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# Dihydrotestosterone regulating apolipoprotein M expression mediates via protein kinase C in HepG2 cells

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## Abstract

**Background:** Administration of androgens decreases plasma concentrations of high-density lipid cholesterol (HDL-C). However, the mechanisms by which androgens mediate lipid metabolism remain unknown. This present study used HepG2 cell cultures and ovariectomized C57BL/6 J mice to determine whether apolipoprotein M (ApoM), a constituent of HDL, was affected by dihydrotestosterone (DHT).

**Methods:** HepG2 cells were cultured in the presence of either DHT, agonist of protein kinase C (PKC), phorbol-12-myristate-13-acetate (PMA), blocker of androgen receptor flutamide together with different concentrations of DHT, or DHT together with staurosporine at different concentrations for 24 hrs. Ovariectomized C57BL/6 J mice were treated with DHT or vehicle for 7d or 14d and the levels of plasma ApoM and livers *ApoM* mRNA were measured. The mRNA levels of ApoM, ApoAI were determined by real-time RT-PCR. ApoM and ApoAI were determined by western blotting analysis.

**Results:** Addition of DHT to cell culture medium selectively down-regulated *ApoM* mRNA expression and ApoM secretion in a dose-dependent manner. At 10 nM DHT, the *ApoM* mRNA levels were about 20% lower than in untreated cells and about 40% lower at 1000 nM DHT than in the control cells. The secretion of ApoM into the medium was reduced to a similar extent. The inhibitory effect of DHT on ApoM secretion was not blocked by the classical androgen receptor blocker flutamide but by an antagonist of PKC, Staurosporine. Agonist of PKC, PMA, also reduced ApoM. At 0.5  $\mu$ M PMA, the *ApoM* mRNA levels and the secretion of ApoM into the medium were about 30% lower than in the control cells. The mRNA expression levels and secretion of another HDL-associated apolipoprotein AI (ApoAI) were not affected by DHT. The levels of plasma ApoM and liver *ApoM* mRNA of DHT-treated C57BL/6 J mice were lower than those of vehicle-treated mice.

**Conclusions:** DHT directly and selectively down-regulated the level of *ApoM* mRNA and the secretion of ApoM by protein kinase C but independently of the classical androgen receptor.

**Keywords:** Androgen, DHT, ApoM, PKC

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## Introduction

Men exhibit a higher incidence of cardiovascular diseases than women, and men have lower circulating levels of antiatherogenic high-density lipoprotein cholesterol (HDL-C). Evidence indicates that the cardiovascular actions of sex steroids are primary factors in mediating this gender-related difference. Because androgen administration lowers HDL-C levels in both genders [1], particularly at supraphysiological plasma concentrations, endogenous androgens, such as testosterone, have been implicated in influencing the lipoprotein profile and risk for cardiovascular diseases (CVD). However, the relationship between androgens and lipid metabolism CVD risk factors is highly complex, and the results of different studies are contradictory [2]. A likely explanation for the complex relationship between androgens and CVD is that androgens affect many risk factors. For example, androgens can increase muscle mass, decrease visceral fat mass in some subjects, improve coronary blood flow, increase mood and motivation (perhaps leading indirectly to health benefits), reduce lipoprotein (a) and leptin, improve insulin sensitivity, and provide other potential benefits, such as anti-inflammatory effects [3]. To understand the role of endogenous and therapeutic androgens in CVD, it will be necessary to identify the mechanisms responsible for the changes in HDL-C levels.

Apolipoprotein M (ApoM) is mainly expressed by hepatocytes and tubular epithelial cells in the kidney and is associated mainly with high-density lipoprotein (HDL) in human plasma [4-7]. Mice deficient in ApoM are impaired in their ability to produce pre $\beta$ -HDL. Further, overexpression of ApoM in LDL-receptor knockout mice protects against atherosclerosis in mice fed a cholesterol-rich diet. These findings indicate that ApoM is important for pre $\beta$ -HDL formation and may exert a protective effect on the development and progression of atherosclerosis [8]. Moreover, evidence indicates that ApoM levels are possibly regulated by several cytokines in the human HepG2 cell line, which was derived from hepatocellular carcinoma [9-14]. However, the pathophysiological importance of ApoM in humans is still unknown.

The androgen receptor (AR) is expressed in the liver, the primary site of lipoprotein regulation, in which it could conceivably alter the expression of genes controlling HDL metabolism. Apolipoprotein AI (ApoAI) levels are reduced after treatment with androgens, suggesting the decreased synthesis or increased catabolism of this core constituent of HDL particles [15,16]. ApoM, which is one of the main constituents of HDL particles, is involved in HDL metabolism and formation of pre $\beta$ -HDL. Whether androgens regulate the secretion of apoM and further mediate lipid metabolism remains unknown.

To further understand the possible effect of androgens on ApoM secretion, we investigated the effects of 5-

dihydrotestosterone (DHT) on the regulation of ApoM expression by HepG2 cells. DHT is a potent natural androgen that, unlike testosterone, cannot be converted to estradiol by aromatase.

## Results

### Effects of DHT on *ApoM* mRNA levels and secretion of ApoM by HepG2 cells

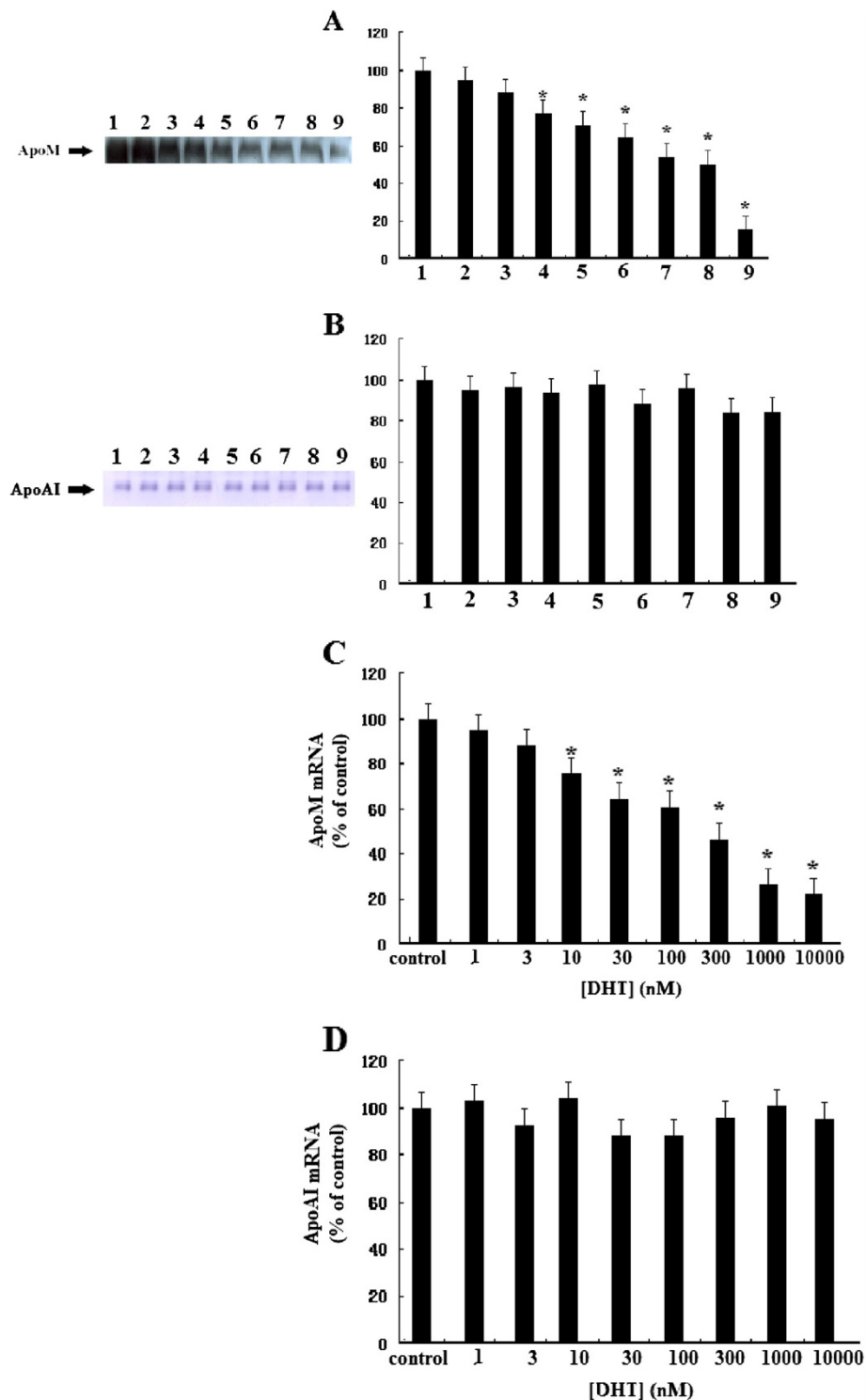
We first investigated whether DHT could modulate the levels of *ApoM* mRNA and ApoM secretion from HepG2 cells. As shown in Figure 1, DHT significantly inhibited secretion and mRNA levels of ApoM. At 10 nM DHT, ApoM secretion was decreased by 20% ( $P < 0.05$ ), and at 1000 nM DHT, ApoM secretion was decreased by 60% ( $P < 0.01$ ) compared with the control media (A). To test the specificity of the effect of DHT on ApoM secretion, we determined the effect of DHT on ApoAI secretion in the same cell culture supernatants. DHT did not affect ApoAI secretion at any concentration tested within the levels of detection of the assays (B). DHT also significantly suppressed the levels of *ApoM* mRNA expression in a dose-dependent manner. At 10 nM, the reduction in *ApoM* mRNA was about 20%, and at 1000 nM, it was reduced by more than 70% ( $P < 0.01$ ) compared with control cells (C). However, the levels of *ApoAI* mRNA were not affected by any concentration of DHT (D).

### DHT-suppressed secretion and the mRNA levels of ApoM are not blocked by flutamide

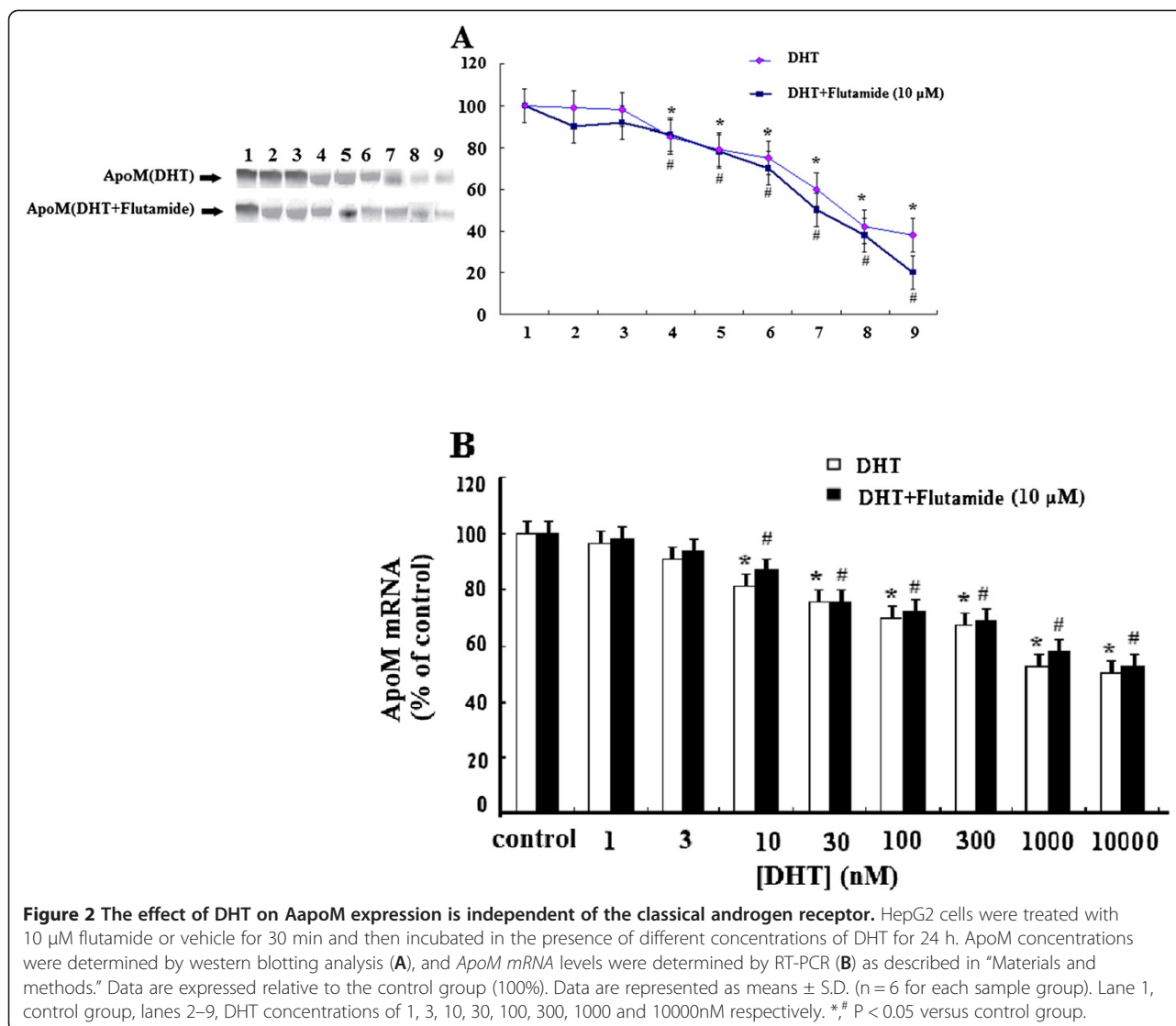
To test if the effect of DHT on ApoM secretion and *ApoM* mRNA levels is mediated by the classical androgen receptor, we performed incubations in the presence or absence of the androgen receptor antagonist, flutamide (Figure 2). After 30 min of incubation with flutamide, HepG2 cells were incubated with different concentrations of DHT for 24 h, thereby resulting in the suppression of the secretion of ApoM (A) and the levels of *ApoM* mRNA (B) in a dose-dependent manner. This demonstrated that flutamide did not change the effects of DHT on ApoM secretion or *ApoM* mRNA levels, although HepG2 cells express the classical androgen receptor.

### PKC is involved in DHT-mediated apoM secretion

The PKC superfamily comprises 9 protein kinases. To determine whether PKC is involved in DHT-mediated ApoM secretion, HepG2 cells were incubated with PMA or Staurosporine in the presence or absence of DHT (Figure 3). PMA decreased the expression and secretion of ApoM (Figure 3A, C). Staurosporine alone had no effect on the levels of ApoM and *ApoM* mRNA (Figure 3B, C). Staurosporine abolished the DHT-mediated decrease in ApoM secretion and expression (Figure 3D, E). These results indicate that PKC affects the DHT-mediated decrease in ApoM secretion and *ApoM* mRNA expression.



**Figure 1** Effect of DHT on secretion and expression of ApoM and ApoAI. HepG2 cells were incubated in 2 ml of DMEM containing 10% FBS until subconfluency was achieved. Medium was then changed to 2 ml of medium containing 10% CTBFS and various concentrations of DHT (0, 1, 3, 10, 30, 100, 300, 1000, 10000nM) and incubated for 24 h. ApoM and ApoAI levels were determined by western blotting analysis (**A**, **B**), *ApoM* and *ApoAI* mRNA levels were determined by RT-PCR (**C**, **D**) as described in "Materials and methods." Data are expressed relative to the control group (100%). Data are represented as means  $\pm$  S.D. (n = 6 for each sample group). Lane 1, control group, lanes 2–9, DHT concentrations of 1, 3, 10, 30, 100, 300, 1000 and 10000nM respectively. \*P < 0.05 vs. control group.



To determine whether PI3-K is involved in the DHT-mediated reduction of ApoM secretion and the decrease in the levels of its mRNA, HepG2 cells were also incubated with the wortmannin, an inhibitor of PI3-K. The PI3-K inhibitor wortmannin did not detectably alter the effects of DHT on ApoM mRNA levels or its secretion (data not shown).

#### Plasma ApoM and hepatic ApoM mRNA levels from mice

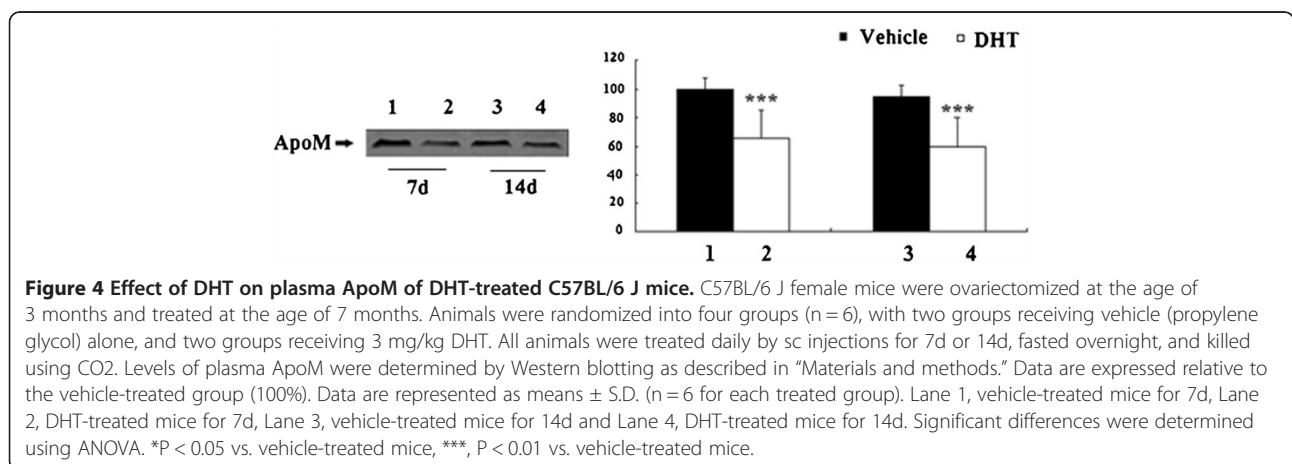
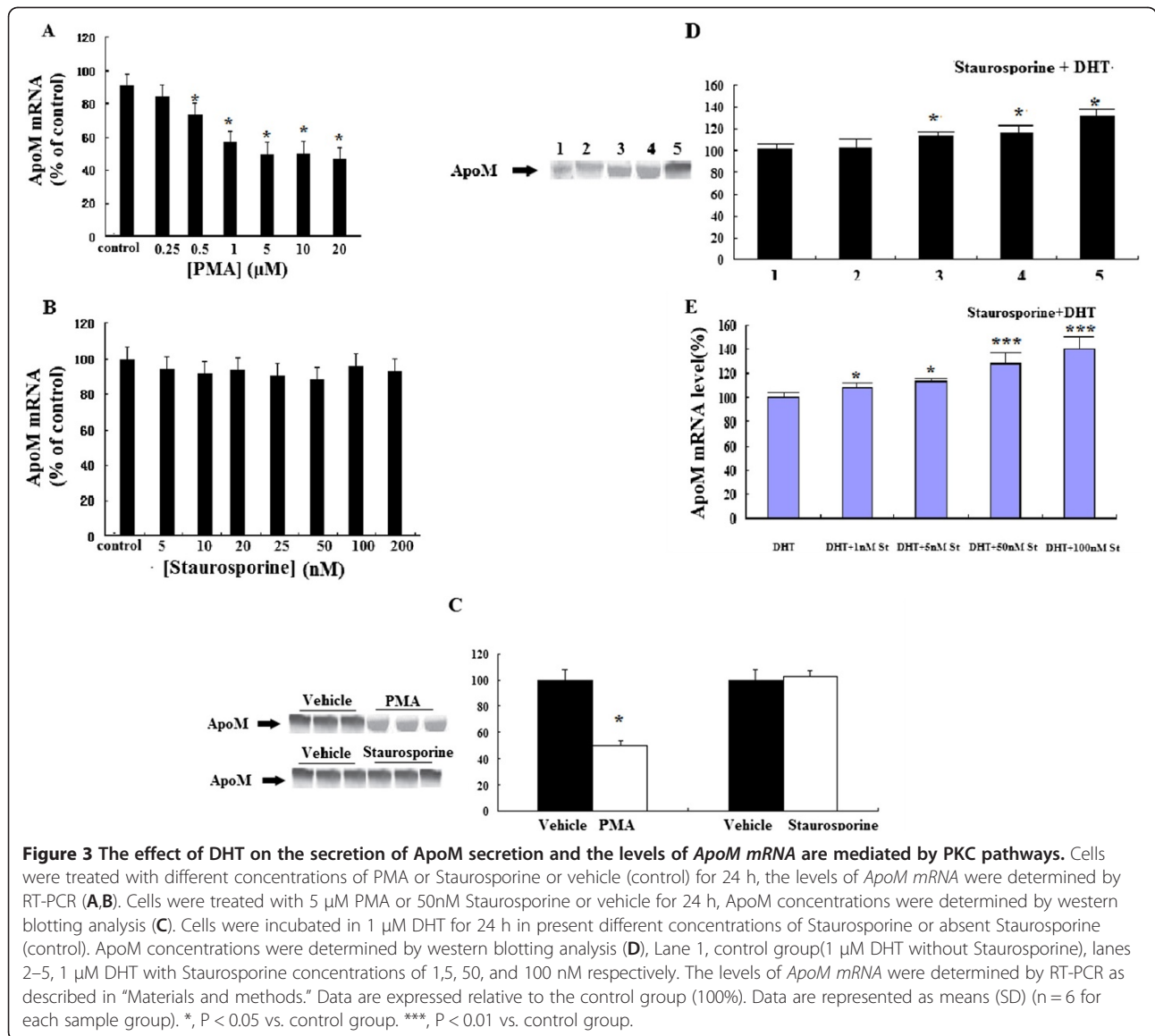
To analyze the effect of androgens on ApoM expression and secretion, levels of plasma ApoM and liver ApoM mRNA of DHT-treated mice were measured and compared with those of vehicle-treated mice. Levels of plasma ApoM was analyzed by Western Blot. Levels of plasma ApoM were reduced in DHT-treated mice significantly (Figure 4). Livers were frozen in liquid nitrogen for ApoM RNA analysis after mice were killed using CO<sub>2</sub>. Levels ApoM mRNA were measured with RT-PCR.

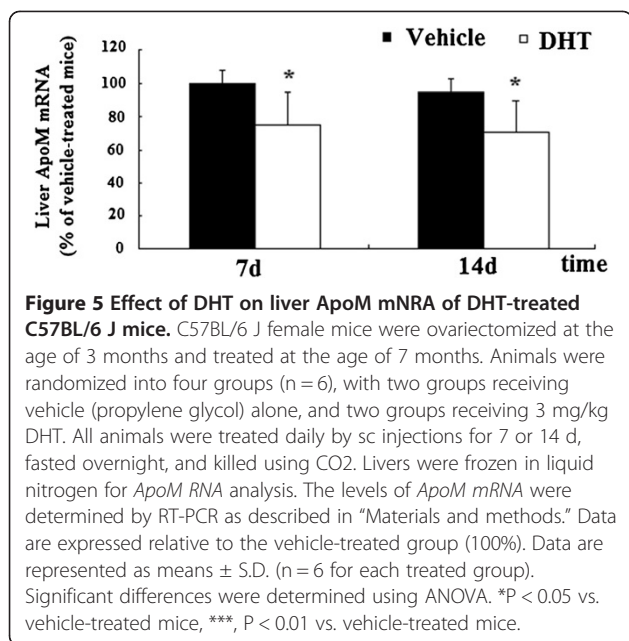
Levels of liver ApoM mRNA were reduced in DHT-treated mice significantly (Figure 5).

#### Discussion

In the present study, we demonstrate that ApoM expression is regulated by DHT in HepG2 cells in a dose-dependent manner and that inhibition of ApoM expression by DHT is mediated by PKC. Moreover, DHT did not affect ApoAI expression and we demonstrated further that inhibition of PI3K did not influence DHT-mediated apoM expression.

Androgens administration lowers HDL-C levels in both genders [1], but androgens modulate cholesterol metabolism in poorly understood ways. Most studies indicate that administration of natural or synthetic androgens produces a plethora of effects, some of which appear negative, such as reduction of HDL-C levels, and others positive, such as increased lean mass and reduced





visceral fat, lower total cholesterol, and improved sensitivity to insulin [17]. To understand the role of endogenous and therapeutic androgens in CVD, it will be necessary to identify the mechanisms responsible for the reduction in HDL-C. One hypothesis considers reduced synthesis of ApoAI, ApoM, or HDL. The second hypothesis considers that there is decreased donation of cholesterol from peripheral tissues to HDL particles, and the third proposes that there is increased clearance of HDL-C. The fourth, a more complex hypothesis, is that androgens lead to HDL remodeling [15,18], cholesterol redistribution [19], or changes in lipoprotein catabolism.

ApoAI and apoM are constituents of HDL. In some studies, ApoAI levels are reduced after androgen treatment, suggesting decreased synthesis or increased catabolism of HDL [15,16]. ApoM was first identified as a component of human postprandial lipoproteins in 1999 [4]. It was estimated that ~5% of HDL particles contain ApoM [20]. ApoM content in the healthy human plasma pool was 0.94 mM. This roughly corresponds to 1/50<sup>th</sup> of the mean molar concentration of apoA-I in plasma [21]. However, the physiopathological functions of ApoM are not fully elucidated. Studies of the regulation of ApoM expression may reveal the clinical importance of ApoM.

The AR is highly expressed in adipocytes and regulates their function by a variety of mechanisms, including local transcriptional regulation of lipases and increased levels of adrenergic receptors as well as inhibition of adipogenesis [19]. The AR is also expressed in liver, a primary site of lipoprotein regulation, in which it could conceivably alter the expression of genes controlling HDL metabolism [22]. ApoM is mainly expressed in the hepatocytes. Here, we administered DHT to examine its

effect on ApoM secretion and *ApoM* mRNA levels in HepG2 cells. The present study supports the view that DHT affects lipoprotein production by demonstrating that DHT significantly decreased *ApoM* mRNA levels in hepG2 cells and the secretion of ApoM secretion into the medium, whereas there was no effect on the *ApoAI* mRNA levels. These findings suggest that there are different mechanisms for regulating ApoAI and ApoM expression in this particular cell line. To analyze the effect of androgens on ApoM expression and secretion in vivo, we administered DHT to the ovariectomized C57BL/6 J mice. Plasma levels of ApoM and liver *ApoM* mRNA of DHT-treated mice were measured and compared with those of vehicle-treated mice. It demonstrated that DHT reduced the levels of plasma ApoM and liver *ApoM* mRNA in DHT-treated mice. The present findings, therefore, might partially indicate a mechanism underlying the reduction of plasma HDL cholesterol during administration of DHT in vivo.

Sphingolipids are a large family of glycolipids and phospholipids that share a common sphingoid base backbone. These once called 'structural' lipids are now well-established signaling molecules that play multiple roles in a vast number of cellular processes. A growing body of literature has demonstrated the reciprocal interaction between bioactive sphingolipids and steroid hormones. Sphingolipids serve as second messengers in steroidogenic regulatory pathways [23,24], and meanwhile steroid hormones regulates the metabolism of sphingolipids [25-27]. Plasma sphingosine-1-phosphate (S1P), which maintains vascular integrity, is associated with HDL (~65%) and albumin (~35%) [28,29]. HDL induced vasorelaxation as well as barrier-promoting and pro-survival actions on the endothelium have been attributed to S1P signaling [30-32]. ApoM is a lipocalin that resides mainly in the plasma HDL fraction [4]. The retained hydrophobic NH<sub>2</sub>-terminal signal peptide anchors ApoM in the phospholipid layer of the lipoprotein and prevents filtration of the ~22-kDa protein in the kidney [33]. Studies in ApoM gene-modified mice suggest that apoM has anti-atherogenic effects, possibly related in part to ApoM's ability to increase cholesterol efflux from macrophage foam cells, to increased pre $\beta$ -HDL formation, and to anti-oxidative effects [8,20,34]. ApoM is a carrier of S1P in HDL and the HDL-associated ApoM-S1P complex mediates vasoprotective actions on the endothelium. This signaling axis may be critical in normal vascular homeostasis and perturbed in vascular diseases [35]. Whether DHT affected HDL-associated function via regulation of ApoM and ApoM-S1P signaling axis is still to be elucidated.

It is well known that androgens exert both transcriptional and non-transcriptional actions [36-38]. The transcriptional actions of androgens are mediated through the classic androgen receptor. The ligand-bound classic

androgen receptor mainly functions as a transcription factor modulating the expression of androgen-receptor target genes. In contrast, non-transcriptional actions of androgens include increasing the concentration of intracellular calcium, and activation of protein tyrosine kinase, such as Src(c-Src), extracellular signal-regulated kinase-1/2 (ERK-1/2), and phosphatidylinositol 3-kinase (PI3K) [39-44]. In our present study, we found that flutamide, a classical androgen receptor blocker, did not modify DHT-mediated ApoM secretion. Although these data may suggest that the action of DHT on ApoM secretion is non-transcriptional, the differentiation between non-transcriptional vs. transcriptional effects is much more complex and cannot be firmly concluded from the present study.

We also investigated the intracellular signaling mechanisms by which DHT mediates ApoM secretion by HepG2 cells. Our present study shows that PMA, a PKC agonist, increased ApoM secretion. Staurosporin, a PKC superfamily inhibitor, abolished the DHT-mediated decrease in ApoM secretion. The intracellular signaling mechanisms by which DHT act through PKC to affect ApoM secretion remains unknown. It is reported that *ApoM* gene expression is affected by nuclear receptors such as hepatocyte nuclear factor-1a (HNF-1a) [45], hepatocyte nuclear factor-4a (HNF-4a), liver receptor homolog-1 (LRH-1) [46], and liver X receptor (LXR) [47].

Leptin is the first identified endocrine product of adipose tissue and was found to regulate vascular function through local and central mechanisms [48]. There is some evidence supporting the effects of leptin on the cardiovascular system and Type 2 diabetes mellitus (T2DM). It was shown that a high leptin level predicts subsequent development of T2DM [49]. Plasma leptin levels positively correlated with TG, Lp (a), Apo-A1, glucose, BMI, insulin resistance (HOMA-IR), SBP and DBP levels and negatively with HDL-C levels in T2DM patients [50,51]. Studies suggest that both leptin and leptin-receptor are essential for ApoM expression in vitro and vivo [9,52]. In the present study we demonstrated that DHT down-regulated the expression and the secretion of ApoM. Whether DHT-affected ApoM expression is mediated by specific nuclear receptors or leptin remains to be investigated.

It has been previously reported that ApoM expression is regulated by PI3-kinase in HepG2-cells [53]. In the present study, we used the PI3-K antagonist (wortmannin) to study DHT-treated HepG2 cells. We found that wortmannin could not abolish DHT-mediated inhibition of ApoM expression, which indicates that PI3-K might not be involved in the DHT-induced inhibition of ApoM expression. Our present results indicate that PKC is involved in DHT-mediated ApoM secretion. However, the participation of PKC family members whose identities remain to be determined.

## Conclusions

DHT directly and selectively down-regulated the level of *ApoM* mRNA and the secretion of ApoM by protein kinase C but independently of the classical androgen receptor.

## Materials and methods

### Materials

The human cell line HepG2, which was derived from hepatocellular carcinoma, was obtained from the American Type Culture Collection (ATCC). Dulbecco's modified Eagle's medium (DMEM) and benzylpenicillin and streptomycin from Gibco (Shanghai, China). Dihydrotestosterone (DHT) and flutamide were purchased from Sigma Chemical Co. Ltd. (Shanghai, China). Staurosporine, PMA and wortmannin were purchased from ENZO (Shanghai, China). Six-well cell culture clusters and 25-cm<sup>2</sup> vented cell culture flasks were purchased from Costar (Shanghai, China). Fetal bovine serum (FBS) and charcoal-treated fetal bovine serum (CTFBS) were obtained from Invitrogen (Shanghai, China). E.Z.N.A. Total RNA Kit II for total RNA purification was from Omega (Shanghai, China). First strand cDNA synthesis kits were obtained from Invitrogen (Shanghai, China). Taqman Universal PCR Master Mix was purchased from TAKARA Bio-Science and Technology Company (Dalian, China). The LightCycler real-time RT-PCR System was purchased from Roche Applied Science (Shanghai, China). Rabbit monoclonal antibodies against human ApoM, ApoAI,  $\beta$ -actin, and horseradish peroxidase-conjugated goat polyclonal secondary antibody to rabbit IgG (ab6721) were obtained from Abcam.

### Cell cultures

HepG2 cells were maintained in DMEM with 10% FBS (w/v) in the presence of benzylpenicillin (0.1 iu l<sup>-1</sup>) and streptomycin (0.1 g l<sup>-1</sup>) under standard culture conditions (5% CO<sub>2</sub>, 37°C). Cells were seeded in 25-cm<sup>2</sup> cell culture flasks or in 6-well cell-culture clusters and allowed to grow to 50–70% confluence. Before the experiment, cells were washed twice with phosphate buffered saline (PBS) and once with DMEM with 10% CTFBS(w/v). When inhibitors were used, they were added in fresh media 30 min prior to adding the other reagents. At the end of the incubation period, media were removed and saved for ApoM and ApoAI assays and the cells for determining *ApoM* and *ApoAI* mRNA levels.

### Effect of the androgen receptor antagonist flutamide on DHT-mediated ApoM secretion and *ApoM* mRNA levels

To evaluate whether the effect of DHT on *ApoM* mRNA levels and the secretion of ApoM from HepG2 cells was mediated via the androgen receptor, cells were incubated

in the presence or absence of flutamide. The medium was changed when the cells grew to subconfluence, and flutamide (10  $\mu$ M) was then added to the media. After 30 min of incubation with flutamide, different concentrations of DHT were added, and the media and cells were harvested 24 h later for determining ApoM or ApoAI levels.

#### **Effect of protein kinase C or phosphatidylinositol 3-kinase on DHT-mediated ApoM secretion and ApoM mRNA levels**

To evaluate whether the effect of DHT on ApoM secretion from human HepG2 cells was mediated via protein kinase C (PKC), cells were incubated with agonist or antagonist of PKC in the presence or absence of DHT. The medium was changed at subconfluence, after 30 min of incubation with an antagonist of the PKC superfamily (staurosporine, 50 nM) or agonist of PKC (PMA), varying concentrations of DHT were added, and media and cells were harvested 24 h later for the determination of ApoM or ApoAI levels.

To evaluate whether the effect of DHT on ApoM secreted by HepG2 cells was mediated via phosphatidylinositol 3-kinase (PI3-K), cells were incubated in the presence or absence of an inhibitor of PI3-K (wortmannin). After 30 min of incubation with wortmannin (50 nM), different concentrations of DHT were added, and the media and cells were harvested 24 h later for the determination of ApoM.

#### **Mice**

C57BL/6 J female mice were obtained from the Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China) and maintained in a 12-h/12-h light/dark cycle with unlimited access to chow and water. Mice were ovariectomized at the age of 3 months and treated at the age of 7 months. Animals were randomized into four groups (n = 6), with two groups receiving vehicle (propylene glycol) alone, and two groups receiving 3 mg/kg DHT. All animals were treated daily by sc injections for 7 d or 14 d, fasted overnight, and killed using CO<sub>2</sub>. Plasmas were collected for ApoM analysis, and livers were frozen in liquid nitrogen for ApoM RNA analysis.

#### **Extraction of total RNA and real time RT-PCR assays**

Total HepG2 RNA was extracted using the E.Z.N.A. Total RNA Kit II according to the manufacturer's instructions. For reverse transcription 5  $\mu$ g total RNA was incubated with 0.5  $\mu$ g T12VN and Superscript III following the manufacturer's suggested protocol. Human ApoM primers (forward: 5'-TACCAGCCCTTCTGCACTG-3', reverse: 5'-ATCGAGGGAAGAGTGGGG-3') and human ApoAI primers (forward: 5'-GGCTGTCATCTCTCAGGGAGTTAG-3', reverse: 5'-ATTTGAACCTGCCTGACCCCTTAG-3')

and  $\beta$ -actin primers (forward: 5'-ACTTACGGTAAA TGGCCCG -3', reverse: 5'-TAGGGGGCGTACTTGG CATA -3') and mouse ApoM primers (forward: 5'-CCAAATAGGCTGTCCCAGAA-3', reverse: 5'-CGAGTC ACTTTCCTGGCTTC-3') were designed with Primer Express software (Applied Biosystems). Quantification of ApoM mRNA levels or ApoAI mRNA levels is relative to  $\beta$ -actin mRNA levels and was performed on a LightCycler in a final volume of 20  $\mu$ l. Optimal conditions were obtained with 2.0  $\mu$ l of Taqman Universal PCR Master Mix, 22.5 pmol of both forward and reverse primers and 1  $\mu$ l of RT product. The thermal cycling conditions for human or mouse ApoM, ApoAI, and  $\beta$ -actin included the following steps: 2 min at 50°C and 1 min 95°C to activate Taq polymerase, 40 cycles of 15 sec at 95°C and 1 min at 60°C. Samples were amplified simultaneously in triplicates in one-assay run. The threshold cycle (CT) is defined as the fractional cycle number at which the reporter fluorescence reaches a certain level. The ratio expression of each gene in experimental vs. control samples was calculated as  $2^{-(\text{mean}\Delta\Delta\text{CT})}$ . Significant differences were determined using ANOVA.

#### **Apolipoproteins M and AI protein mass determinations**

The relative molecular masses of ApoM and ApoAI were determined by western blotting analysis. Cell culture medium containing CTFBS or plasma from mice was fractionated by SDS-polyacrylamide gel electrophoresis, and the proteins were transferred to a nitrocellulose membrane, which was incubated with rabbit monoclonal antibodies and goat polyclonal secondary antibody. Bands corresponding to the different apolipoproteins were visualized using an ECL Plus Western blotting detection system (GE Healthcare Life Science) or using the peroxidase staining method and quantified using Quantity One software.

#### **Statistical analysis**

Results are expressed as means  $\pm$  S.D. Two groups were compared using Student's *t*-test, and multiple groups were analyzed by factorial ANOVA followed by Newman-Keuls' post hoc comparisons. Statistical calculations were performed with Statistical software package version 7.1. Differences were considered significant at  $P < 0.05$ .

#### **Competing interests**

The authors declare that they have no competing interests.

#### **Authors' contributions**

RJ and JY participated in the assay of RT-PCR. JZH and WLY participated in the assay of Western blotting. WRX performed the statistical analysis. JZH and LMQ participated in cell culture. YYZ, CB, ZJ, WW and JGQ participated in the project design. All authors read and approved the final manuscript.

#### **Author's information**

Cao Bing is a co-first author.



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