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Expanding the Use of Fluorogenic Enzyme Reporter Substrates to Imaging Metabolic Flux Changes: the Activity Measurement of 5α-Steroid Reductase in Intact Mammalian Cells

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ABSTRACT The study of dynamic properties of metabolic and signaling networks is hindered by the lack of methods for imaging metabolic fluxes in individual intact cells. We describe a novel optical approach for measuring the changes of metabolic fluxes in cells, based on a two-substrate competition between a physiological substrate and a fluorogenic reporter substrate. We have constructed a model cell system for a two-step metabolic pathway involved in the metabolism of testosterone. Potent androgen testosterone is converted by steroid 5α-reductase to DHT (5α-dihydrotestosterone), which is subsequently metabolized to 3α-diol (3α,17β-androstanediol) by the reductase AKR1C2 (aldo-ketoreductase 1C2), for which we have previously developed the fluorogenic reporter substrate Coumberone. Despite the medicinal importance of 5α-reductase, there are presently no probes or methods for the continuous activity readout of this enzyme in cells. We show that the activity of 5α-R1 (5α-reductase type 1) can be measured in COS-1 cells *via* the changes of DHT flux. Our system enables a measurement of 5α-reductase activity in cells, *via* either fluorimetry or fluorescence microscopy, with a wide dynamic range of activities, and provides a continuous optical assay for evaluation of small molecule inhibitors for this important enzyme. Furthermore, this paper demonstrates a novel optical approach to measuring metabolic flux changes in living cells and expands the utility of fluorogenic enzyme reporter substrates: optical reporters can measure not only the activity of the target enzyme but also the activity of other enzymes upstream in the pathway, for which there are no probes available.

To enable the study of dynamic properties of metabolism, methods for the measurement of metabolic fluxes in individual intact cells are needed. Metabolic flux is defined as the rate of processing of a metabolite through a defined metabolic pathway (*1*). The measurement of flux changes has classically been achieved by flash freezing the tissue or cells, followed by extraction, separation, and analysis of relevant metabolites. Modern high-throughput metabolite analysis provides valuable information about large sections of metabolome (*2*); however, these methods are discontinuous and destructive. Nuclear magnetic resonance spectroscopy (especially ^{13}C and ^{31}P NMR) has become an important tool for the measurement of metabolic fluxes in living cells as it is continuous and noninvasive, but NMR lacks the spatial resolution required for imaging single cells and subcellular compartments (*3*). In contrast, optical sensors, either synthetic (*4*) or genetically encoded (*5, 6*), that measure metabolite concentrations *via* a binding equilibrium offer major promise for determining the concentration of small molecule metabolites in individual cells *via* fluorescence microscopy. We here describe a novel optical approach for measuring the changes of metabolic fluxes in intact cells, based on a two-substrate competition between a fluorogenic reporter substrate (*7–14*) and a physiological substrate at a downstream enzyme of a selected metabolic pathway. This is a proof-of-concept study as the experimental system was engineered by transfecting mammalian cells with the enzymes that constitute the metabolic pathway involved in the reduction of testosterone.

Concept for Optical Imaging of Metabolic Fluxes. The concept for our approach is depicted in Figure 1; in a two-step metabolic pathway, a fluorogenic reporter

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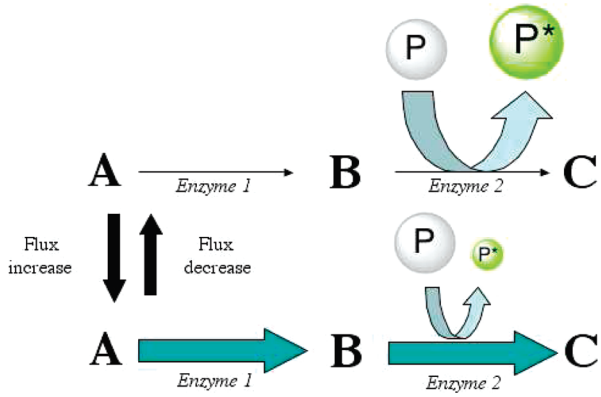

Figure 1. The concept for optical imaging of metabolic fluxes in intact cells is based on a competition between the physiological substrate (B) and the fluorogenic reporter substrate (P) for the active site of enzyme 2. In a low flux state, the rate of probe metabolism is relatively high (fast optical output); in a high flux state, the rate of probe metabolism is relatively low (slow optical output). A is a physiological metabolite entering the pathway. C is a physiological metabolite exiting the pathway.

substrate (P) competes with a physiological substrate (B) in the second enzymatic step. The key assumption is that the flux moving through the entire pathway is equivalent or similar to the flux going through the first enzyme (in this case, metabolic flux is the rate of metabolite A processing through the pathway defined by enzyme 1 and enzyme 2). In a low flux state, the reporter substrate is converted to a fluorescent product (P*) with a relatively high rate. In a high flux state, the reporter substrate is competed out by the physiological substrate, leading to a relatively low rate of formation of the fluorescent product. Thus, a change of the flux in the entire pathway is detected *via* either fluorimetric assay or fluorescence microscopy by measuring the relative rates of the reporter substrate metabolism (Figure 1).

Enzymologists often use the "coupled enzyme assay" to measure the rate of an enzymatic reaction where there are no spectroscopic or other means for continuous monitoring of the product formation or substrate consumption (*15*). The addition of a second indicator enzyme is required for a chromogenic reaction (*e.g.*, horseradish peroxidase to monitor the hydrogen peroxide side product of an oxidative process), and thus this assay is not suitable for measuring the metabolic fluxes in intact cells. Binding competition between the reporter ligand and the analyte of interest is the basis of widely used detection assays (immunoassays such as ELISA or fluorescence assays) (*16, 17*). However, our approach uses a competitive reporter substrate for an enzyme that is a native component of the metabolic pathway, and thus the assay is suitable for intracellular imaging.

Steroid 5α-Reductase: Physiological Roles. Steroid 5α-reductase, of which two isozymes are known (type 1 and type 2), is a key enzyme in the metabolism of androgens, progestins, and glucocorticoids, catalyzing the stereospecific reduction of the 4,5-double bond of the A-ring enone (Figure 2) (*18, 19*). 5α-Reductase

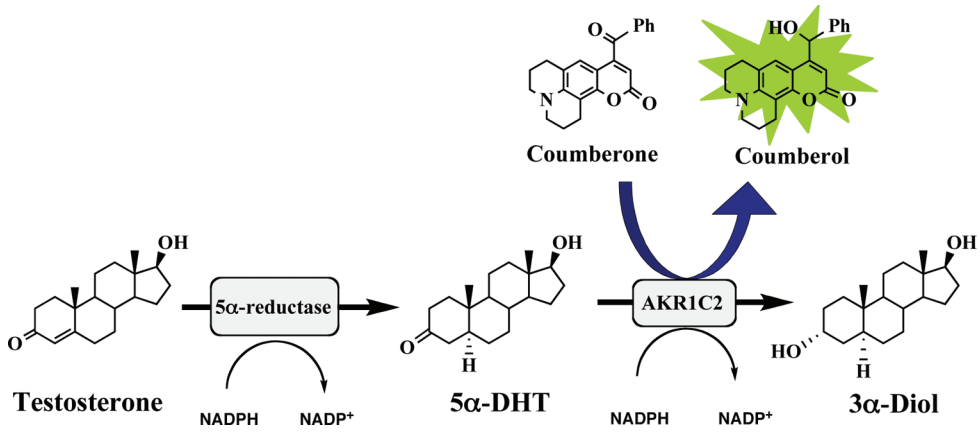

Figure 2. Potent androgens testosterone and DHT (5α-dihydrotestosterone) are metabolized to 3α-diol (3α,17β-androstanediol) *via* a two-step pathway. Coumberone is a fluorogenic substrate that measures the activity of AKR1C2 in cells. In the presence of testosterone, the same probe measures the activity of 5α-reductase through the changes of metabolic flux.

modulates the steroid ligand potency both in the periphery and in the brain, and it has been suggested that genetic variants of these proteins may play a role in the predisposition for a number of disorders including prostate cancer, baldness, and depression (*20–22*). Despite the importance of this enzyme, there are no probes or methods for continuous monitoring of 5α-reductase in intact cells.

RESULTS AND DISCUSSION

In the physiological context, 5α-reductase converts testosterone to DHT (5α-dihydrotestosterone), which is subsequently converted to 3α-diol (3α,17β-androstanediol) by the reductase AKR1C2 (aldoketoreductase 1C2) (*23, 24*), for which we have previously developed the fluorogenic substrate Coumberone (Figure 2) (*25, 26*). Instead of developing a new reporter probe for 5α-reductase, we considered the possibility of measuring the activity of this enzyme through a change of the metabolite flux: increasing the 5α-reductase activity will increase the flux of DHT, which in turn will compete with the reporter substrate Coumberone and result in a decreased rate of the fluorescent output.

Relative Affinities of Steroid Metabolites for AKR1C2. For this approach to succeed, we had to ensure that testosterone's affinity for AKR1C2 was substantially lower than that of DHT. Monkey kidney COS-1 cells were selected for these experiments as they provide a favorable metabolic background. COS-1 cells were transfected with the AKR1C2 plasmid, and the required kinetic parameters were obtained by measuring the effect of each steroid on the Coumberone metabolism rate in intact cells using the competition assay between the reporter substrate and the dark substrate (Figure 3) (*25–27*). Importantly, the apparent K_i value of testosterone was 8-fold higher than the value of the apparent K_m of DHT ($K_{i(T)} = 8.7 \pm 1.8$ μM; $K_{m(DHT)} = 1.1 \pm 0.1$ μM). Also, the affinity of the final product 3α-diol was determined for AKR1C2 ($K_{i(3\alpha\text{-diol})} = 3.3 \pm 0.4$ μM). The hydrophobic steroids are soluble in aqueous media (and growth medium containing 0.5% v/v of DMSO) at these relatively high concentrations (0−50 μM) as evident by homogeneity of the solution. This is consistent with the reported solubility of testosterone in water and aqueous media (24−66 μg mL^{-1}, 85−229 μM concentration) (*28*).

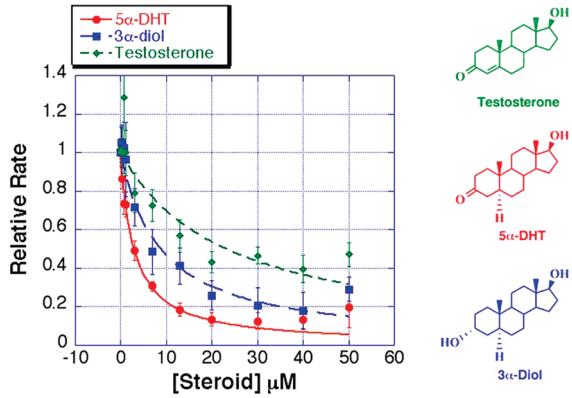

Figure 3. Effect of substrate DHT and ligands testosterone and 3α-diol on the AKR1C2-catalyzed rate of Coumberone metabolism in intact COS-1 cells. The large difference between testosterone and DHT in the competitive inhibition of AKR1C2 is the key prerequisite for the measurement of 5α-reductase activity through the formation of DHT. For experimental measurements, after washing the cells and replacing the media, AKR1C2 transfected cells were incubated with 5 μM Coumberone and varying concentrations (0−50 μM) of the corresponding steroid. Fluorescence of the media was measured over time (20 h) by fluorimetric analysis. Data shown is the average ± SE of four independent assays.

Expression of 5α-R1 Affects the Steroid Flux and the Optical Output. To put the concept to test, COS-1 cells expressing AKR1C2 were transfected with varying amounts of 5α-reductase type 1 (5α-R1, 0−2 μg of DNA plasmid). After growing these cells to confluency, testosterone (20 μM) and Coumberone (5 μM) were added, and the fluorescence of the medium was monitored over time *via* fluorimetry. Indeed, the expression of 5α-R1 led to the suppression of Coumberone metabolism. A plot of the relative rate of fluorescence increase versus the amount of 5α-R1 plasmid used for transfection revealed an exponential decay curve (Figure 4, panel A; the relative rate is defined as the rate of fluorescence increase in the presence of 5α-R1 divided by the rate in the absence of 5α-R1). The expression of 5α-R1 protein in cells was confirmed by Western blot (Figure 4, panel B).

In the absence of testosterone, there was no significant change in the rate of Coumberone reduction, confirming that the suppression of the probe metabolism is dependent on the flux of the DHT metabolite (Figure 4, panel A). This control experiment also showed that increasing the expression of 5α-reductase had no effect on the activity of AKR1C2. The shape of the curve in the presence of testosterone (Figure 4, panel A) closely resembles the one obtained in a two-substrate competi-

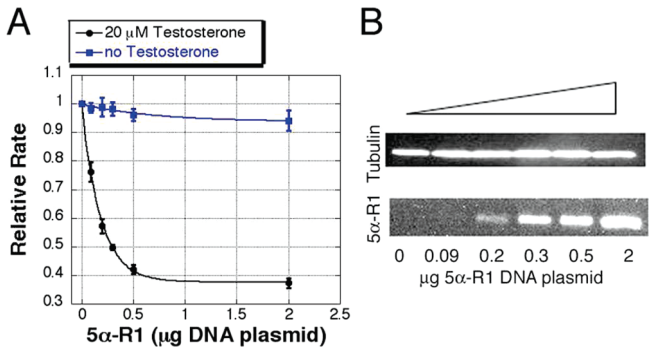

Figure 4. Expression of 5α-R1 (5α-reductase type 1) in COS-1 cells suppresses the metabolism of Coumberone. A) Correlation of the relative fluorimetric rate of Coumberone metabolism to the amount of 5α-R1 plasmid used for transfection, both in the presence and in the absence of testosterone. The relative rate is defined as the ratio of Coumberone metabolism rate when 5α-R1 is expressed to the rate under no 5α-R1 expression. For experimental measurements, cells were transfected with AKR1C2 and varying amounts of 5α-R1 DNA plasmid (0−2 μg). Twenty-four hours after transfection, the cells were washed, media was replaced, and cells were incubated with 5 μM Coumberone and 20 μM testosterone (parallel experiments were performed in the absence of testosterone). Aliquots (100 μL) of the culture media were removed over time (4 h), and fluorescence was measured by fluorimetric analysis. Data shown is the average ± SE of seven independent assays performed in duplicates. B) The expression of 5α-R1 protein was confirmed by Western blot analysis.

tion assay where an increasing amount of DHT was directly added to the cells (Figure 3). The suppression of the probe metabolism levels off at approximately 0.5 μg of 5α-R1 plasmid, which is also the amount where the protein expression begins to saturate (Figure 4, panels A and B), suggesting that in COS-1 cells, 5α-reductase activity closely correlates with the protein expression level. To ensure that the decrease in fluorescence was due to competitive inhibition and not a result of cell death, cellular viability was tested *via* the lactate dehydrogenase (LDH) assay (Supplementary Figure 1).

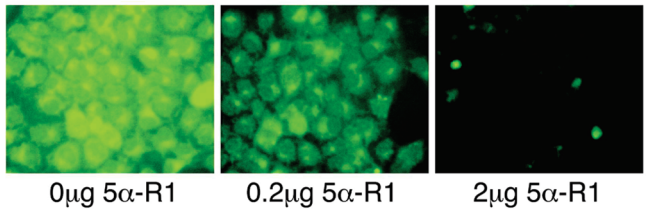

Figure 5. A decrease in fluorescence is observed with the increasing 5α-R1 expression. After washing the AKR1C2 and 5α-R1 transfected cells and replacing the media, metabolic studies were initiated by addition of 5 μM Coumberone and 20 μM testosterone (parallel experiments were performed in the absence of testosterone). Shown here are fluorescent images of COS-1 cells taken 4 h after the start of metabolic studies. Cells were imaged using a customized Chroma filter set for 350/50 nm excitation and 525/50 emission. As shown *via* fluorimetry, in the absence of testosterone, there was no significant change in the rate of Coumberone metabolism (Supplementary Figure 3).

Importantly, we were also able to image the changes in the metabolic flux in intact cells *via* fluorescence microscopy (Figure 5 and Supplementary Figure 2). In analogy to fluorimetry, the microscopy imaging showed a decrease in Coumberone metabolism in cells with increasing expression of 5α-reductase. Indeed, the changes in metabolic flux were observed optically in a continuous assay and with the resolution of individual cells!

At the high protein expression levels of 5α-R1 (>0.5 μg of DNA plasmid), the metabolism of Coumberone is largely suppressed (Figure 5 and Supplementary Figure 2). The microscopy imaging was performed 4 h after the addition of substrates to the cells, which allows for high conversion of testosterone to DHT and DHT to 3α-diol (*e.g.*, 5 μM DHT is completely metabolized in 4 h) (25). Inhibition of Coumberone metabolism at this point is thus ascribed to competitive binding of the substrate (DHT) and the product (3α-diol) to the AKR1C2 enzyme. This is consistent with the rate suppression measured by fluorimetry (Figure 3); the Coumberone metabolism rate is reduced by 80% in the presence of 10 μM DHT (corresponding to 50% conversion of testosterone) and by 50% in the presence of 10 μM 3α-diol.

Direct Read-Out of 5α-R1 Inhibition. The activity of 5α-reductase and the resulting DHT flux was also manipulated pharmacologically with the clinically used inhibitor dutasteride (GG745, a dual inhibitor of both 5α-reductase isozymes) (*29*). Increasing the concentration of dutasteride (0−1 μM) led to a faster metabolism of Coumberone, as measured by fluorescence microscopy, indicating the inhibition of 5α-reductase and the decrease of the metabolic flux. At 1 μM of dutasteride, the images were identical to the null-transfected cells (Figure 6). Importantly, it was verified that the difference in fluorescence was due to 5α-reductase inhibition and not due to inhibition of the reporter enzyme AKR1C2 (Supplementary Figure 4).

These results show that our system enables a measurement of 5α-reductase activity in cells with a wide dynamic range of activities and provides a continuous fluo-

rescence assay for evaluation of small molecule inhibitors for this important medicinal target.

Detection Limit with Respect to Metabolite Concentration. Furthermore, this paper makes a conceptual contribution by demonstrating the ability to optically measure the changes in metabolic fluxes in living cells. In this regard, we examined the detection limits of the assay with respect to testosterone concentration (Figure 7). The rate of Coumberone metabolism was measured in COS-1 cells expressing both 5α-reductase and AKR1C2, in the presence of a wide range of testosterone concentrations. These measurements showed that concentration of testosterone higher than 3 μM is needed to observe a statistically significant decrease in fluorescence. This concentration limit is consistent with and determined by the optimal substrate concentration range of AKR1C2 ($K_{m(DHT)} = 1.1 \pm 0.1$ μM), the enzyme where the two-substrate competition takes place. Coumberone's kinetic parameter ($K_{m(Coum)} = 3.0 \pm 0.4$ μM) is nearly ideal as it approximates that of the physiological substrate DHT. However, it is the mismatch between the optimal concentration range of AKR1C2 (low micromolar) and the physiological concentration of androgens (low nanomolar) that makes this assay unsuitable for measuring physiologically relevant changes in steroid fluxes. It is not an inherent problem of the method; in fact, the detection limits of the flux dynamic range are imposed by the functional parameters of the enzyme target (K_m for the physiological substrate).

Conclusions. This paper describes a proof-of-principle study that demonstrates a novel optical approach to measuring metabolic flux changes in living cells. This work, although based on established two-substrate competition assays, demonstrates for the first time the optical indication of metabolic flux changes in intact cells *via* competition between a fluorogenic reporter substrate and a physiological substrate. A model system of the testosterone metabolic pathway was constructed in COS-1 cells to demonstrate the feasibility of this method in intact mammalian cells. We show that a broad range of 5α-R1 activity could be measured *in situ* by examining the flux changes at the down stream enzyme AKR1C2. We also show that the detection limits of the metabolite flux dynamic range can be estimated by the functional parameters of the enzyme target (K_m for the physiological and reporter substrate).

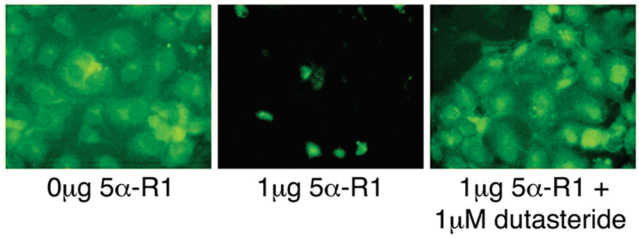

Figure 6. Microscopy imaging of 5α-reductase inhibition on COS-1 cells. The inhibitor dutasteride (1 μM) restores the fluorescence to the level observed in cells with no 5α-R1. After the AKR1C2 and 5α-R1 transfected cells were washed and the media was replaced, cells were incubated with 1 μM dutasteride, 5 μM Coumberone, and 20 μM testosterone. Fluorescence of cells was imaged 4 h after the start of inhibition studies using a customized Chroma filter set for 350/50 nm excitation and 525/50 emission.

Applicability of the Concept to Other Metabolic Pathways: the Key Functional Parameters. In principle, this approach is applicable to other metabolic pathways, as long as a fluorogenic substrate is available for a suitable enzyme of the pathway. In each case, the chosen system will have to be evaluated along the lines illustrated in this paper with the key requirements that the K_m for the reporter substrate is similar to that of the

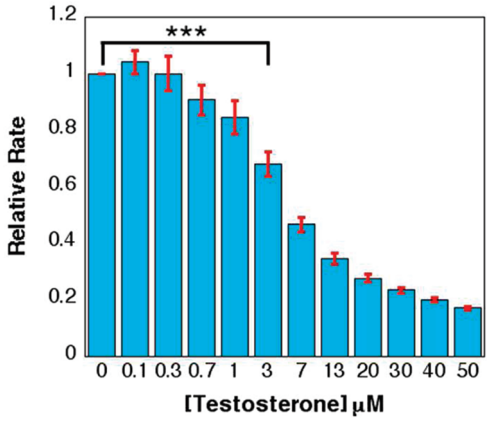

Figure 7. Determination of the effective metabolite concentration range. Coumberone fluorimetric rates under varying testosterone concentrations (0−50 μM) indicate that a minimum concentration of 3 μM of testosterone is needed to observe a statistically significant difference in fluorescence. For experimental measurements, after the cells were washed and the media was replaced, AKR1C2 and 5α-R1 transfected cells were incubated with 5 μM Coumberone and varying concentrations of testosterone (0−50 μM). Fluorescence of the media was measured over time (20 h) by fluorimetric analysis. ***$P < 0.0001$, $n = 8$.

physiological substrate, and for endogenous pathways, that the endogenous concentration range of the physiological substrate is close to its K_m value. Additional requirement is that the upstream metabolites do not directly inhibit the enzyme targeted by the reporter substrate (see Figure 3). Our approach is complementary to the ratiometric optical sensors that measure the metabolite concentration changes *via* a binding equilibrium (*5, 6*). We envision that the simultaneous use of enzyme reporters (this work) and metabolite sensors (*5, 6*) will provide powerful optical means for the examination of dynamic properties of metabolic pathways in intact cells. One important goal of analyzing metabolic networks is the determination of flux ratios at key nodes of divergent or convergent pathways. This may be achievable in intact cells *via* optical means, for example, by having the reporter substrate in one arm of the metabolic pathway and the ratiometric metabolite sensor in the other arm, both indicating flux changes in the corresponding branches of the system related to a chosen perturbation. This represents a new frontier that depends chiefly on the development of fluorogenic reporter substrates and metabolite sensors, which is an active area of research (*5–14*).

It should be noted that even if the application of the new optical method illustrated in this paper to the endogenous metabolic systems remains challenging, the method is still of considerable value in the context of engineered systems (for example, continuous cell-based assay for high-throughout screening of inhibitors, as indicated here). This work expands the utility of fluorogenic enzyme reporter substrates (*7–14*). Due to the connectivity of multiple enzymes through the shared metabolites, optical reporters can measure not only the activity of the target enzyme but also the activity of other enzymes upstream in the pathway, for which there are no probes available, *via* the change of metabolic flux.

METHODS

Chemicals and Materials. All steroids used in the study, 17β-hydroxy-5α-androstan-3-one (DHT, dihydrotestosterone), 5α-androstan-3α,17β-diol (3α-diol), 17β-hydroxy-4-androsten-3-one (testosterone), were purchased from Steraloids. Dutasteride (dual 5α-reductase inhibitor) was purchased from AKSci. LDH cytotoxicity assay kit was purchased from TaKaRa Bio Inc. Coumberone was synthesized as described (*25*). The (pCMV)-SDR5A1 construct (5α-reductase type 1, 5α-R1) was generously provided by Prof. D. W. Russell (UT Southwestern Medical Center). The (pcDNA3)-AKR1C2 construct was a kind gift from Prof. T. M. Penning (University of Pennsylvania School of Medicine).

Cell Culture. African green monkey kidney fibroblast cells (COS-1 cells) were purchased from the American Tissue Culture Collection. COS-1 cells were maintained at 37 °C and 5% CO_2 in DMEM supplemented with 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 2 mM L-glutamine (Gibco Invitrogen Corporation), and 10% heat-inactivated fetal bovine serum (Atlanta Biologicals). They were passaged twice a week at 1:5 to 1:10 dilutions.

Competitive Binding of Testosterone and Its Metabolites to AKR1C2. COS-1 cells were seeded at a density of 2×10^6 cells per 10 cm plate. After 24 h, cells were transfected with FuGENE6 transfection reagent (Roche Diagnostics) according to the manufacturer's protocol; each 10 cm plate was transfected using 18 μL of FuGENE6 to 6 μg of pcDNA3-AKR1C2. After 24 h, cells were washed, trypsinized, and seeded in white FALCON 96-well flat bottom tissue culture-treated plates at a density of 1×10^4 cells per well. After 48 h, the cells were washed, and the media was replaced with DMEM minus phenol red supplemented with 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 2 mM L-glutamine, and 1% charcoal/dextran treated-FBS (Atlanta Biologicals). Approximately 2 h after the media was changed, each well was incubated with 5 μM Coumberone along with varying concentrations (0−50 μM) of the corresponding steroid (testosterone, DHT, and 3α-diol). The probe and steroids were added in DMSO at a constant final concentration of 0.5% v/v, which had no effect on cell viability. Fluorescence of each well was measured over time through a MicroMax 384 connected to a Jobin Yvon Fluorolog through F-3000 fiber optic cables upon excitation at 385 nm and emission at 510 nm. To compare the effects of the competitive "dark" substrate (DHT), its precursor (testosterone), and its metabolite (3α-diol), on the metabolism of Coumberone, the probe metabolism rate was related to the metabolic rate in the presence of the different steroids (*23*). These relative rates were then fit to the equation $v_i/v_0 = [\,(1+K_M/S)\,]/[1 + K_M/S(1 + S'/K'_M)\,]$ by using KaleidaGraph to yield K'_M of DHT (the Michaelis constant for the dark substrate) as well as the K'_i of the ligands (testosterone and 3α-diol) as described (*27*).

Protocol for the Readout of 5α-Reductase Activity in Cells *via* Fluorimetry. COS-1 cells were seeded at 2×10^6 cells per 10 cm plate. After 24 h, cells were transfected using 18 μL of FuGENE6 to 6 μg of pcDNA3-AKR1C2. Twenty-four hours post-transfection, COS-1 (+AKR1C2) cells were plated in 6-well dishes at a density of 2.5×10^5 cells. After 48 h, cells were transfected with varying amounts of 5α-reductase (0−2 μg of DNA plasmid), using FuGENE6 at a 3 μL FuGENE6 to 1 μg of (pCMV)-SDR5A1 ratio.

Metabolism studies were initiated 24 h after cellular transfection. Approximately 2 h prior to metabolism studies, the media was replaced with DMEM minus phenol red supplemented with 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 2 mM L-glutamine, and 1% charcoal/dextran treated-FBS. Metabolism studies commenced with the addition of 5 μM AKR1C2 reporter Coumberone and 20 μM testosterone (added in DMSO) to the cell growth media. Parallel fluorimetric metabolism experiments were performed in the absence of testosterone. The DMSO added was at a constant final concentration of 0.25% v/v, which had no effect on cell viability as judged by the LDH viability assay. For fluorimetric analysis, aliquots (100 μL) of the culture media were removed over time, collected on 96-well plates and measured by a MicroMax 384 connected to a Jobin Yvon Fluorolog through F-3000 fiber optic cables by detecting fluores-

cence upon excitation at 385 nm. Initial reaction velocities were obtained from plots of fluorescence emission at 510 nm versus time. The slope was divided by the change in fluorescence corresponding to complete reduction of the probe (metabolism in the absence of 5α-reductase DNA plasmid transfection) to obtain relative rates.

Western Blot Analysis of 5α-Reductase Protein Expression. Twenty micrograms of COS-1 cell lysates were resolved on 12% SDS/PAGE gels. The gel was electrophoresized at 120 V for 90 min and transferred onto a Millipore Immobilon-P membrane (prewet in methanol) in 190 mM glycine, 25 mM Tris buffer, and 20% methanol for 30 min at 400 mA. The membrane was blocked for 1 h at RT in 10% powdered milk in TBS with 0.2% Tween-20 and then reacted 2 h with a 5α-reductase type 1 isozyme antibody diluted 1/1000 in 10% TBS-T. The antibody was raised in a rabbit against the N-terminal region of 5α-reductase type 1 and was a generous gift from Prof. D. W. Russell (University of Texas, Southwestern Medical Center). The membrane was then stringently washed for 10 min three times in TBS with 0.1% SDS, 0.1% NP-40, and 0.5% deoxycholic acid (RIPA buffer). The membrane was then incubated with antirabbit, horseradish peroxidase-linked secondary antibody from donkey diluted 1/2000 in 10% powdered milk in TBS-T for 20 min. The membrane was then washed again three times in RIPA buffer and then developed using PIERCE ECL Detection Reagents according to the manufacturer's protocol. Tubulin loading control was similarly detected using a tubulin antibody (Sigma) diluted 1/2000. Protein levels were estimated after resolution on an Imagestation 400CF system (Kodak).

Imaging 5α-Reductase Activity in Cells through Fluorescence Microscopy. COS-1 cells were imaged over time using a Leica DMI4000 inverted fluorescent microscope equipped with a BGR internal filter wheel system for UV, blue, and green excitation and a customized Chroma filter set for 350/50 nm excitation and 525/50 emission. Cells were visualized with a 10x/0.40 objective. The fluorescent reporter Coumberone was monitored using an exposure time of 340 ms and a lamp intensity of 17%. Fluorescent images were taken 4 h after probe addition, time at which the largest difference in protein expression was observed. For each experiment, there were 2 wells per DNA plasmid concentration and two images per well. Images of each representative experiment were adjusted using the same contrast ratios and the mean fluorescent intensity of each image was determined using Image J (National Institutes of Health).

5α-Reductase Expression Has No Effect on the Enzymatic Activity of AKR1C2. COS-1 cells were grown and transfected with constant amount of AKR1C2 while the amount of 5α-reductase DNA plasmid was varied as described for the expression studies. The metabolic studies were also performed as stated above with the exception that no testosterone was added to the cells. Metabolism was followed *via* fluorimetry and microscopy as stated above.

Imaging 5α-Reductase Inhibition in COS-1 Cells. COS-1 (+AKR1C2) cells were transfected with 1 μg of mock or 5α-reductase DNA plasmid per well of a 6-well plate. Approximately 48 h after transfection, the cells were washed, and the media was refreshed with DMEM minus phenol red and incubated for 2 h. The cells were then incubated with 5 μM Coumberone, 20 μM testosterone, and 1 nM−2.5 μM Dutasteride (GG745, a potent dual inhibitor of both 5α-reductase isozymes) (*29*), all dissolved in DMSO. The DMSO added was at a constant final concentration of 0.5% v/v, which had no effect on cell viability. Inhibition was monitored *via* fluorescence microscopy. COS-1 (+AKR1C2) transfectant (no 5α-reductase) was used as a standard for fully inhibited 5α-reductase.

Dutasteride Inhibition of AKR1C2 (Control). COS-1 (+AKR1C2) cells were incubated with 5 μM Coumberone along with varying concentrations of dutasteride (0−2.5 μM). The probe and inhibitor were added in DMSO at a constant final concentration of 0.5% v/v, which had no effect on cell viability. For fluorimetric analysis, aliquots (100 μL) of the culture media were removed over time, collected on 96-well plates, and measured by detecting fluorescence upon excitation at 385 nm. Percent inhibition was calculated *via* the direct assay of AKR1C2 activity using Coumberone.

Determination of Cell Viability *via* the Lactate Dehydrogenase Assay. LDH cytotoxicity assay was run according to manufacturer's protocol.

Determination of the Effective Testosterone Concentration Range. COS-1 cells were seeded at a density of 1×10^6 cells per 10 cm plate. After 48 h, cells were transfected with 6 μg of pcDNA3-AKR1C2 and 6 μg of (pCMV)-SDR5A1 using 36 μL of FuGENE6 to 12 μg of total DNA plasmid. After 24 h, cells were trypsinized and seeded in white FALCON 96-well tissue culture-treated plates at a density of 1×10^4 cells per well. After 72 h, media was replaced with DMEM minus phenol red supplemented with 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 2 mM L-glutamine, and 1% charcoal/dextran treated-FBS. Approximately 2 h after the media was changed, each well was incubated with 5 μM Coumberone along with varying concentrations of testosterone (0−50 μM). The probe and steroid were added in DMSO at a constant final concentration of 0.5% v/v, which had no effect on cell viability. Fluorescence of each well was measured over time and initial reaction velocities were obtained from plots of fluorescence versus time. The rate of probe metabolism in the presence of testosterone was normalized to the rate in the absence of testosterone. The Student's *t* test was used to determine the minimum amount of testosterone needed to observe a statistically significant difference in fluorescence (comparison of no testosterone with different testosterone concentrations).

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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