Characterization of 5α-reductase activity and isoenzymes in human abdominal adipose tissues

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ABSTRACT

The substrate for the generation of 5α-dihydrotestosterone (DHT) is either androstenedione (4-dione) which is first converted to androstanedione and then to DHT through 17-oxoreductase activity, or testosterone, which is directly converted to DHT. Three 5α-reductase isoenzymes have been characterized and designated as types 1, 2 and 3 (SRD5A1, 2 and 3). **Objective:** To define the predominant source of local DHT production in human adipose tissues, identify 5α-reductase isoenzymes and test their impact on preadipocyte differentiation. **Methods:** Cultures of omental (OM) and subcutaneous (SC) preadipocytes were treated for 0, 6 or 24h with 30 nM ^{14}C-4-dione or ^{14}C-testosterone, with and without 500 nM 5α-reductase inhibitors 17-N,N-diethylcarbamoyl-4-methyl-4-aza-5-androstan-3-one (4-MA) or finasteride. Protein level and mRNA abundance of 5α-reductase isoenzymes/transcripts were examined in whole SC and OM adipose tissue. HEK-293 cells stably transfected with 5α-reductase type 1, 2 or 3 were used to test 5α-reductase inhibitors. We also assessed the impact of 5α-reductase inhibitors on preadipocyte differentiation. **Results:** Over 24h, DHT formation from 4-dione increased gradually ($p<0.05$) and was significantly higher compared to that generated from testosterone ($p<0.001$). DHT formation from both 4-dione and testosterone was blocked by both 5α-reductase inhibitors. In whole adipose tissue from both fat compartments, SRD5A3 was the most highly expressed isoenzyme followed by SRD5A1 ($p<0.001$). SRD5A2 was not expressed. In HEK-293 cells, 4-MA and finasteride inhibited activity of 5α-reductases type 2 and type 3 but not type 1. In preadipocyte cultures where differentiation was inhibited by 4-dione ($p<0.05$, $n=7$) or testosterone ($p<0.05$, $n=5$), the inhibitors 4-MA and finasteride abolished these effects. **Conclusion:** Although 4-dione is the main source of DHT in human preadipocytes, 5α-reductase-mediated DHT production mediates the inhibitory effect of both 4-dione and testosterone on preadipocyte differentiation.

Keywords: 5α-reductase, adipose tissue, dihydrotestosterone, testosterone and androstenedione.

1. INTRODUCTION

5α-reductases are microsomal enzymes responsible for the formation of 5α-dihydrotestosterone (DHT), either through conversion of androstenedione (4-dione) to androstanedione (A-dione) and subsequent 17-oxoreduction, or through direct conversion of testosterone (testo) to DHT [1]. Three isoenzymes have been identified which are the products of three different genes: SRD5A1, SRD5A2, SRD5A3 [2]. The type 1 and 2 isoenzymes of SRD5A have a low homology; they have different chromosomal localizations and kinetic parameters; they also differ in their distribution patterns in androgen target tissues [3]. A third 5α-reductase isoenzyme designated as type 3 was detected in prostate tissue and was reported to be poorly inhibited by dutasteride at high androgen concentrations *in vitro* [4]. SRD5A3 also plays a role in N-linked protein glycosylation, and mutations in this gene cause a rare Mendelian disease [5].

In adipose tissue, androgens such as DHT and testo inhibit preadipocyte differentiation [6, 7]. They have also been reported to decrease triglyceride synthesis and increase lipolysis in some studies (reviewed in [7]). Testo treatment decreases abdominal fat accumulation in men with the metabolic syndrome [8], whereas DHT has little effects on this parameter [9]. The role of 5α-reductase isoenzymes in adipose tissue androgen homeostasis remains to be determined. Our group reported that DHT formation from 4-dione was significantly decreased by *in vitro* induction of preadipocyte differentiation [10]. The isoforms responsible for this activity have never been clearly identified (reviewed in [11]).

Our objective was to define the predominant source of local DHT production in human adipose tissues, identify 5α-reductase isoenzymes responsible for this reaction and test the impact of 5α-

reductase inhibition on preadipocyte differentiation. We tested the hypothesis that adrenal androgen 4-dione is the primary source of DHT in adipose tissue compared to testo. We also postulate that 5α-reductase isoenzymes mediate the effect of testo and 4-dione on preadipocyte differentiation.

2. SUBJECTS AND METHODS

2.1 Subjects

The study sample included obese (n=3, age: 46.4±2.4 years, BMI: 37.7±1.4 kg/m2) and non-obese (n=7, age: 48.0±7.3 years, BMI: 26.5±1.8 kg/m2) men recruited through the elective general surgery schedule of the CHU de Québec Medical Center as well as severely obese men undergoing bariatric surgery (n=24, age: 48.8±12.6 years, BMI: 51.6±10.6 kg/m2). The latter patients were recruited through the Biobank of the *Institut universitaire de cardiologie et de pneumologie de Québec* according to institutionally-approved management modalities. The project was approved by the ethics committees of both institutions. All participants provided written, informed consent.

2.2 Adipose tissue sampling

Subcutaneous (SC) and omental (OM) adipose tissue samples were collected during surgery and immediately carried to the laboratory. A fresh portion of the biopsy sample was used for adipocyte isolation and primary cultures. The remaining tissue was immediately frozen at -80°C for gene expression measurements.

2.3 Preadipocyte isolation

Adipose tissue samples were digested for 45 minutes at 37°C using type I collagenase in Krebs-Ringer-Henseleit (KRH) buffer according to a modified version of the Rodbell method [12]. After tissue digestion, the cell suspension was filtered through nylon mesh and floating adipocytes were washed three times with KRH buffer. The stromal-vascular fraction was isolated using a modification of the Van Harmelen method [13]. The residual buffer containing the

stromal-vascular fraction was centrifuged and the pellet was washed in DMEM/F12 supplemented with 10% calf serum. Cells were filtered through 140 μm nylon mesh and placed in culture plates at 37°C under 5% CO_2 atmosphere. Preadipocytes were maintained in DMEM/F12 supplemented with 2.5% fetal bovine serum, 10 ng/ml endothelial growth factor, 1 ng/ml fibroblast growth factor, 6 μM insulin and antibiotics.

2.4 Preadipocyte differentiation

For gene expression, differentiation of fully confluent preadipocyte cultures was induced in 12-well plates for 14 days using a commercial differentiation medium from Zen-Bio (Durham, NC, USA). This medium consisted of DMEM/F12 supplemented with a PPAR-γ agonist, insulin, dexamethasone and 3-isobutyl-1-methylxanthine. For glycerol-3-phosphate dehydrogenase (G3PDH) activity measurements, subcutaneous preadipocyte cultures in 96-well plates were pre-incubated 2h with 500 nM of 5α-reductase inhibitors 17-N,N-diethylcarbamoyl-4-methyl-4-aza-5-androstan-3-one (4-MA) or finasteride (Sigma, Oakville, ON, Canada). ^{14}C-4-dione or ^{14}C-testo (0, 10 nM and 30 nM) was added for a 24h incubation period at 37°C. The next day, preadipocyte differentiation was induced for 14 days using serum-free Zen-Bio medium (Durham, NC, USA) supplemented with charcoal-treated serum. Inhibitors and 4-dione or testo were added to the medium prior to preadipocyte differentiation induction. G3PDH activity was measured as described previously [14]. Briefly, 14 days differentiated cells from two separate wells of 96-well plates were washed with PBS and homogenized in cold homogenization solution (100 μl/well; 20 mM Tris, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 7.3). Activity was assessed in reaction buffer (90 μL/well; 100 mM triethanolamine, 2.5 mM EDTA, 0.1 mM β-mercaptoethanol and 353 μM NADH, pH 7.7) for 10 min at 37°C and the assay was initiated

by the addition of 8 mM dihydroxyacetone phosphate (DHAP). Optical density at 340 nm was measured at repeated intervals for 3-5 min with a Spectramax340pc (Molecular Device, Sunnyvale, CA, USA) and milliunits of G3PDH activity was calculated using a standard curve of purified G3PDH enzyme (Sigma, Oakville, ON, Canada). DNA content per well was quantified with a NanoVue spectrophotometer (GE Healthcare, Baie D'Urfe, QC, Canada) and used to normalize. G3PDH activity was expressed as percentage of control wells (% of control). A total of 7 cultures showed significant response to 4-dione out of 10 cultures, and 5 cultures responded to testo out of 9.

2.5 5α-reductase activity in preadipocytes

OM and SC preadipocyte cultures were seeded in 24-well plates and incubated in a time course experiment (0, 6 and 24h) with 30 nM of either ^{14}C-4-dione or ^{14}C-testo (American Radiolabeled Chemicals, St. Louis, MO, USA). For enzyme inhibition, preadipocytes were pre-incubated 2h with 500 nM of 4-MA or finasteride (Sigma, Oakville, ON, Canada). After the pre-incubation period, 30 nM of ^{14}C-4-dione or ^{14}C-testo was added for 24h incubation at 37°C under 5% CO_2 atmosphere. Steroids from OM and SC preadipocyte culture media were extracted twice with diethyl ether. The dried steroids were dissolved with dichloromethane and migrated on thin layer chromatograms (TLC) with toluene-acetone (4:1). TLC plates were scanned on a Storm 860 PhosphorImager (GE Healthcare, Baie D'Urfe, QC, Canada). Densitometric analyses were performed using ImageJ software (NIH, Bethesda, MD, USA). The conversion rates of androgen substrates (4-dione or testo) were expressed as percentage of metabolite formation divided by protein amounts in the culture well (% formation/μg protein).

2.6 5α-reductase activity in stably transfected HEK-293 cells

Human embryonic kidney cells (HEK-293) expressing each of the 3 subtypes of the human SRD5A genes were kindly provided by Dr. Van Luu-The from the CHU de Québec Research Center, Québec, Canada. Briefly, the cDNA fragments for the 3 enzyme isoforms were subcloned into a pCMV-Neo expression vector to generate stably transfected HEK-293 cells (**Table 1**). Transfected cells were maintained in DMEM high glucose medium, supplemented with 10% fetal bovine serum and 400 µg/ml of G418. Untransfected HEK-293 and SRD5A-transfected cells were pretreated for 2h with 500 nM of 4-MA or finasteride. After the pre-incubation period, 30 nM of ^{14}C-4-dione was added for 24h at 37°C. Steroids from the culture media were extracted with diethyl ether and resolved on TLC as described above. The results were expressed as percentage of total radioactive density of each steroid on protein concentration (% of total density/µg protein).

2.7 Messenger RNA expression by real-time RT-PCR

Total RNA was extracted from whole OM and SC tissue, from non-differentiated and differentiated preadipocyte cultures and from stably transfected HEK-293 cells using the RNeasy lipid tissues mini kit and on-column DNase treatment (Qiagen, Hilden, DE) following the manufacturer's instructions. Total RNA quality was assessed on an Agilent BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). First-strand cDNA synthesis was accomplished using Superscript III RNase H-RT (Invitrogen Life Technologies, Burlington, ON, Canada), oligo-dT_{18}, random hexamers and purified (PCR purification Qiagen, Hilden, DE). cDNA corresponding to 20 ng of total RNA was used to perform fluorescent-based real-time RT-PCR quantification using the Light Cycler 480 (Roche Diagnostics, Mannheim, DE). The Light

Cycler 480 SYBR Green I Master Reagent (Roche Diagnostics, Indianapolis, IN, USA) was used as recommended by the manufacturer. The conditions for PCR reactions were: 45 cycles, denaturation at 95°C for 10 sec, annealing at 57-60°C for 10 sec, elongation at 72°C for 14 sec and then 72-74°C for 5 sec (reading). Normalization was performed using the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantitative real-time RT-PCR measurements were performed by the *CHU de Québec* Research Center Gene Expression Platform. Primer sequences used to assess expression of PPARγ and GAPDH or SRD5A1, SRD5A2 and SRD5A3 in tissues, preadipocytes or HEK-293 transfected cells are listed in **Table 2**. All primer sequences were designed using Gene Tool (Biotools, Jupiter, FL, USA).

2.8 Western blotting

Following to RNA extraction from whole OM and SC adipose tissues, one volume of ethanol was added to the lower phase of the trizol extraction. DNA traces were extracted by centrifugation at 2000×g for 2 min. Two volumes of isopropanol were added to the supernatant followed by vortexing and incubation for 10 min at room temperature. Proteins were precipitated by centrifugation at 5000×g for 10 min. Guanidine in ethanol (0.3 M) was used for washing the pellet three times for 20 min followed by one wash with 100% ethanol. The pellet was resuspended using heat (65°C) and sonication (10 s) cycles in 25 mM Tris, pH 7.4, 1.5 % SDS buffer containing protease inhibitors. Protein homogenate (30 µg) was diluted in SDS buffer 4X (200 mM Tris, pH 6.8, 8% SDS, 5 mM EDTA, 40% glycerol, 0.2 mM DTT) and heated at 95^{o}C for 3 min and separated on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (1 h at 100 V) and 5% non-fat milk diluted in 1X TBS (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) was used to block non-specific sites for 1h at room

temperature. Membranes were then incubated overnight at 4°C with rabbit primary antibody against SRD5A1 protein (kindly provided by Dr Van Luu-The, CHU de Quebec Research Center, Quebec, Canada), SRD5A3 protein (Abcam, Cambridge, MA, USA) and β-tubulin (Cell Signaling Technology, Danvers, MA, USA) as loading control. Membranes were washed and incubated 1h with anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Finally, membranes were washed and proteins were visualized by chemiluminescence (Western Lightning Plus-ECL, Perkin Elmer, MA, USA). ImageJ software (NIH, Bethesda, MD, USA) was used for densitometric analysis.

2.9 Statistical analyses

Student's paired *t*-tests were performed to assess the depot difference in mRNA and protein expression of the three 5α-reductase isoenzymes in OM vs. SC whole adipose tissues. These tests were also performed to compare DHT formation in preadipocyte cultures treated with 4-dione or testo. Repeated-measures analysis of variance in each fat compartment was used to compare mean mRNA expression of PPARγ, SRD5A1 and SRD5A3 in non-differentiated and differentiated preadipocytes. This procedure was also used to assess the formation of steroid metabolites generated in the time course experiment with OM and SC preadipocyte cultures with or without 5α-reductase inhibitors or the dose-response effect of steroids and inhibitors on differentiation. Pearson correlations were computed to assess associations between SRD5A1 or SRD5A3 mRNA expression in adipose tissue and the body mass index (BMI). Log_{10} or Box-Cox transformation was used to normalize non-normally distributed variables. All data are presented as mean ± SEM. Statistical analyses were performed using JMP software (SAS Institute, Cary, NC, USA).

3. RESULTS

The potential pathways for DHT synthesis in human adipose tissue are either direct conversion of testo to DHT through 5α-reductase activity or indirect conversion of 4-dione to A-dione through 5α-reductase activity followed by subsequent conversion of A-dione to DHT through 17-oxoreductase activity **(Figure 1A)**. To identify the primary source of DHT in adipocytes, we treated OM (n=4) and SC (n=5) preadipocyte cultures with 30 nM of ^{14}C-4-dione or ^{14}C-testo. Upon treatment with ^{14}C-4-dione, we detected progressive and significant increases in DHT ($p<0.05$) and testo ($p<0.01$) formation over time (0h, 6h, 24h) in both OM **(Figure 1B)** and SC **(Figure 1D)** cultures. Upon treatment with ^{14}C-testo, we found an increase of 4-dione formation through oxidative 17-oxoreductase activity ($p<0.01$), but DHT formation did not significantly increase over time in both OM **(Figure 1C)** and SC **(Figure 1E)** cultures. After 24h of treatment, we detected higher DHT formation in preadipocyte cultures treated with ^{14}C-4-dione than in those treated with ^{14}C-testo ($p<0.01$). This analysis remained significant in each compartment analyzed separately ($p<0.05$ for both).

The impact of 5α-reductase inhibitors (4-MA and finasteride) was tested in the same model (**Figure 2)**. Upon treatment of OM (n=4) (**Figure 2A)** and SC (n=5) (**Figure 2C)** preadipocyte cultures for 24h with ^{14}C-4-dione with or without the inhibitors, we detected major inhibition of androstanedione (A-dione) and DHT formation with both 4-MA ($p<0.005$, for both depots) and finasteride ($p<0.05$, for both depots). There was a trend for a treatment-depot interaction for DHT formation with both 4-MA and finasteride treatment ($p\leq0.09$). We found an increase in testo formation from 4-dione with 4-MA ($p<0.01$) or finasteride ($p<0.05$) incubation. When OM

(Figure 2B) and SC **(Figure 2D)** preadipocyte cultures were treated for 24h with ^{14}C-testo and 4-MA or finasteride, we found significant inhibition of DHT formation with 4-MA and finasteride ($p \leq 0.05$, for both inhibitors). An increase in 4-dione formation from testo was found with 4-MA and finasteride ($p < 0.01$, for both inhibitors).

Figure 3A shows that only SRD5A1 and SRD5A3 mRNAs are expressed in whole OM and SC adipose tissues, with significantly higher expression of SRD5A3 than SRD5A1 in both depots ($p \leq 0.001$). SRD5A2 mRNA was not detected in these samples. No statistically significant difference was detected in SRD5A1 or SRD5A3 mRNA expression between the two compartments. We also measured protein expression of SRD5A1 and SRD5A3 and did not detect any depot difference **(Figure 3B)**. We assessed gene expression in OM and SC preadipocytes before and after induction of differentiation. As expected, PPAR-γ expression increased significantly over 14 days of culture and a depot difference was noted ($p < 0.05$, in each compartment) (**Figure 4A)**. Expression of SRD5A isoforms was approximately 10- to 15-fold lower than that of PPAR-γ. SRD5A1 mRNA expression appeared to increase slightly with differentiation, but this trend did not reach significance ($p = 0.11$). A trend for a depot difference was observed ($p = 0.07$) **(Figure 4B)**. There was no statistically significant difference in SRD5A3 mRNA expression before and after differentiation ($p = 0.24$), but a significant depot difference was detected ($p < 0.05$) **(Figure 4C)**.

To assess the effects of 5α-reductase inhibitors 4-MA and finasteride on each 5α-reductase isoenzyme, we used HEK-293 stably transfected with each of the three known human enzymes. Expression of each subtype was confirmed by real-time RT-PCR with specific primers.

Untransfected HEK-293 cells were used as negative control as we detected very low expression of SRD5A1 and 3, and no expression of SRD5A2 (data not shown). Stably transfected cell lines overexpressing each 5α-reductase isoenzyme were pre-treated for 2h with or without 500 nM 4-MA or finasteride and then further incubated for 24h with 30 nM ^{14}C-4-dione and each inhibitor or vehicle. In these conditions, untransfected cells showed little formation of A-dione from 4-dione **(Figure 5A)**. Cells overexpressing 5α-reductase type 1 showed very strong 4-dione-to-A-dione activity which was slightly inhibited by 4-MA, but not finasteride **(Figure 5B)**. Strong activity was also detected in the 5α-reductase type 2 clone, but was blunted by both inhibitors, with slightly higher inhibition rates using finasteride **(Figure 5C)**. Finally, cells overexpressing 5α-reductase type 3 had lower activity which was completely abolished by both 4-MA and finasteride **(Figure 5D)**.

To assess the impact of 5α-reductase inhibition on preadipocyte differentiation, SC preadipocyte cultures undergoing differentiation were treated with testo (10 and 30 nM) or 4-dione (10 and 30 nM) with and without inhibitors. G3PDH activity was measured to assess the extent of differentiation **(Figure 6)**. Cultures that were sensitive to 4-dione ($n=7$, $p<0.05$, **Figure 6A-C**) or testo ($n=5$, $p<0.05$, **Figure 6D-F**) were analyzed. The dose-response inhibition observed upon treatment with either 4-dione or testo was completely abolished by 4-MA and finasteride.

We investigated correlations between SRD5A1 and SRD5A3 mRNA expression level in each adipose compartment and age, body mass index (BMI) or adipocyte diameter. A significant, positive correlation between SRD5A1 mRNA expression level in whole SC adipose tissue and

BMI was found ($n=20$, $p<0.05$). No other significant correlations were noted in this sample (data not shown).

4. DISCUSSION

The aim of this study was to to define the predominant source of local DHT production in human adipose tissues, identify 5α-reductase isoenzymes responsible for this reaction and test the impact of 5α-reductase inhibition on preadipocyte differentiation. In time-course experiments, we showed that 4-dione is the main source of DHT in human preadipocytes, and that 4-MA and finasteride inhibit these reactions. Our results also indicated that mRNA of SRD5A1 and SRD5A3, but not SRD5A2 are expressed in whole OM and SC adipose tissues in men, SRD5A3 being the most highly expressed isoform. In preadipocyte cultures, mRNA expression of SRD5A1 and SRD5A3 was detectable, albeit at lower levels than adipocyte gene PPAR-γ. Expression of SRD5A1 and 3 transcripts was increased after differentiation but this trend did not reach significance. Finally, our results indicated that DHT formation by 5α-reductase isoenzymes mediates the inhibitory effect of both 4-dione and testo on preadipocyte differentiation. Our results clarify the dynamics of DHT formation and action in adipocytes and provide indication that 5α-reductase type 3 may be relevant for adipose tissue physiology.

Regarding the dynamics of DHT formation in preadipocyte cultures, we detected significant increases in DHT production from 4-dione up to 24 h, at which point significantly higher DHT production was found than with equimolar treatment with testo. These results are consistent with what was found in other tissues, specifically that DHT formation likely results mostly from 4-dione transformation [1, 15, 16]. These findings are also consistent with those of Perel et al. [17],

demonstrating formation of 5α-reduced androgen metabolites such as A-dione, androsterone and DHT in stromal cells from breast adipose tissue incubated with 4-dione. Production of 5α-reduced metabolites exceeded estrone production by 100 fold. The same research group reported no statistical difference in 5α-reductase activity between flank and abdominal adipose tissue cell cultures [18]. In our study, formation of DHT was slightly higher in SC cells, which was consistent with the significant depot difference in SRD5A3 in preadipocytes undergoing differentiation. The significance of these depot differences needs to be further investigated.

Our description of SRD5A isoform expression patterns in whole adipose tissues is consistent with that of Upreti et al. [19], who reported that SRD5A1, but not SRD5A2, was expressed in human SC adipose tissue. For a long time, it was assumed that one out of two 5α-reductase isoenzymes mediated local DHT production. However, a third type of 5α-reductase was identified [20]. It was reported to be expressed in many tissues or organs [20]. Consistent with these findings, we report here that it is the most highly expressed 5α-reductase subtype in human adipose tissues, although this difference was not apparent at the protein level. Our experiments in HEK-293 cells also show that common inhibitors such as 4-MA and finasteride inhibit activity of this isoform. Apart from the type 2 isoenzyme which is not expressed in adipose cells, inhibitors were mostly effective with the type 3 isoenzyme. Considering that most of the DHT formed by preadipocytes was also blocked by both inhibitors, we conclude that the type 3 isoenzyme may be relevant for DHT formation in these cells.

In a similar manner, the 5α-reductase inhibitors completely reversed the inhibition of preadipocyte differentiation that was induced by 4-dione and testo treatment. We have

previously reported that testo and DHT both inhibit preadipocyte differentiation in OM and SC primary preadipocyte cultures of men and women [14]. Although not all cultures responded to the hormonal treatment in the present experiment, our results support the notion that DHT formation through 5α-reductase activity mediates a vast portion of the effects of both 4-dione and testo on preadipocyte differentiation. The magnitude of the inhibitory effect on differentiation with each steroid (4-dione vs. testo) must be interpreted with caution. The relative impact of each steroid cannot be directly compared in this experiment because results are expressed as a function of the control condition and only responsive cultures were examined. This may partly explain why we do not observe a stronger effect of 4-dione despite a higher potential for DHT production by this steroid compared to testo.

Messenger RNAs of SRD5A1 and SRD5A3 were not dramatically modulated by induction of differentiation. These results are consistent with those of Blouin et al. [10], in which we reported that SRD5A1 was not influenced by differentiation in both abdominal adipose tissue depots. The slight increase in SRD5A1 mRNA expression with differentiation potentially explains the positive correlation between whole adipose tissue expression of this gene and BMI of the patients examined. Increased adiposity is, indeed, generally associated with higher numbers of large adipocytes, especially in the subcutaneous compartment. The significance of this correlation and the lack of association reported for SRD5A3 need to be interpreted with caution. The fact that the sample included mostly obese individuals may have underestimated potential associations in whole adipose tissue. Tsilchorozidou et al. [21] reported that 5α-reductase activity toward cortisol was positively correlated with BMI in a cohort of PCOS women. On the other

hand, Wake et al. [22] reported that SRD5A1 mRNA level in human SC adipose tissue did not predict the amount of body fat or its distribution.

Upon treatment with testo, we observed accumulation of 4-dione likely formed by 17β-HSD type 2 [23]. This reaction was in fact largely predominant over DHT formation. This finding suggests that in adipose tissue, testo will more likely be converted to 4-dione than to DHT. Conversely, our results suggest that 4-dione is partly converted to 5α-reduced metabolites and testo, the latter reactions occurring most likely through 17β-HSD type 3 and/or 5 (reviewed in [11]). We have also previously reported that DHT may be further inactivated through aldo-ketoreductase type 2 (AKR1C2) into either 3α- or 3β-androstanediol [10].

Formation of 5α-reduced metabolites of other steroids may also be relevant in adipose tissue. Our group reported that 5α-pregnane-3α/β-ol-20-one, 5α-pregnanedione and 5α-pregnane-20α-ol-3-one were major metabolites of progesterone in OM and SC preadipocyte cultures [24]. Regarding cortisol, Tomlinson et al. [25] reported decreased 5α-reductase activity after weight loss, which was based on the ratio of circulating 5α-THF over THF. Although the liver was likely a major contributor to this change, a contribution by adipose tissue cannot be excluded. The relevance of 5α-reductase isoenzymes in a possible local, depot-specific modulation of the availability of androgens, progesterone and glucocorticoids needs to be further studied.

5. CONCLUSION

We report that 4-dione is the main source of DHT in human preadipocytes and that DHT formation through 5α-reductase activity mediates most of the inhibitory effects of 4-dione and

testo on preadipocyte differentiation. Messenger RNA and protein of SRD5A1 and SRD5A3 are detected in adipose tissue, with SRD5A3 being the most highly expressed in both fat compartments examined. Considering that the inhibitors were effective in blunting activity of 5α-reductase type 3 but not type 1, we conclude that 5α-reductase type 3 may play a role in adipose tissue.

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CONFLICT OF INTEREST

No conflict of interest to disclose.

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REFERENCES

1. Andersson, S. and D.W. Russell, *Structural and biochemical properties of cloned and expressed human and rat steroid 5 alpha-reductases.* Proc Natl Acad Sci U S A, 1990; **87**:3640-3644.
2. Li, J., et al., *Androgen regulation of 5alpha-reductase isoenzymes in prostate cancer: implications for prostate cancer prevention.* PLoS One, 2011; **6**:e28840.
3. Occhiato, E.G., et al., *Selective non-steroidal inhibitors of 5 alpha-reductase type 1.* J Steroid Biochem Mol Biol, 2004; **88**:1-16.
4. Titus, M.A., et al., *5alpha-reductase type 3 enzyme in benign and malignant prostate.* Prostate, 2014; **74**:235-249.
5. Stiles, A.R. and D.W. Russell, *SRD5A3: A surprising role in glycosylation.* Cell, 2010; **142**:196-198.
6. Blouin, K., A. Boivin, and A. Tchernof, *Androgens and body fat distribution.* J Steroid Biochem Mol Biol, 2008; **108**:272-280.
7. Zerradi, M., et al., *Androgens, body fat Distribution and Adipogenesis.* Current Obesity Reports, 2014; 3:396–403.
8. Traish, A.M., *Testosterone and weight loss: the evidence.* Curr Opin Endocrinol Diabetes Obes, 2014; **21**:313-322.
9. Marin, P., et al., *Androgen treatment of abdominally obese men.* Obes Res, 1993; **1**:245-251.
10. Blouin, K., et al., *Pathways of adipose tissue androgen metabolism in women: depot differences and modulation by adipogenesis.* Am J Physiol Endocrinol Metab, 2009; **296**:E244-E255.
11. Tchernof, A., et al., *Updated survey of the steroid-converting enzymes in human adipose tissues.* J Steroid Biochem Mol Biol, 2014; **147c**:56-69.
12. Rodbell, M., *metabolism of isolated fat cells. i. effects of hormones on glucose metabolism and lipolysis.* J Biol Chem, 1964; **239;** 375-380.
13. Van Harmelen, V., K. Rohrig, and H. Hauner, *Comparison of proliferation and differentiation capacity of human adipocyte precursor cells from the omental and subcutaneous adipose tissue depot of obese subjects.* Metabolism, 2004; **53**:632-637.
14. Blouin, K., et al., *Effects of androgens on adipocyte differentiation and adipose tissue explant metabolism in men and women.* Clin Endocrinol, 2010; **72**:176-188.
15. Samson, M., et al., *Biosynthesis of dihydrotestosterone by a pathway that does not require testosterone as an intermediate in the SZ95 sebaceous gland cell line.* J Invest Dermatol, 2010; **130**:602-604.
16. Sugimoto, Y., et al., *Cations inhibit specifically type I 5 alpha-reductase found in human skin.* J Invest Dermatol, 1995; **104**:775-778.
17. Perel, E., et al., *The formation of 5 alpha-reduced androgens in stromal cells from human breast adipose tissue.* J Clin Endocrinol Metab, 1986; **62**:314-318.
18. Killinger, D.W., et al., *Influence of adipose tissue distribution on the biological activity of androgens.* Ann N Y Acad Sci, 1990; **595**:199-211.
19. Upreti, R., et al., *5alpha-reductase type 1 modulates insulin sensitivity in men.* J Clin Endocrinol Metab, 2014; **99**:E1397-E406.
20. Uemura, M., et al., *Novel 5 alpha-steroid reductase (SRD5A3, type-3) is overexpressed in hormone-refractory prostate cancer.* Cancer Sci, 2008; **99**:81-86.
21. Tsilchorozidou, T., J.W. Honour, and G.S. Conway, *Altered cortisol metabolism in polycystic ovary syndrome: insulin enhances 5alpha-reduction but not the elevated adrenal steroid production rates.* J Clin Endocrinol Metab, 2003; **88**:5907-5913.
22. Wake, D.J., et al., *Intra-adipose sex steroid metabolism and body fat distribution in idiopathic human obesity.* Clin Endocrinol, 2007; **66**:440-446.

23. Fouad Mansour, M., et al., *Oxidative activity of 17beta-hydroxysteroid dehydrogenase on testosterone in male abdominal adipose tissues and cellular localization of 17beta-HSD type 2.* Mol Cell Endocrinol, 2015; **414**:168-176.
24. Zhang, Y., et al., *Progesterone metabolism in adipose cells.* Mol Cell Endocrinol, 2009; **298**:76-83.
25. Tomlinson, J.W., et al., *Reduced glucocorticoid production rate, decreased 5alpha-reductase activity, and adipose tissue insulin sensitization after weight loss.* Diabetes, 2008; **57**:1536-1543.

FIGURE LEGENDS

Figure 1: Time course of DHT formation from 4-dione or testo in SC or OM preadipocyte cultures. (A) Pathways of DHT synthesis through 5α-reductase isoenzymes. 5α-reductase activity likely mediated by isoenzymes types 1 and/or 3; 17β-HSD activity likely mediated by 17β-HSD type 2; 17-oxoreductase activity likely mediated by 17β-HSD types 3 and/or 5. Time course (0h, 6h and 24h) of androgen metabolites after treatment of preadipocyte cultures with 30 nM of ^{14}C-4-dione **(B)** and **(D)** or with 30 nM ^{14}C-testo **(C)** and **(E)**. OM (n=4), SC (n=5). A-dione: androstanedione; 4-dione: androstenedione; Testo: testosterone. Mean ± SEM. Statistically significant differences indicated in text.

Figure 2: Effect of 5α-reductase inhibitors on DHT formation in preadipocytes. Inhibition of DHT and A-dione formation after 24h incubation of preadipocyte cultures with 30 nM ^{14}C-4-dione and 500 nM of 4-MA or finasteride **(A)** and **(C)**. Inhibition of DHT formation after 24h treatment of preadipocyte cultures with 30 nM ^{14}C-testo and 500 nM of 4-MA or finasteride **(B)** and **(D)**. OM (n=4), SC (n=5). A-dione: androstanedione; 4-dione: androstenedione; Testo: testosterone; FINA: finasteride. Mean ± SEM. Statistically significant differences indicated in text.

Figure 3: SRD5A isoenzymes mRNA and protein expression in whole human adipose tissues. (A) mRNA expression levels were normalized according to GAPDH expression, n=20. **(B)** Protein expression levels relative to tubulin expression, n=7. Mean ± SEM. ** $p \leq 0.001$ compared to type 1 in respective compartment.

Figure 4: PPARγ, SRD5A1 and SRD5A3 mRNA expression in preadipocyte cultures before and after induction of differentiation. PPARγ **(A),** SRD5A1 **(B)** and SRD5A3 **(C)** mRNA expressions in OM (n=5) and SC (n=7) cells before and 14 days after differentiation. Messenger RNA normalized according to GAPDH mRNA expression. ND: non-differentiated preadipocytes; D: differentiated preadipocytes. Mean ± SEM.

Figure 5: Activity of 5α-reductases types 1, 2 or 3 and inhibitory effects of 4-MA or finasteride in HEK-293 stably overexpressing each isoenzyme. (A) Untransfected cells, **(B)** 5α-reductase type 1-expressing cells, **(C)** 5α-reductase type 2-expressing cells and **(D)** 5α-reductase type 3-expressing cells. TLC images and corresponding densitometric analyses are shown for each cell line. A-dione: androstanedione; 4-dione: androstenedione; FINA: finasteride. Mean ± SEM.

Figure 6. Effect of 5α-reductase inhibitors on preadipocyte differentiation. G3PDH activity in differentiating subcutaneous preadipocytes treated with **(A)** androstenedione (4-dione, n=7) and **(B)** 500 nM of 4-MA or **(C)** finasteride (FINA) over 14 days. G3PDH activity in differentiating subcutaneous preadipocytes treated with **(D)** testosterone (Testo, n=5) and **(E)** 500 nM of 4-MA or **(F)** finasteride (FINA) over 14 days. G3PDH activity expressed as % of control (CTL). Mean ± SEM. *P<0.05.

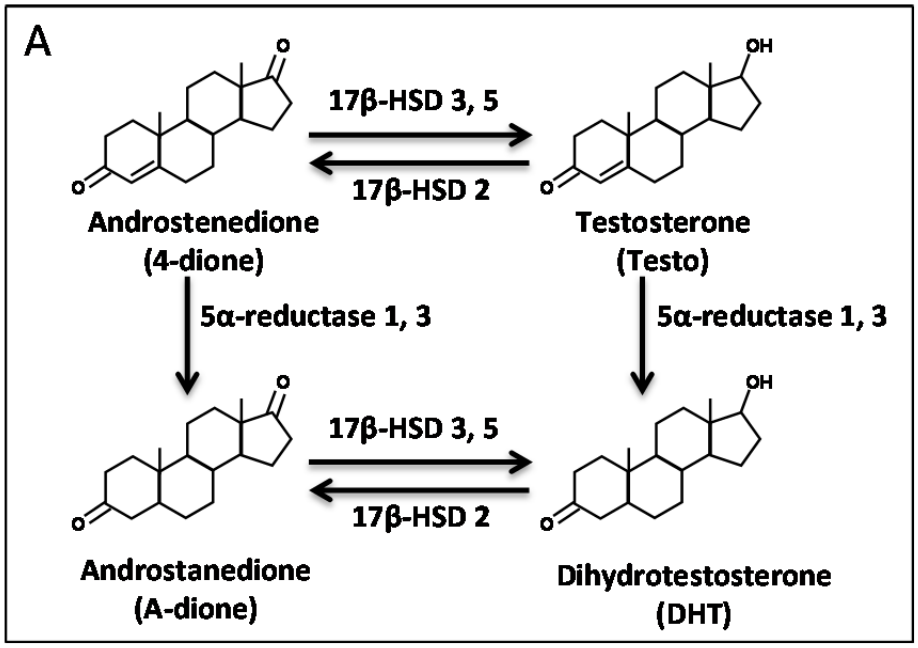
Table 1: Vector, subcloning sites, GenBank accession numbers and primer sequences used to generate HEK-293 cells stably expressing each 5α-reductase isoenzyme

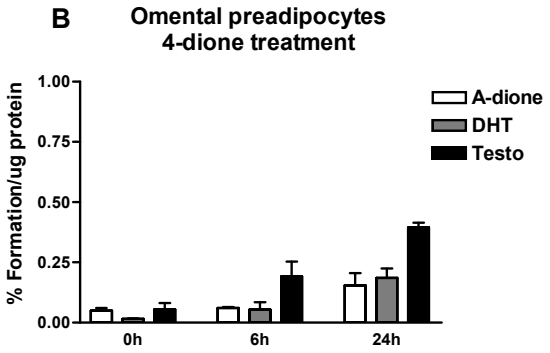
Description	Vector	Sub-cloning site	GenBank	Primer sequence 5'→3' S/AS
Human 5α-reductase 1	pCMV-Neo	EcoR1-EcoR1	NM_001047	GAATTCCACCATGGCAAC GGCGACGGGGGT/GAATTC GCACTTAAAACAAAAATG GAAT
Human 5α-reductase 2	pCMV-Neo	EcoR1-Blunt End	NM_000348	GAATTCGGCGCGATGCAG GTTCAGTGCCAG/AATCCC CAGGCCAGCTGGCAG
Human 5α-reductase 3	pCMV-Neo	Sal1-Xba1	NM_024592	GTCGACCATGGCTCCCTGG GCGGAGGCCGAG/TCTAGA TTAAAACAAAAATGGTAG GAAAGCTT

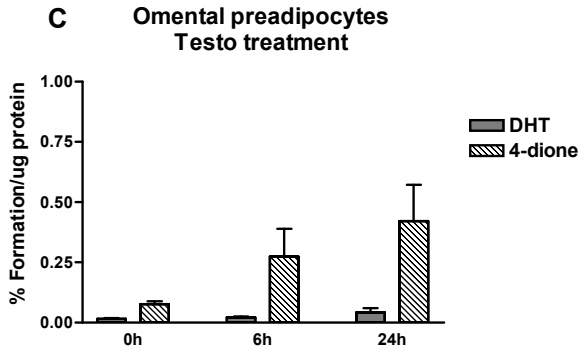
Table 2: GenBank accession numbers, amplicon sizes and primer sequences used for expression measurement in the present study

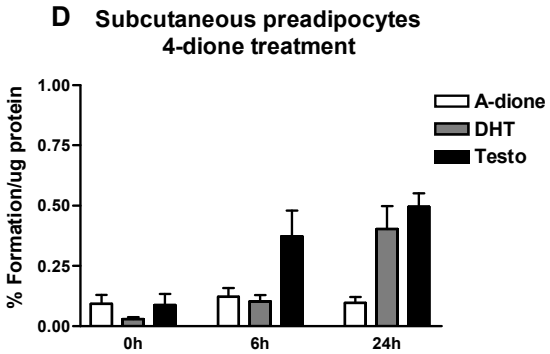
Gene Symbol	Description	GenBank	size (pb)	Primer sequence 5'→3' S/AS
SRD5A1	Homo sapiens steroid-5-α-reductase, α polypeptide 1 (3-oxo-5 α-steroid delta 4-dehydrogenase α1)	NM_001047	121	TGGCGATTATGTTCTGT ACCTGTA/AACCACAAG CCAAAACCTATTAGA
SRD5A2	Homo sapiens steroid-5-α-reductase, α polypeptide 2 (3-oxo-5 α-steroid delta 4-dehydrogenase α2)	NM_000348	145	CGGTTTAGCTTGGGTGT CTTCTTATT/TGGCTCCA GAAACATACGTAAACA AG
SRD5A3	Homo sapiens steroid 5α-reductase 3	NM_024592	170	CCCAAGAGTTCGAGACC AGCCT/CCTCGGCTCACT GTACCCTTG
SRD5A1 (HEK-293 5α-type 1)	Homo sapiens steroid-5-α-reductase, α polypeptide 1 (3-oxo-5 α-steroid delta 4-dehydrogenase α1)	NM_001047	121	TGGCGATTATGTTCTGT ACCTGTA/AACCACAAG CCAAAACCTATTAGA
SRD5A2 (HEK-293 5α-type 2)	Homo sapiens steroid-5-α-reductase, α polypeptide 2 (3-oxo-5 α-steroid delta 4-dehydrogenase α2)	NM_000348	145	CGGTTTAGCTTGGGTGT CTTCTTATT/TGGCTCCA GAAACATACGTAAACA AG
SRD5A3 (HEK-293 5α-type 3)	Homo sapiens steroid 5α-reductase 3	NM_024592	175	TTTGGAGACTGGTTTGA ATATGTTT/TGCTTTTGT AGAATTGGTGGCT
PPARG	Homo sapiens peroxisome proliferator-activated receptor γ	NM_138712	229	GTCGGATCCACAAAAA AAGTAGAA/AGCGGGAA GGACTTTATGTATGA
GAPDH	Homo sapiens glyceraldehyde-3-phosphate dehydrogenase	NM_002046	194	GGCTCTCCAGAACATCA TCCCT/ACGCCTGCTTCA CCACCTTCTT

Figure 1


A
17β-HSD 3, 5
17β-HSD 2
Androstenedione
(4-dione)
Testosterone
(Testo)
5α-reductase 1, 3
5α-reductase 1, 3
17β-HSD 3, 5
17β-HSD 2
Androstanedione
(A-dione)
Dihydrotestosterone
(DHT)


B
Omental preadipocytes
4-dione treatment
% Formation/ug protein
A-dione
DHT
Testo
0h
6h
24h


C
Omental preadipocytes
Testo treatment
% Formation/ug protein
DHT
4-dione
0h
6h
24h


D
Subcutaneous preadipocytes
4-dione treatment
% Formation/ug protein
A-dione
DHT
Testo
0h
6h
24h

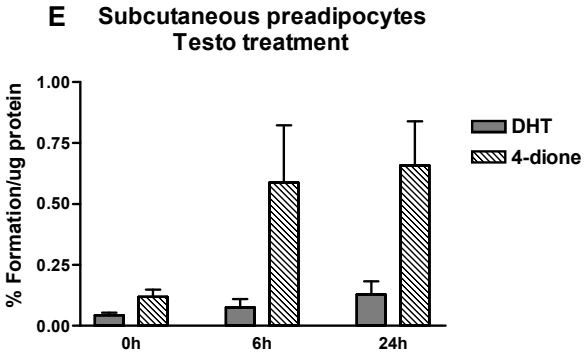

E
Subcutaneous preadipocytes
Testo treatment
% Formation/ug protein
DHT
4-dione
0h
6h
24h

Figure 2

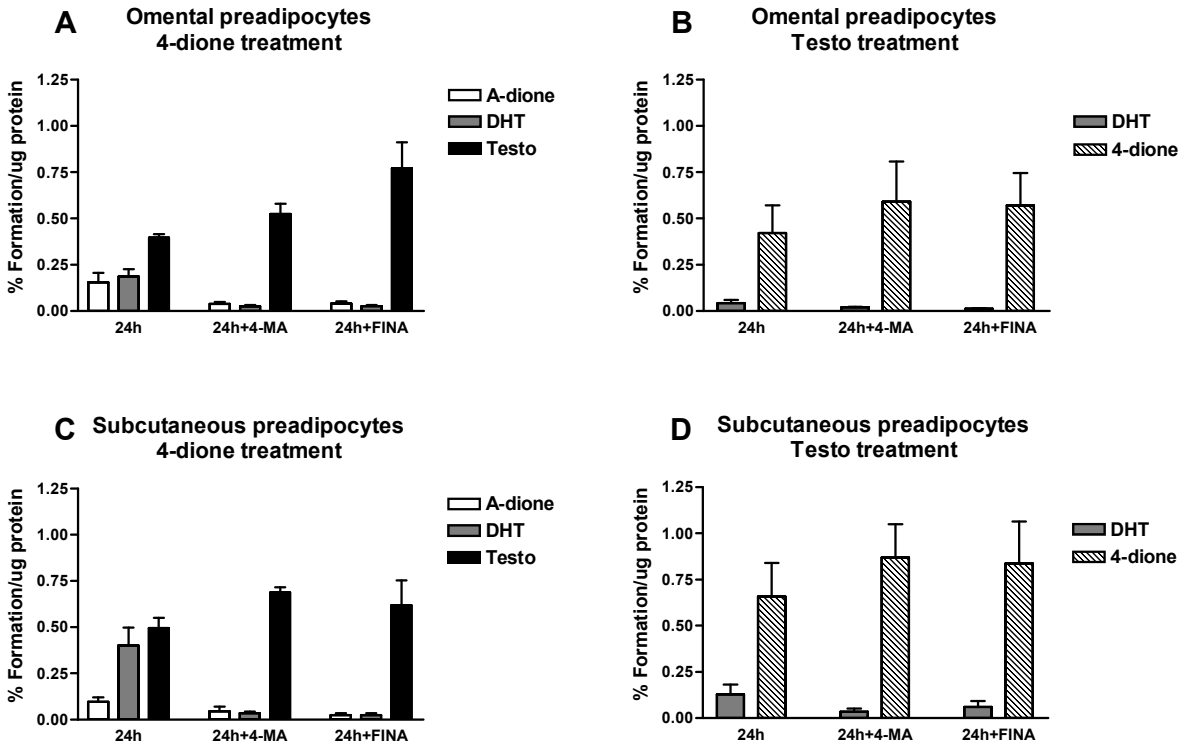

A
Omental preadipocytes
4-dione treatment
% Formation/ug protein
1.25
1.00
0.75
0.50
0.25
0.00
24h
24h+4-MA
24h+FINA
A-dione
DHT
Testo
B
Omental preadipocytes
Testo treatment
DHT
4-dione
C
Subcutaneous preadipocytes
4-dione treatment
D
Subcutaneous preadipocytes
Testo treatment

Figure 3

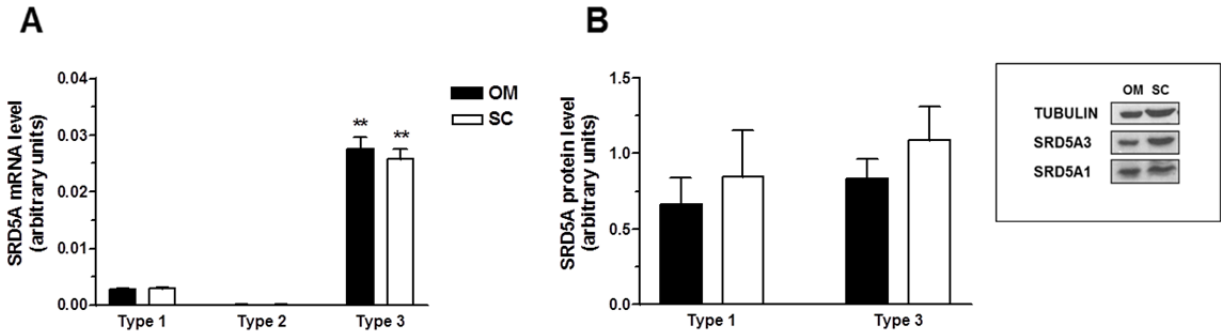

A
SRD5A mRNA level (arbitrary units)
0.04
0.03
0.02
0.01
0.00
OM
SC
**
**
Type 1
Type 2
Type 3
B
SRD5A protein level (arbitrary units)
1.5
1.0
0.5
0.0
Type 1
Type 3
OM SC
TUBULIN
SRD5A3
SRD5A1

Figure 4

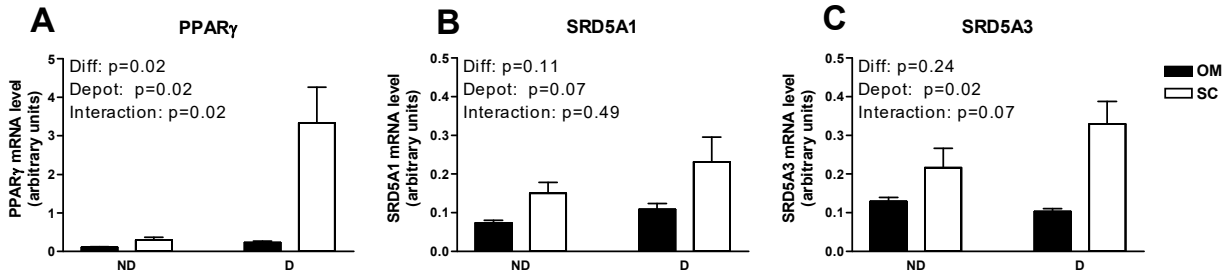

A
PPARγ
Diff: p=0.02
Depot: p=0.02
Interaction: p=0.02
PPARγ mRNA level (arbitrary units)
ND
D
B
SRD5A1
Diff: p=0.11
Depot: p=0.07
Interaction: p=0.49
SRD5A1 mRNA level (arbitrary units)
C
SRD5A3
Diff: p=0.24
Depot: p=0.02
Interaction: p=0.07
SRD5A3 mRNA level (arbitrary units)
OM
SC

Figure 5

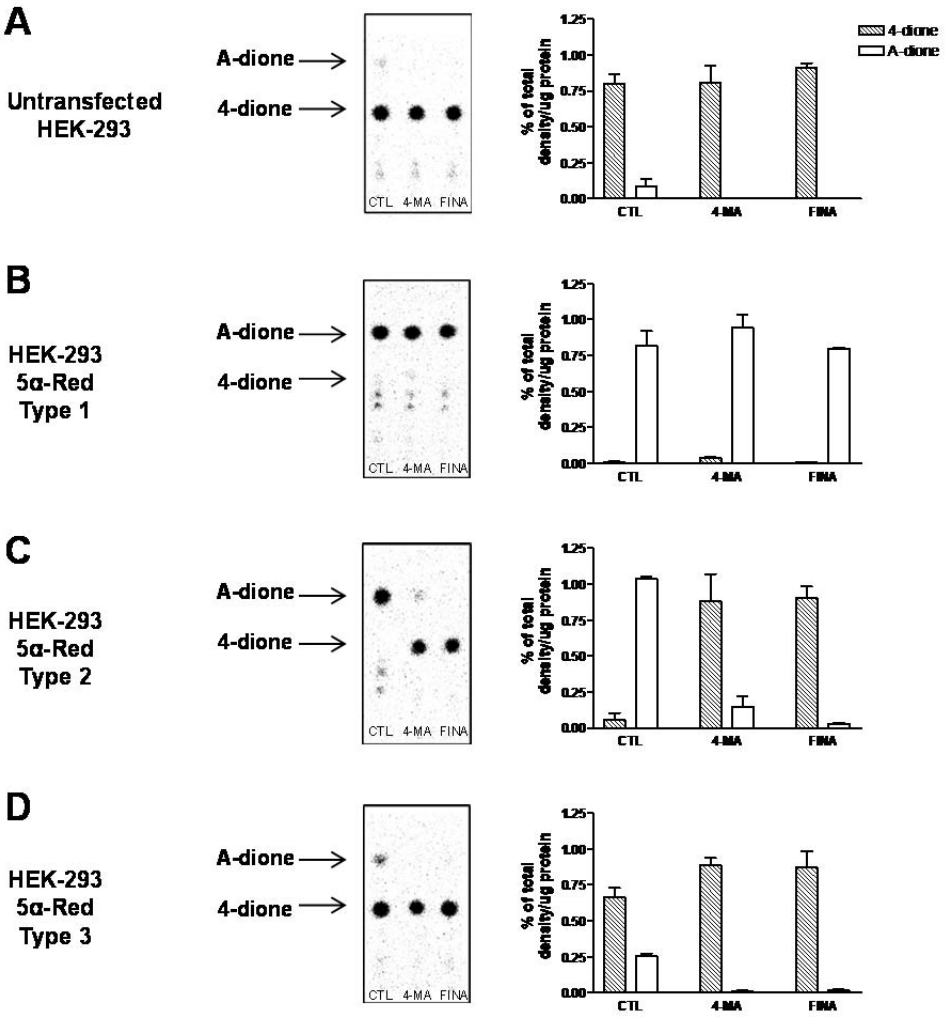
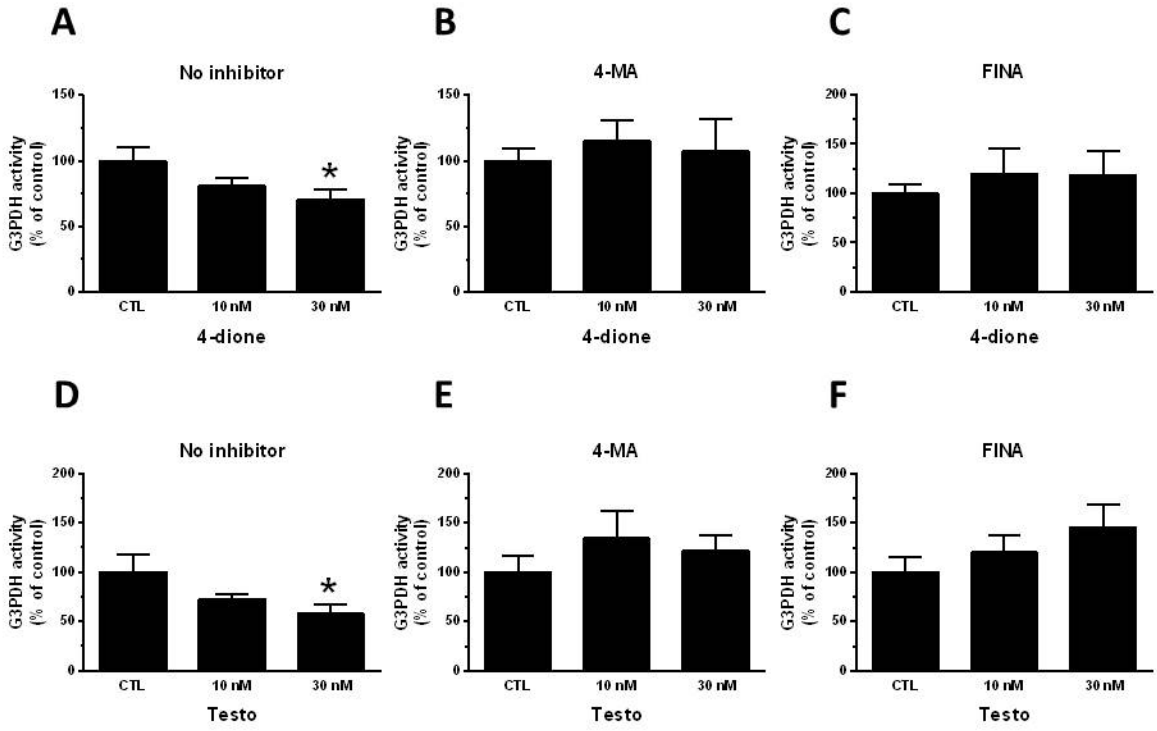

A
Untransfected HEK-293
A-dione
4-dione
CTL 4-MA FINA
% of total density/ug protein
4-dione
A-dione
CTL
4-MA
FINA
B
HEK-293 5α-Red Type 1
C
HEK-293 5α-Red Type 2
D
HEK-293 5α-Red Type 3

Figure 6


A
No inhibitor
G3PDH activity (% of control)
CTL
10 nM
30 nM
4-dione
B
4-MA
C
FINA
D
Testo
E
F