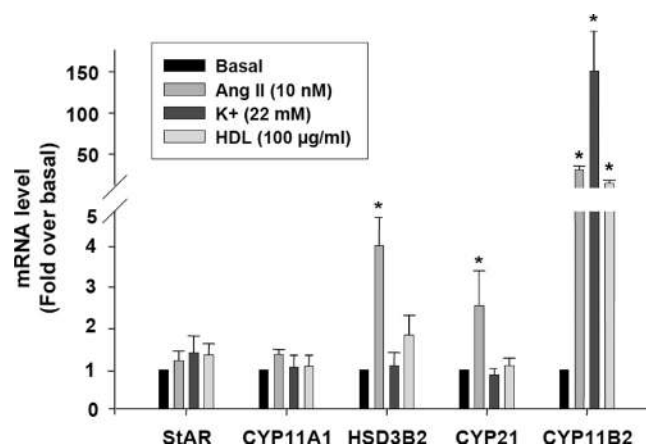

FIG. 3. HDL effects on steroidogenic enzyme transcript levels. H295R cells were treated with AngII (10 nM), K^+ (22 mM) or HDL (100 μg/ml) for 24 h. RNA was isolated and reverse transcribed for qPCR quantification. Data were normalized to *18S* rRNA and shown as the fold change compared with basal. Statistical significances were determined using one-way ANOVA followed by a Dunnett test. *, $P < 0.05$.

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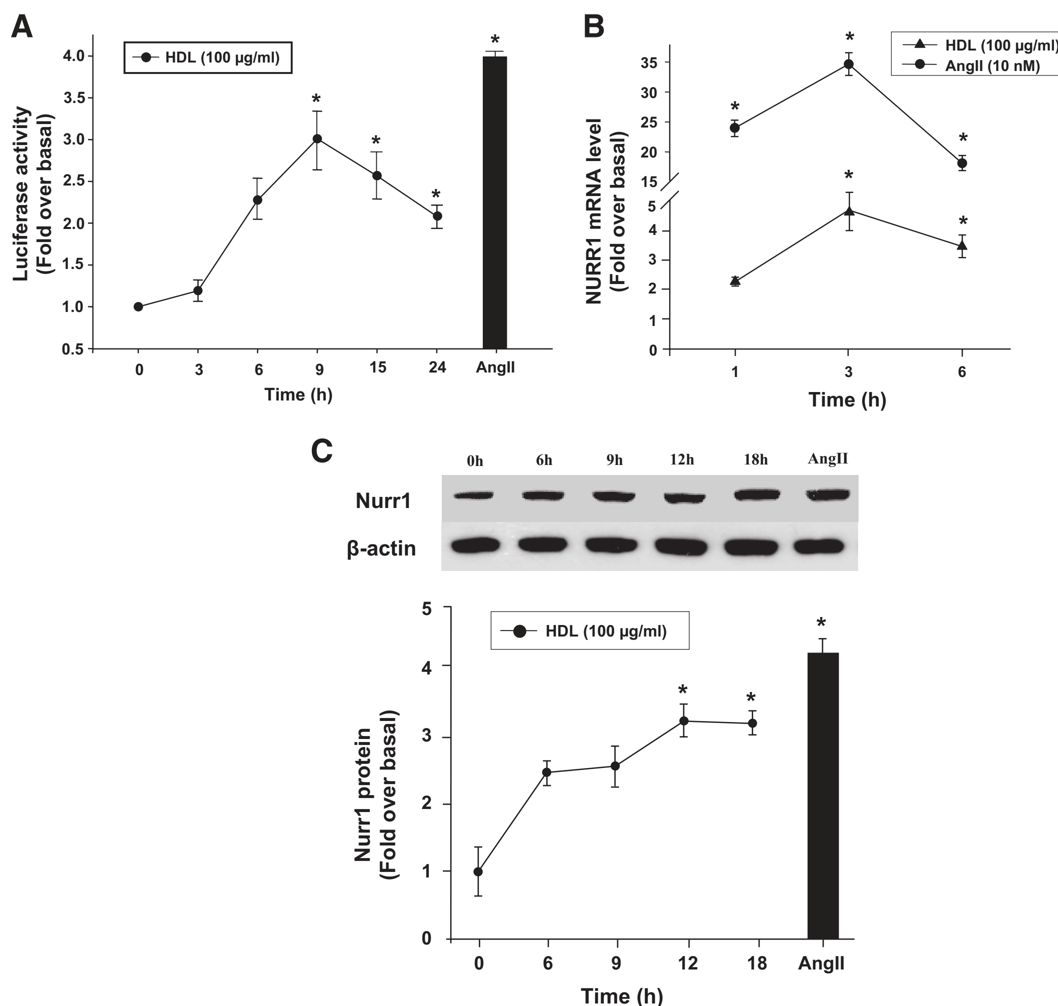

FIG. 4. HDL effects on *CYP11B2* promoter activity and NURR1 expression. A, HDL effects on *CYP11B2* promoter activity. H295R cells were transfected with the *CYP11B2* promoter construct (1 μg/ml) and allowed to recover overnight. Luciferase activity was tested after treatment with or without HDL treatment (100 μg/ml) for the indicated time. Data were normalized to cotransfected β-galactosidase vector and are shown as the fold induction over the untreated group. As a positive control, cells were treated with AngII (10 nM) for 9 h. Results represent the means ± SEM from at least three independent experiments each performed in triplicate. Statistical differences were determined using one-way ANOVA followed by Dunnett test. *, $P < 0.05$. B and C, HDL stimulates both transcript (B) and protein (C) expression of NURR1. H295R cells were treated with AngII (10 nM) or HDL (100 μg/ml) for the indicated times (1–18 h). qPCR was used to quantify *NURR1* mRNA level, and Western analysis was used for NURR1 protein quantification. qPCR results were normalized to *18S* rRNA, and Western data were normalized to β-actin protein levels. Results represent the means ± SEM from at least three independent experiments. Statistical significances were determined using one-way ANOVA followed by a Dunnett test. *, $P < 0.05$.

HDL stimulates CYP11B2 promoter activity and NURR1 expression

H295R cells were transfected with a vector possessing the 5′ flanking *CYP11B2* promoter region and treated with HDL or AngII for 3–24 h. HDL significantly stimulated promoter activity in a time-dependent manner, starting from 6 h and peaking around 9 h, with a maximal stimulation of 2.8-fold (Fig. 4A). We have previously demonstrated a role for the transcription factor NURR1 in the transcription of *CYP11B2* (11, 39). HDL caused a time-dependent increase in *NURR1* transcript (4.5-fold at 3 h) (Fig. 4B) and protein (3-fold at 12 h) (Fig. 4C), suggesting that the HDL-triggered signaling cascade activates the expression of NURR1 and thus stimulates *CYP11B2* transcription.

HDL stimulation is mediated by SR-BI

The adrenal cortex of several species expresses high levels of the HDL receptor SR-BI (40–44). The human adrenal is no exception, and transcripts and protein expression have been documented (27, 28, 45). To examine the role of SR-BI in the HDL-mediated signaling pathway, we used a SR-BI-specific inhibitor, BLT-1. As shown in Fig. 5A, although BLT-1 itself increased the basal level of *CYP11B2* mRNA, preincubating with BLT-1 blocked the HDL stimulatory effects. The inhibition appeared to be specific, because BLT-1 did not decrease AngII activation of cellular *CYP11B2* expression.

The involvement of SR-BI was further confirmed by siRNA knockdown of SR-BI expression. Transient deliv-

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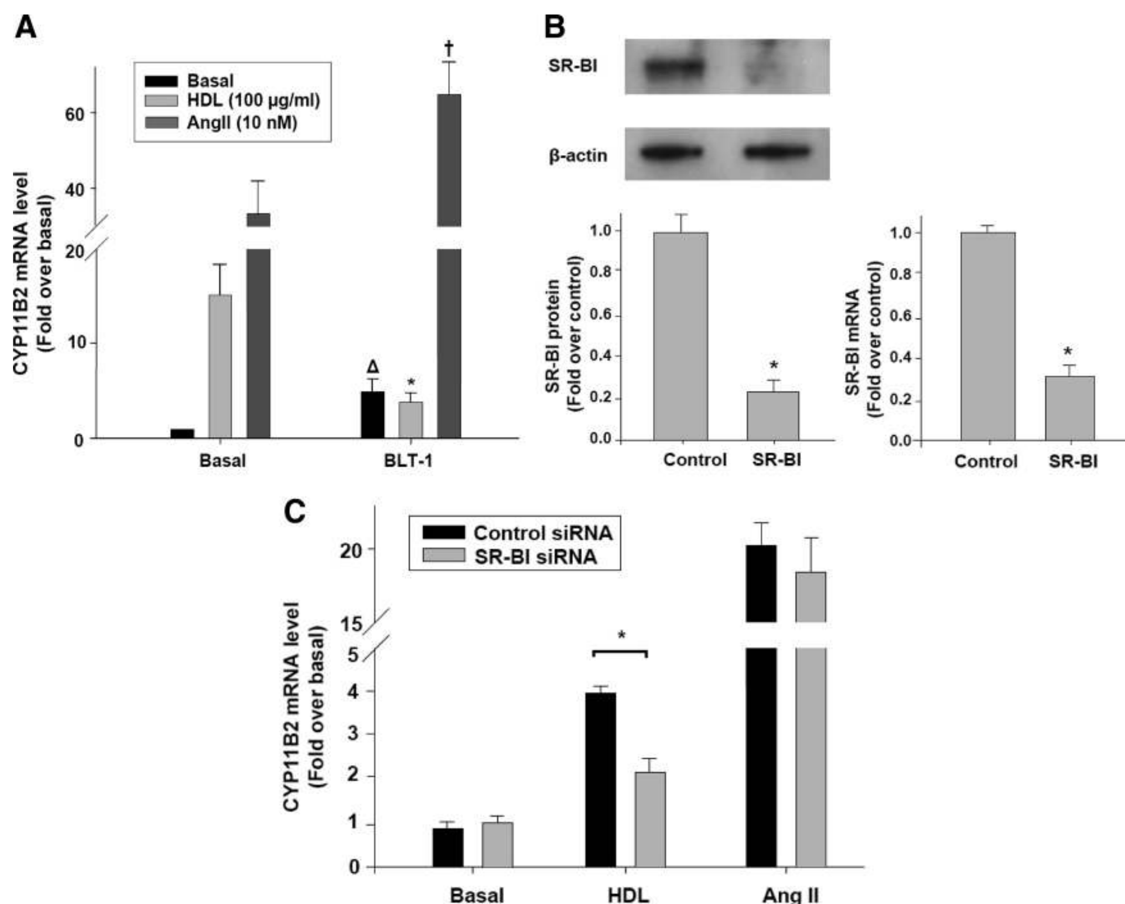

FIG. 5. The role of SR-BI receptor in HDL regulation of *CYP11B2*. A, BLT-1 effects on HDL stimulation of *CYP11B2*. H295R cells were preincubated with BLT-1 (3 μM) for 20 min, followed by basal, HDL (100 μg/ml), or AngII (10 nM) treatment for 24 h. RNA were isolated and reverse transcribed for qPCR quantification. qPCR data were normalized to *18S* rRNA and shown as the fold change compared with untreated basal cells. Results represent the means ± SEM from at least three independent experiments. Statistics were calculated using one-way ANOVA followed by a Dunnett test. Δ, $P < 0.05$, compared with basal; *, $P < 0.05$, compared with HDL treatment; †, $P < 0.05$, compared with AngII treatment. B and C, SR-BI knockdown effects on HDL stimulation of *CYP11B2* mRNA. H295R cells were allowed to grow to 80–90% confluence. Electronucleation was performed as described in *Materials and Methods*, and cells were plated for 2 d before treatment with HDL (100 μg/ml) for 24 h. RNA was isolated, and qPCR was performed for *CYP11B2* mRNA quantification. Data were normalized to *18S* rRNA and shown as the fold change compared with control siRNA-transfected cells (B). Western blotting was performed as described in *Materials and Methods*. Western data were normalized to β-actin protein level and shown as the fold change compared with control siRNA-transfected cells (C). Data represent the means ± SEM from at least three independent experiments. Statistical significances were determined using either Student's paired *t* test (B) or one-way ANOVA followed by a Dunnett test (C). *, $P < 0.05$.

ery of siRNA for SR-BI decreased the endogenous expression of *SR-BI* mRNA to less than 30% and the protein level to 25% when compared with cells transfected with scrambled siRNA (Fig. 5B). After 24 h treatment with HDL, the knockdown group exhibited decreased HDL-stimulating effects on *CYP11B2* by 50%, although AngII effects were not influenced (Fig. 5C). However, we did notice a decrease in overall fold stimulation in response to both ligands as a result of the AMAXA electroporation.

HDL stimulates adrenal cell CYP11B2 expression through calcium signaling pathways

The importance of calcium signaling for HDL-stimulated *CYP11B2* expression was investigated with the help of the calcium channel blocker nifedipine and calmodulin kinase inhibitor KN93 (Fig. 6A). Preincubation with 10 μM nifedipine completely blocked HDL effects on *CYP11B2* and 5 μM KN93 inhibited 80% of HDL activity. The inhibition of both HDL and K^+ activity by nifedipine and KN93 suggests that like K^+, HDL signals mainly through calcium. In agreement with previous results, both nifedipine and KN93 only partially inhibit AngII-stimulating effects, indicating the involvement of another signaling cascade (likely protein kinase C) in AngII effects on *CYP11B2* expression.

This result was confirmed by examination of intracellular calcium concentrations in H295R cells (Fig. 6B). As published previously (46–48), AngII caused a significant but transient increase in intracellular calcium concentrations. HDL triggered a lower-magnitude peak Ca^{2+} increase, but this increase was maintained for more than 100 sec. Both a calcium chelator (BAPTA-AM) and an SR-BI inhibitor (BLT-1) blocked this change in calcium concentration, suggesting that HDL stimulated calcium influx

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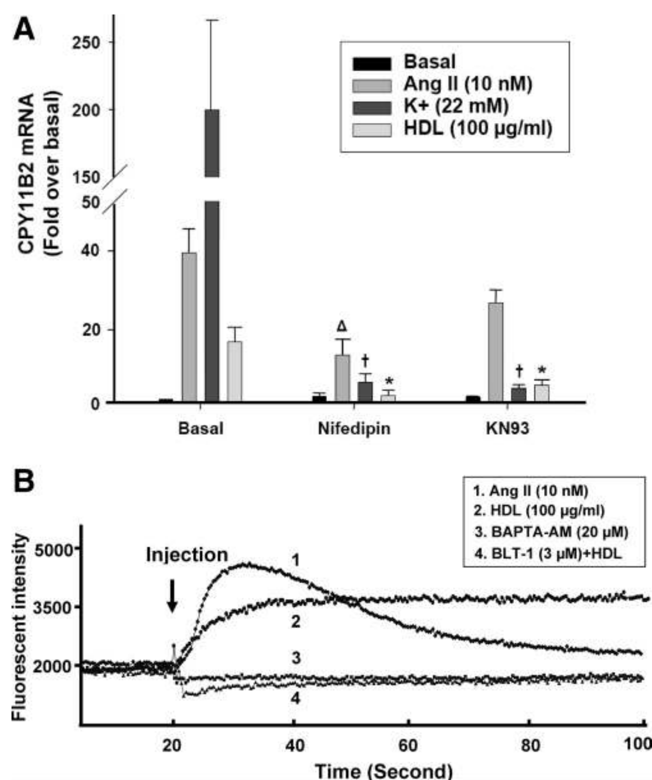

FIG. 6. Role of calcium signaling in HDL stimulation of *CYP11B2* expression. A, Calcium signaling antagonists inhibited HDL-induced stimulation of *CYP11B2*. H295R cells were preincubated with nifedipine (10 μM) or KN93 (5 μM) for 20 min followed by treatment with agonists (24 h). *CYP11B2* qPCR data were normalized to *18S* ribosome RNA and shown as the fold change compared with untreated basal cells. Results represent the means ± SEM from at least three independent experiments. Statistical significances were determined using Student's paired *t* test. Δ, $P < 0.05$, compared with AngII treatment; †, $P < 0.05$, compared with K^+ treatment; *, $P < 0.05$, compared with HDL treatment. B, HDL increased the intracellular calcium concentration in H295R cells. Cells were allowed to grow to 80–90% confluence on a 96-well dish and incubated with fluorescent dye (100 μl) for 40 min before use. Cells were preincubated with and without BAPTA-AM or BLT-1 for 30 min. Fluorescent intensity was recorded for the 20 sec before and 80 sec after agonist addition.

and this process was mediated by SR-BI. Preincubation with AT1 receptor block losartan completely abolished the calcium-stimulating effects of AngII but did not influence HDL function (data not shown).

HDL2 is more potent than HDL3 in stimulating adrenal cells

When comparing HDL from different sources, we observed a range of adrenal cell *CYP11B2* responses. All three commercially obtained samples (from Calbiochem, AbD Serotec, and Millipore) showed similar significant effects on aldosterone and *CYP11B2* stimulation, whereas samples by our collaborators had only minor activity (Fig. 7A). To clarify the differences in HDL function, FPLC was performed to determine the HDL components of an HDL preparation with high stimulatory activity (sample 2, from Calbiochem) and low stimulatory activity (sample 1, prepared within our group). As shown in Fig. 7B, HDL from our group was mainly composed of the HDL3 subfraction, whereas Calbiochem HDL had more of an HDL2 component. This result suggested that different HDL subfractions might influence the aldosterone/*CYP11B2*-stimulating effect. This hypothesis was tested by specifically isolating HDL2 and HDL3 subfractions, with the total HDL isolated from the same sample as control, and using them to treat H295R cells. HDL2 (100 μg/ml) significantly increased *CYP11B2* transcript levels, whereas HDL3 (100 μg/ml) did not influence *CYP11B2* expression (Fig. 8A).

To confirm the above finding, a similar set of experiments was performed comparing HDL2- and HDL3-stimulating effects on aldosterone production, *CYP11B2* transcripts, promoter activity, intracellular calcium concentration, and *NURR1* mRNA level (Fig. 8). HDL2 was more active than HDL3, and this idea suggested that HDL2 might be a more potent initiator of the calcium signaling pathway in human adrenocortical cells.

Discussion

Like all steroid-producing glands, the adrenal synthesizes steroid hormones using cholesterol as a precursor. There are three primary cholesterol sources for steroidogenic tissues: *de novo* synthesis, LDL, and HDL. Different animal species have their own preferred source of cholesterol. For humans, LDL is the main cholesterol source for adrenal steroidogenesis (14–17), whereas rats and mice use HDL as the major source of cholesterol in steroid synthesis (49, 50). The varied role of lipoproteins in steroid production may relate in part to the apoprotein content in HDL that has been shown to be quite different between those species. Although direct incubation of HDL with rat adrenocortical cells did not increase steroid production *in vitro*, HDL has been shown to enhance steroidogenesis with ACTH (50). However, Leydig cells from rats can use HDL to regulate testosterone production via an apolipoprotein A1-dependent pathway (51). Furthermore, in mouse, HDL has been shown to stimulate StAR protein expression (52) and act as a potential growth factor for adrenocortical cells (53).

Bovine steroidogenic cells, on the other hand, appear to use both LDL and HDL for steroidogenesis (29, 54), and bovine glomerulosa cells respond to HDL with increases in aldosterone production in a mechanism that may involve a calcium signaling pathway (55). We also observed a significant stimulation of bovine glomerulosa cell aldosterone production by HDL in the current study (data not shown). Similar effects were reported by Capponi's group,

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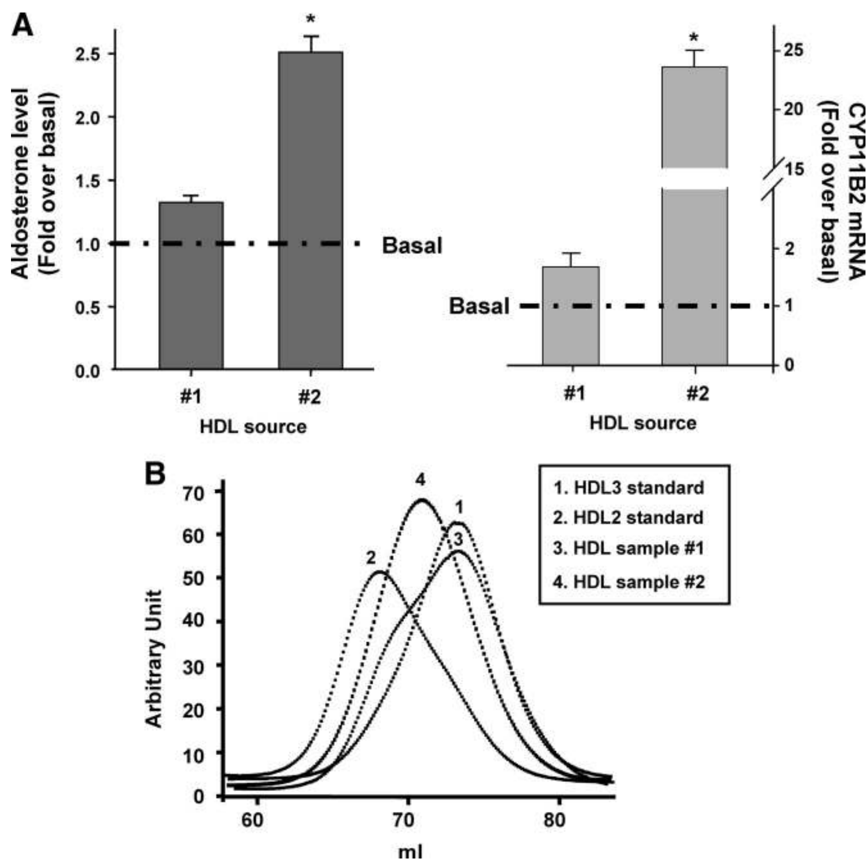

FIG. 7. Variation in HDL2 *vs.* HDL3 ratio in HDL from different sources and their effects on aldosterone production and *CYP11B2* expression. A, Variable effects of HDL on aldosterone production and *CYP11B2* expression. H295R cells were treated with the indicated concentration of HDL for 24 h. RIA was performed to determine the concentration of aldosterone produced by cells, and qPCR was performed for quantifying *CYP11B2* mRNA level. All data are shown as the fold change compared with basal. Results represent the means ± SEM from at least three independent experiments. Statistical significances were determined using one-way ANOVA followed by a Dunnett test. *, $P < 0.05$. B, Representative diagram of FPLC comparison of the HDL components from different sources. Purified HDL2 and HDL3 were used as controls. HDL sample 1 was isolated by our research laboratory, and sample 2 was purchased from Calbiochem.

and they further extended the stimulating effects of HDL to H295 human adrenocortical cells (29). However, the latter article made the assumption that the HDL effect was associated with its cholesterol-providing function. For the current study, we replicated the aldosterone-stimulating effects in H295R cells and proposed a novel mechanism that HDL may initiate a calcium signaling pathway leading to increased expression of the late rate-limiting step enzyme *CYP11B2*.

HDL is used by most cells mainly through binding to SR-BI. SR-BI is an 82-kDa membrane protein with 509 amino acids (56, 57). It contains one putative membrane-spanning hydrophobic domain near each end of the protein and a large extracellular region with a cysteine-rich carboxyl-terminal half and multiple potential *N*-glycosylation sites. SR-BI is highly glycosylated and can also be modified by fatty acylation (58). As opposed to the receptor-mediated endocytosis pathway used by LDL receptors in lipid uptake, SR-BI uses a selective uptake mechanism, which efficiently transports lipid but not the carrier protein into cells (57, 59). This process can be divided into two independent steps: receptor-mediated surface binding and lipid transportation across the membrane. With the use of the specific inhibitor of SR-BI, BLT-1 (60), we were able to separate these two steps and show that lipid uptake rather than receptor binding is required to trigger aldosterone production and CYP11B2 stimulation in adrenocorticoid cells.

As a lipoprotein family member, HDL can be further divided into two subfractions depending on particle size and density, namely HDL2 and HDL3. Although lower levels of both HDL subfractions have been shown to be markers of coronary heart disease (61), HDL3 exerts a greater preventive effect on Cu^{2+}-induced LDL oxidation (62). HDL2 and HDL3 differ in both lipoprotein and lipid components (63–65). A study conducted by Rothblat and colleagues (30) suggested that the phospholipid composition of HDL is a major determinant of the bidirectional flux and net movement of cellular free cholesterol mediated by SR-BI, raising the possibility that HDL2 and HDL3 have diverse effects on SR-BI-mediated cholesterol transport. The HDL2/HDL3 ratio can be shifted by both physiological (exercise, menopause, and gender) (66–69) and pathological (familial hypercholesterolemia, obesity, alcohol consumption, smoking, and substance abuse) conditions (70–75). Our data suggest that the changes in HDL level (especially HDL2/HDL3 ratio) might modulate adrenal aldosterone production. Although aldosterone levels are primarily regulated by RAAS, most humans who are on a high-sodium diet have very low renin levels. Under these conditions, HDL2 may play a role in regulating aldosterone production. Furthermore, our study suggested that HDL2 has the potential to act with ACTH to increase aldosterone production. In addition, the high expression level of SR-BI in adrenal steroid-producing tumors (76) further emphasized the need to examine the role of HDL in the dysregulation of steroidogenesis seen in adrenal disease.

One mechanism for HDL activation of adrenal cell signaling would be through lipids or proteins associated with

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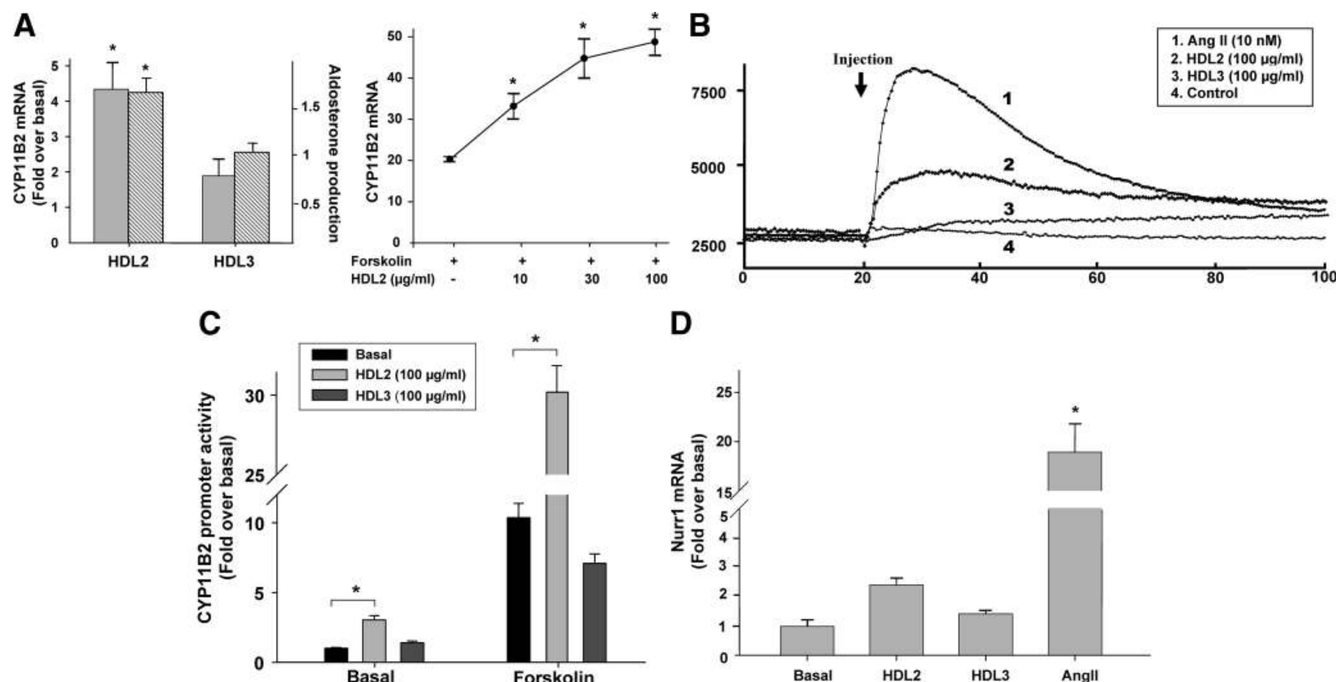

FIG. 8. Potency of HDL2 *vs.* HDL3 in stimulating adrenal cells. A, HDL2 had stronger stimulatory effects on both aldosterone production and *CYP11B2* mRNA expression. H295R cells were treated with HDL2 or HDL3 (100 μg/ml) with or without forskolin (10 μM) for 24 h in 0.1% low-serum medium. RIA was performed to determine the concentration of aldosterone produced by cells, and qPCR was performed for quantifying *CYP11B2* mRNA level. All data are shown as the fold change compared with basal (*solid bar*, *CYP11B2* mRNA; *dashed bar*, aldosterone production). Results represent the means ± SEM from at least three independent experiments. Statistical differences were determined using one-way ANOVA followed by Dunnett test. *, $P < 0.05$. B, HDL2 increased the intracellular calcium concentration in H295R cells. Cells were allowed to grow to 80–90% confluence on a 96-well dish and incubated with fluorescent dye (100 μl) for 40 min before use. Cells were exposed to the indicated agonists, and the plate was shaken for 2 sec to allow quick mixing. Fluorescent intensity was recorded for the 20 sec before and 80 sec after agonist addition. C, HDL2 was more potent than HDL3 in stimulating *CYP11B2* promoter activity and was additive with forskolin. H295R cells were transfected with 1 μg of *CYP11B2* promoter construct and allowed to recover overnight. Luciferase activity was tested after treatment with or without HDL2, HDL3 (100 μg/ml), or forskolin (1 μM) for 6 h. Data were normalized to cotransfected β-galactosidase vector and are shown as the fold induction over the untreated group. Results represent the means ± SEM from at least three independent experiments. Statistical differences were determined using ANOVA on rank. *, $P < 0.05$. D, HDL2 stimulates the expression of *NURR1*. H295R cells were treated with AngII (10 nM) or HDL2 or HDL3 (100 μg/ml) for 3 h. qPCR was used to quantify cellular *NURR1* mRNA level, and data were normalized to *18S* rRNA. Results represent the means ± SEM from at least three independent experiments. Statistical differences were determined using one-way ANOVA. *, $P < 0.05$.

the HDL particle. Based on previous studies (77–82), potential candidates for the active component of HDL were examined using adrenocortical cells. Sphingosine 1 phosphate (S1P) has been shown, in multiple studies, to account for many of the antiatherogenic activities of HDL (77, 78, 83–86). Mounting evidence supports the hypothesis that HDL, by binding to SR-BI, increases endothelial nitric oxide synthase phosphorylation via the activation of both phosphatidylinositol 3-kinase and MAPK pathways (79, 87). Recent results also suggest the involvement of S1P receptors (especially type 1 and type 3) in this signaling process (24). However, treatment of H295R cells with S1P or its analog, FTY720, did not stimulate H295R cells and preincubation of a S1P receptor inhibitor (VPC 23019) failed to block HDL stimulation of *CYP11B2* (data not shown). In addition, HDL3 has twice the amount of S1P than the more steroidogenically active HDL2 (88), further suggesting that S1P is not the stimulatory agent for adrenal cells.

Another candidate is 12-hydroxyeicosatetraenoic acid (12-HETE), which has been proposed to have a role in mediating AngII-induced aldosterone secretion. Nadler and co-workers (81, 82, 89–91) reported that AngII treatment increases the expression of 12-lipoxygenase in human cultured glomerulosa cells and stimulates 12-HETE production in H295R cells. Elevated 12-lipoxygenase levels raised aldosterone secretion under both basal and AngII-stimulated conditions, accompanied by an increase in *CYP11B2* transcript levels (89). In addition, in both bovine and rat glomerulosa cell models, treatment of 12-HETE causes an influx of calcium (80, 92). However, because of the fact that no 12-HETE receptor has been identified, the mechanisms for 12-HETE stimulation of aldosterone production have not been determined. In the present study, we were not able to observe the effects of 12-HETE on H295R *CYP11B2* and aldosterone (data not shown), suggesting that this lipid is not the active component found in HDL.

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Recently, an interesting finding from a phase III clinical trial was published, in which a potent inhibitor of cholesteryl ester transfer protein was studied as a potential treatment for cardiovascular disease. Noticeably, treatment caused a 20% decrease in LDL and a 61% increase in HDL in patients after torcetrapib/statin administration (93–95). The increase in circulating HDL was mainly due to elevated HDL2 concentrations (93, 96). However, treatment also caused an elevation in blood pressure and aldosterone levels in subjects given torcetrapib. Current evidence suggests that the effects of torcetrapib were related to a nonspecific action that was unrelated to the inhibition of cholesteryl ester transfer protein (97–99). Although there are no clinical data indicating that individuals with high HDL have more hypertension or higher aldosterone levels (or lower K^+ levels), our *in vitro* observations using cultured adrenal cells suggest that further studies are now needed to determine whether HDL2 can directly influence adrenal gland production of aldosterone.

Acknowledgments

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References

1. **Clark BJ, Soo SC, Caron KM, Ikeda Y, Parker KL, Stocco DM** 1995 Hormonal and developmental regulation of the steroidogenic acute regulatory protein. Mol Endocrinol 9:1346–1355
2. **Clark BJ, Wells J, King SR, Stocco DM** 1994 The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). J Biol Chem 269:28314–28322
3. **Mornet E, Dupont J, Vitek A, White PC** 1989 Characterization of two genes encoding human steroid 11β-hydroxylase (P-450(11)β). J Biol Chem 264:20961–20967
4. **Kater CE, Biglieri EG, Rost CR, Schambelan M, Hirai J, Chang BC, Brust N** 1985 The constant plasma 18-hydroxycorticosterone to aldosterone ratio: an expression of the efficacy of corticosterone methyloxidase type II activity in disorders with variable aldosterone production. J Clin Endocrinol Metab 60:225–228
5. **Ogishima T, Suzuki H, Hata J, Mitani F, Ishimura Y** 1992 Zone-specific expression of aldosterone synthase cytochrome P-450 and cytochrome P-45011β in rat adrenal cortex: histochemical basis for the functional zonation. Endocrinology 130:2971–2977
6. **Wotus C, Levay-Young BK, Rogers LM, Gomez-Sanchez CE, Engeland WC** 1998 Development of adrenal zonation in fetal rats defined by expression of aldosterone synthase and 11β-hydroxylase. Endocrinology 139:4397–4403
7. **Condon JC, Pezzi V, Drummond BM, Yin S, Rainey WE** 2002 Calmodulin-dependent kinase I regulates adrenal cell expression of aldosterone synthase. Endocrinology 143:3651–3657
8. **Pezzi V, Clyne CD, Ando S, Mathis JM, Rainey WE** 1997 Ca^{2+}-regulated expression of aldosterone synthase is mediated by calmodulin and calmodulin-dependent protein kinases. Endocrinology 138: 835–838
9. **Lu L, Suzuki T, Yoshikawa Y, Murakami O, Miki Y, Moriya T, Bassett MH, Rainey WE, Hayashi Y, Sasano H** 2004 Nur-related factor 1 and nerve growth factor-induced clone B in human adrenal cortex and its disorders. J Clin Endocrinol Metab 89:4113–4118
10. **Bassett MH, White PC, Rainey WE** 2004 A role for the NGFI-B family in adrenal zonation and adrenocortical disease. Endocr Res 30:567–574
11. **Bassett MH, Suzuki T, Sasano H, White PC, Rainey WE** 2004 The orphan nuclear receptors NURR1 and NGFIB regulate adrenal aldosterone production. Mol Endocrinol 18:279–290
12. **Bassett MH, Mayhew B, Rehman K, White PC, Mantero F, Arnaldi G, Stewart PM, Bujalska I, Rainey WE** 2005 Expression profiles for steroidogenic enzymes in adrenocortical disease. J Clin Endocrinol Metab 90:5446–5455
13. **Nogueira EF, Bollag WB, Rainey WE** 2009 Angiotensin II regulation of adrenocortical gene transcription. Mol Cell Endocrinol 302: 230–236
14. **Higashijima M, Nawata H, Kato K, Ibayashi H** 1987 Studies on lipoprotein and adrenal steroidogenesis: I. Roles of low density lipoprotein- and high density lipoprotein-cholesterol in steroid production in cultured human adrenocortical cells. Endocrinol Jpn 34: 635–645
15. **Carr BR, Simpson ER** 1981 Lipoprotein utilization and cholesterol synthesis by the human fetal adrenal gland. Endocr Rev 2:306–326
16. **Simpson ER, Carr BR, Parker Jr CR, Milewich L, Porter JC, MacDonald PC** 1979 The role of serum lipoproteins in steroidogenesis by the human fetal adrenal cortex. J Clin Endocrinol Metab 49:146–148
17. **Higashijima M, Kato K, Nawata H, Ibayashi H** 1987 Studies on lipoprotein and adrenal steroidogenesis: II. Utilization of low density lipoprotein- and high density lipoprotein-cholesterol for steroid production in functioning human adrenocortical adenoma cells in culture. Endocrinol Jpn 34:647–657
18. **Kraemer FB** 2007 Adrenal cholesterol utilization. Mol Cell Endocrinol 265–266:42–45
19. **Heikkilä P, Kahri AI, Ehnholm C, Kovanen PT** 1989 The effect of low- and high-density lipoprotein cholesterol on steroid hormone production and ACTH-induced differentiation of rat adrenocortical cells in primary culture. Cell Tissue Res 256:487–494
20. **Carr BR, Parker Jr CR, Milewich L, Porter JC, MacDonald PC, Simpson ER** 1980 The role of low density, high density, and very low density lipoproteins in steroidogenesis by the human fetal adrenal gland. Endocrinology 106:1854–1860
21. **Andersen JM, Dietschy JM** 1978 Relative importance of high and low density lipoproteins in the regulation of cholesterol synthesis in the adrenal gland, ovary, and testis of the rat. J Biol Chem 253: 9024–9032
22. **Boggaram V, Funkenstein B, Waterman MR, Simpson ER** 1984 Lipoproteins and the regulation of adrenal steroidogenesis. Endocr Res 10:387–409
23. **Grewal T, de Diego I, Kirchhoff MF, Tebar F, Heeren J, Rinninger F, Enrich C** 2003 High density lipoprotein-induced signaling of the MAPK pathway involves scavenger receptor type BI-mediated activation of Ras. J Biol Chem 278:16478–16481
24. **Kimura T, Tomura H, Mogi C, Kuwabara A, Damirin A, Ishizuka T, Sekiguchi A, Ishiwara M, Im DS, Sato K, Murakami M, Okajima F** 2006 Role of scavenger receptor class B type I and sphingosine 1-phosphate receptors in high density lipoprotein-induced inhibi-

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tion of adhesion molecule expression in endothelial cells. J Biol Chem 281:37457–37467
25. **Mineo C, Shaul PW** 2003 HDL stimulation of endothelial nitric oxide synthase: a novel mechanism of HDL action. Trends Cardiovasc Med 13:226–231
26. **Miura S, Fujino M, Matsuo Y, Kawamura A, Tanigawa H, Nishikawa H, Saku K** 2003 High density lipoprotein-induced angiogenesis requires the activation of Ras/MAP kinase in human coronary artery endothelial cells. Arterioscler Thromb Vasc Biol 23:802–808
27. **Liu J, Voutilainen R, Heikkilä P, Kahri AI** 1997 Ribonucleic acid expression of the CLA-1 gene, a human homolog to mouse high density lipoprotein receptor SR-BI, in human adrenal tumors and cultured adrenal cells. J Clin Endocrinol Metab 82:2522–2527
28. **Murao K, Terpstra V, Green SR, Kondratenko N, Steinberg D, Quehenberger O** 1997 Characterization of CLA-1, a human homologue of rodent scavenger receptor BI, as a receptor for high density lipoprotein and apoptotic thymocytes. J Biol Chem 272:17551–17557
29. **Cherradi N, Bideau M, Arnaudeau S, Demaurex N, James RW, Azhar S, Capponi AM** 2001 Angiotensin II promotes selective uptake of high density lipoprotein cholesterol esters in bovine adrenal glomerulosa and human adrenocortical carcinoma cells through induction of scavenger receptor class B type I. Endocrinology 142:4540–4549
30. **Yancey PG, de la Llera-Moya M, Swarnakar S, Monzo P, Klein SM, Connelly MA, Johnson WJ, Williams DL, Rothblat GH** 2000 High density lipoprotein phospholipid composition is a major determinant of the bi-directional flux and net movement of cellular free cholesterol mediated by scavenger receptor BI. J Biol Chem 275:36596–36604
31. **Ye P, Mariniello B, Mantero F, Shibata H, Rainey WE** 2007 G-protein-coupled receptors in aldosterone-producing adenomas: a potential cause of hyperaldosteronism. J Endocrinol 195:39–48
32. **Liu L, Bortnick AE, Nickel M, Dhanasekaran P, Subbaiah PV, Lund-Katz S, Rothblat GH, Phillips MC** 2003 Effects of apolipoprotein A-I on ATP-binding cassette transporter A1-mediated efflux of macrophage phospholipid and cholesterol: formation of nascent high density lipoprotein particles. J Biol Chem 278:42976–42984
33. **Holland OB, Mathis JM, Bird IM, Rainey WE** 1993 Angiotensin increases aldosterone synthase mRNA levels in human NCI-H295 cells. Mol Cell Endocrinol 94:R9–R13
34. **Bird IM, Mason JI, Rainey WE** 1998 Protein kinase A, protein kinase C, and Ca^{2+}-regulated expression of 21-hydroxylase cytochrome P450 in H295R human adrenocortical cells. J Clin Endocrinol Metab 83:1592–1597
35. **Lebrethon MC, Jaillard C, Defayes G, Begeot M, Saez JM** 1994 Human cultured adrenal fasciculata-reticularis cells are targets for angiotensin-II: effects on cytochrome P450 cholesterol side-chain cleavage, cytochrome P450 17 alpha-hydroxylase, and 3 β-hydroxysteroid-dehydrogenase messenger ribonucleic acid and proteins and on steroidogenic responsiveness to corticotropin and angiotensin-II. J Clin Endocrinol Metab 78:1212–1219
36. **Nogueira EF, Vargas CA, Otis M, Gallo-Payet N, Bollag WB, Rainey WE** 2007 Angiotensin-II acute regulation of rapid response genes in human, bovine, and rat adrenocortical cells. J Mol Endocrinol 39:365–374
37. **Denner K, Rainey WE, Pezzi V, Bird IM, Bernhardt R, Mathis JM** 1996 Differential regulation of 11 β-hydroxylase and aldosterone synthase in human adrenocortical H295R cells. Mol Cell Endocrinol 121:87–91
38. **Tremblay A, Waterman MR, Parker KL, Lehoux JG** 1991 Regulation of rat adrenal messenger RNA and protein levels for cytochrome P-450s and adrenodoxin by dietary sodium depletion or potassium intake. J Biol Chem 266:2245–2251
39. **Nogueira EF, Xing Y, Morris CA, Rainey WE** 2009 Role of angiotensin II-induced rapid response genes in the regulation of enzymes needed for aldosterone synthesis. J Mol Endocrinol 42:319–330
40. **Hoekstra M, Ye D, Hildebrand RB, Zhao Y, Lammers B, Stitzinger M, Kuiper J, Van Berkel TJ, Van Eck M** 2009 Scavenger receptor class B type I-mediated uptake of serum cholesterol is essential for optimal adrenal glucocorticoid production. J Lipid Res 50:1039–1046
41. **Rajapaksha WR, McBride M, Robertson L, O'Shaughnessy PJ** 1997 Sequence of the bovine HDL-receptor (SR-BI) cDNA and changes in receptor mRNA expression during granulosa cell luteinization in vivo and in vitro. Mol Cell Endocrinol 134:59–67
42. **Ritsch A, Tancevski I, Schgoer W, Pfeifhofer C, Gander R, Eller P, Foeger B, Stanzl U, Patsch JR** 2004 Molecular characterization of rabbit scavenger receptor class B types I and II: portal to central vein gradient of expression in the liver. J Lipid Res 45:214–222
43. **Reaven E, Cortez Y, Leers-Sucheta S, Nomoto A, Azhar S** 2004 Dimerization of the scavenger receptor class B type I: formation, function, and localization in diverse cells and tissues. J Lipid Res 45:513–528
44. **McLean MP, Sandhoff TW** 1998 Expression and hormonal regulation of the high-density lipoprotein (HDL) receptor scavenger receptor class B type I messenger ribonucleic acid in the rat ovary. Endocrine 9:243–252
45. **Imachi H, Murao K, Sato M, Hosokawa H, Ishida T, Takahara J** 1999 CD36 LIMPII analogous-1, a human homolog of the rodent scavenger receptor B1, provides the cholesterol ester for steroidogenesis in adrenocortical cells. Metabolism 48:627–630
46. **Burnay MM, Python CP, Vallotton MB, Capponi AM, Rossier MF** 1994 Role of the capacitative calcium influx in the activation of steroidogenesis by angiotensin-II in adrenal glomerulosa cells. Endocrinology 135:751–758
47. **Quinn SJ, Williams GH, Tillotson DL** 1988 Calcium oscillations in single adrenal glomerulosa cells stimulated by angiotensin II. Proc Natl Acad Sci USA 85:5754–5758
48. **Startchik I, Morabito D, Lang U, Rossier MF** 2002 Control of calcium homeostasis by angiotensin II in adrenal glomerulosa cells through activation of p38 MAPK. J Biol Chem 277:24265–24273
49. **Kraemer FB, Shen WJ, Patel S, Osuga J, Ishibashi S, Azhar S** 2007 The LDL receptor is not necessary for acute adrenal steroidogenesis in mouse adrenocortical cells. Am J Physiol Endocrinol Metab 292:E408–E412
50. **Gwynne JT, Hess B** 1980 The role of high density lipoproteins in rat adrenal cholesterol metabolism and steroidogenesis. J Biol Chem 255:10875–10883
51. **Travert C, Fofana M, Carreau S, Le Goff D** 2000 Rat Leydig cells use apolipoprotein E depleted high density lipoprotein to regulate testosterone production. Mol Cell Biochem 213:51–59
52. **Reyland ME, Evans RM, White EK** 2000 Lipoproteins regulate expression of the steroidogenic acute regulatory protein (StAR) in mouse adrenocortical cells. J Biol Chem 275:36637–36644
53. **Murao K, Imachi H, Cao W, Yu X, Li J, Yoshida K, Ahmed RA, Matsumoto K, Nishiuchi T, Wong NC, Ishida T** 2006 High-density lipoprotein is a potential growth factor for adrenocortical cells. Biochem Biophys Res Commun 344:226–232
54. **Rainey WE, Rodgers RJ, Mason JI** 1992 The role of bovine lipoproteins in the regulation of steroidogenesis and HMG-CoA reductase in bovine adrenocortical cells. Steroids 57:167–173
55. **Simpson HD, Shepherd R, Shepherd J, Fraser R, Lever AF, Kenyon CJ** 1989 Effects of cholesterol and lipoproteins on aldosterone secretion by bovine zona glomerulosa cells. J Endocrinol 121:125–131
56. **Acton SL, Scherer PE, Lodish HF, Krieger M** 1994 Expression cloning of SR-BI, a CD36-related class B scavenger receptor. J Biol Chem 269:21003–21009
57. **Altmann SW, Davis Jr HR, Yao X, Laverty M, Compton DS, Zhu LJ, Crona JH, Caplen MA, Hoos LM, Tetzloff G, Priestley T, Burnett DA, Strader CD, Graziano MP** 2002 The identification of intestinal scavenger receptor class B, type I (SR-BI) by expression cloning and its role in cholesterol absorption. Biochim Biophys Acta 1580:77–93
58. **Viñals M, Xu S, Vasile E, Krieger M** 2003 Identification of the N-linked glycosylation sites on the high density lipoprotein (HDL)

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receptor SR-BI and assessment of their effects on HDL binding and selective lipid uptake. J Biol Chem 278:5325–5332
59. **Gu X, Trigatti B, Xu S, Acton S, Babitt J, Krieger M** 1998 The efficient cellular uptake of high density lipoprotein lipids via scavenger receptor class B type I requires not only receptor-mediated surface binding but also receptor-specific lipid transfer mediated by its extracellular domain. J Biol Chem 273:26338–26348
60. **Nieland TJ, Penman M, Dori L, Krieger M, Kirchhausen T** 2002 Discovery of chemical inhibitors of the selective transfer of lipids mediated by the HDL receptor SR-BI. Proc Natl Acad Sci USA 99: 15422–15427
61. **Lamarche B, Moorjani S, Cantin B, Dagenais GR, Lupien PJ, Després JP** 1997 Associations of HDL2 and HDL3 subfractions with ischemic heart disease in men. Prospective results from the Quebec Cardiovascular Study. Arterioscler Thromb Vasc Biol 17:1098–1105
62. **Yoshikawa M, Sakuma N, Hibino T, Sato T, Fujinami T** 1997 HDL3 exerts more powerful anti-oxidative, protective effects against copper-catalyzed LDL oxidation than HDL2. Clin Biochem 30:221–225
63. **Roche D, Migueres ML, Lequang NT, Burstein M, Ekindjian OG, Girard-Globa A** 1991 Concentrations of high-density lipoprotein subfraction HDL2 and lipoprotein A-I in a random population of healthy subjects. Clin Chem 37:2111–2113
64. **Kontush A, Therond P, Zerrad A, Couturier M, Négre-Salvayre A, de Souza JA, Chantepie S, Chapman MJ** 2007 Preferential sphingosine-1-phosphate enrichment and sphingomyelin depletion are key features of small dense HDL3 particles: relevance to antiapoptotic and antioxidative activities. Arterioscler Thromb Vasc Biol 27:1843–1849
65. **Kennedy AL, Wasan KM** 1999 Preferential distribution of amphotericin B lipid complex into human HDL3 is a consequence of high density lipoprotein coat lipid content. J Pharm Sci 88:1149–1155
66. **Lemieux S, Després JP, Moorjani S, Nadeau A, Thériault G, Prud'homme D, Tremblay A, Bouchard C, Lupien PJ** 1994 Are gender differences in cardiovascular disease risk factors explained by the level of visceral adipose tissue? Diabetologia 37:757–764
67. **Sgouraki E, Tsopanakis A, Tsopanakis C** 2001 Acute exercise: response of HDL-C, LDL-C lipoproteins and HDL-C subfractions levels in selected sport disciplines. J Sports Med Phys Fitness 41: 386–391
68. **Spate-Douglas T, Keyser RE** 1999 Exercise intensity: its effect on the high-density lipoprotein profile. Arch Phys Med Rehabil 80:691–695
69. **Ushiroyama T, Sakuma K, Ikeda A, Ueki M** 2005 The HDL2/HDL3 ratio in menopause. Int J Gynaecol Obstet 88:303–308
70. **Schäfer C, Parlesak A, Eckoldt J, Bode C, Bode JC, März W, Winkler K** 2007 Beyond HDL-cholesterol increase: phospholipid enrichment and shift from HDL3 to HDL2 in alcohol consumers. J Lipid Res 48:1550–1558
71. **Verges B, Brun JM, Vaillant G, Quantin C, Brunet-Lecomte P, Farnier M, Gambert P** 1992 Influence of obesity and hypertriglyceridaemia on the low HDL2-cholesterol level and on its relationship with prevalence of atherosclerosis in type 2 diabetes. Diabetes Metab 18:289–297
72. **Moffatt RJ, Chelland SA, Pecott DL, Stamford BA** 2004 Acute exposure to environmental tobacco smoke reduces HDL-C and HDL2-C. Prev Med 38:637–641
73. **Moorjani S, Dupont A, Labrie F, Lupien PJ, Gagné C, Brun D, Giguère M, Bélanger A, Cusan L** 1988 Changes in plasma lipoproteins during various androgen suppression therapies in men with prostatic carcinoma: effects of orchiectomy, estrogen, and combination treatment with luteinizing hormone-releasing hormone agonist and flutamide. J Clin Endocrinol Metab 66:314–322
74. **Avogaro P, Cazzolato G** 1975 Familial hyper-HDL-(a)-cholesterolemia. Atherosclerosis 22:63–77
75. **Tilly-Kiesi M, Tikkanen MJ** 1992 Effects of lovastatin and gemfibrozil on high-density lipoprotein subfraction density and composition in patients with familial hypercholesterolemia. J Lab Clin Med 120:103–110
76. **Imachi H, Murao K, Sayo Y, Hosokawa H, Sato M, Niimi M, Kobayashi S, Miyauchi A, Ishida T, Takahara J** 1999 Evidence for a potential role for HDL as an important source of cholesterol in human adrenocortical tumors via the CLA-1 pathway. Endocr J 46:27–34
77. **Rodríguez C, González-Díez M, Badimon L, Martínez-González J** 2009 Sphingosine-1-phosphate: A bioactive lipid that confers high-density lipoprotein with vasculoprotection mediated by nitric oxide and prostacyclin. Thromb Haemost 101:665–673
78. **Sattler K, Levkau B** 2009 Sphingosine-1-phosphate as a mediator of high-density lipoprotein effects in cardiovascular protection. Cardiovasc Res 82:201–211
79. **Brizuela L, Rábano M, Peña A, Gangoiti P, Macarulla JM, Trueba M, Gómez-Muñoz A** 2006 Sphingosine 1-phosphate: a novel stimulator of aldosterone secretion. J Lipid Res 47:1238–1249
80. **Stern N, Yanagawa N, Saito F, Hori M, Natarajan R, Nadler J, Tuck M** 1993 Potential role of 12 hydroxyeicosatetraenoic acid in angiotensin II-induced calcium signal in rat glomerulosa cells. Endocrinology 133:843–847
81. **Natarajan R, Dunn WD, Stern N, Nadler J** 1990 Key role of diacylglycerol-mediated 12-lipoxygenase product formation in angiotensin II-induced aldosterone synthesis. Mol Cell Endocrinol 72: 73–80
82. **Natarajan R, Stern N, Hsueh W, Do Y, Nadler J** 1988 Role of the lipoxygenase pathway in angiotensin II-mediated aldosterone biosynthesis in human adrenal glomerulosa cells. J Clin Endocrinol Metab 67:584–591
83. **Argraves KM, Argraves WS** 2007 HDL serves as a S1P signaling platform mediating a multitude of cardiovascular effects. J Lipid Res 48:2325–2333
84. **Tölle M, Schuchardt M, van der Giet M** 2010 Relevance of sphingolipids in the pleiotropic protective effects of high-density lipoproteins. Curr Pharm Des 16:1468–1479
85. **Argraves KM, Gazzolo PJ, Groh EM, Wilkerson BA, Matsuura BS, Twal WO, Hammad SM, Argraves WS** 2008 High density lipoprotein-associated sphingosine 1-phosphate promotes endothelial barrier function. J Biol Chem 283:25074–25081
86. **Keul P, Sattler K, Levkau B** 2007 HDL and its sphingosine-1-phosphate content in cardioprotection. Heart Fail Rev 12:301–306
87. **Frias MA, James RW, Gerber-Wicht C, Lang U** 2009 Native and reconstituted HDL activate Stat3 in ventricular cardiomyocytes via ERK1/2: role of sphingosine-1-phosphate. Cardiovasc Res 82:313–323
88. **Lee MH, Hammad SM, Semler AJ, Luttrell LM, Lopes-Virella MF, Klein RL** 2010 HDL3, but not HDL2, stimulates plasminogen activator inhibitor-1 release from adipocytes: the role of sphingosine-1-phosphate. J Lipid Res 51:2619–2628
89. **Gu J, Wen Y, Mison A, Nadler JL** 2003 12-Lipoxygenase pathway increases aldosterone production, 3′,5′-cyclic adenosine monophosphate response element-binding protein phosphorylation, and p38 mitogen-activated protein kinase activation in H295R human adrenocortical cells. Endocrinology 144:534–543
90. **Natarajan R, Yang DC, Lanting L, Nadler JL** 2002 Key role of P38 mitogen-activated protein kinase and the lipoxygenase pathway in angiotensin II actions in H295R adrenocortical cells. Endocrine 18: 295–301
91. **Stern N, Natarajan R, Tuck ML, Laird E, Nadler JL** 1989 Selective inhibition of angiotensin-II-mediated aldosterone secretion by 5-hydroxyeicosatetraenoic acid. Endocrinology 125:3090–3095
92. **Shibata H, Kojima I** 1991 Involvement of protein kinase C in angiotensin II-mediated release of 12-hydroxyeicosatetraenoic acid in bovine adrenal glomerulosa cells. Endocrinol Jpn 38:611–617
93. **Brousseau ME, Schaefer EJ, Wolfe ML, Bloedon LT, Digenio AG, Clark RW, Mancuso JP, Rader DJ** 2004 Effects of an inhibitor of cholesteryl ester transfer protein on HDL cholesterol. N Engl J Med 350:1505–1515

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94. **Nissen SE, Tardif JC, Nicholls SJ, Revkin JH, Shear CL, Duggan WT, Ruzyllo W, Bachinsky WB, Lasala GP, Lasala GP, Tuzcu EM** 2007 Effect of torcetrapib on the progression of coronary atherosclerosis. N Engl J Med 356:1304–1316
95. **Barter PJ, Caulfield M, Eriksson M, Grundy SM, Kastelein JJ, Komajda M, Lopez-Sendon J, Mosca L, Tardif JC, Waters DD, Shear CL, Revkin JH, Buhr KA, Fisher MR, Tall AR, Brewer B** 2007 Effects of torcetrapib in patients at high risk for coronary events. N Engl J Med 357:2109–2122
96. **Athyros VG, Kakafika A, Tziomalos K, Karagiannis A, Mikhailidis DP** 2008 Cholesteryl ester transfer protein inhibition and HDL increase: has the dream ended? Expert Opin Investig Drugs 17:445–449
97. **Hu X, Dietz JD, Xia C, Knight DR, Loging WT, Smith AH, Yuan H, Perry DA, Keiser J** 2009 Torcetrapib induces aldosterone and cortisol production by an intracellular calcium-mediated mechanism independently of cholesteryl ester transfer protein inhibition. Endocrinology 150:2211–2219
98. **Forrest MJ, Bloomfield D, Briscoe RJ, Brown PN, Cumiskey AM, Ehrhart J, Hershey JC, Keller WJ, Ma X, McPherson HE, Messina E, Peterson LB, Sharif-Rodriguez W, Siegl PK, Sinclair PJ, Sparrow CP, Stevenson AS, Sun SY, Tsai C, Vargas H, Walker 3rd M, West SH, White V, Woltmann RF** 2008 Torcetrapib-induced blood pressure elevation is independent of CETP inhibition and is accompanied by increased circulating levels of aldosterone. Br J Pharmacol 154: 1465–1473
99. **Stein EA, Stroes ES, Steiner G, Buckley BM, Capponi AM, Burgess T, Niesor EJ, Kallend D, Kastelein JJ** 2009 Safety and tolerability of dalcetrapib. Am J Cardiol 104:82–91

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