Intracrine Testosterone Activation in Human Pancreatic β -Cells Stimulates Insulin Secretion

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Testosterone (T) affects β -cell function in men and women. T is a prohormone that undergoes intracrine conversion in target tissues to the potent androgen dihydrotestosterone (DHT) via the enzyme 5α -reductase (5α -R) or to the active estrogen 17β -estradiol (E2) via the aromatase enzyme. Using male and female human pancreas sections, we show that the 5α -R type 1 isoform (SRD5A1) and aromatase are expressed in male and female β -cells. We show that cultured male and female human islets exposed to T produce DHT and downstream metabolites. In these islets, exposure to the 5α -R inhibitors finasteride and dutasteride inhibited T conversion into DHT. We did not detect T conversion into E2 from female islets. However, we detected T conversion into E2 in islets from two out of four male donors. In these donors, exposure to the aromatase inhibitor anastrozole inhibited E2 production. Notably, in cultured male and female islets, T enhanced glucose-stimulated insulin secretion (GSIS). In these islets, exposure to 5α -R inhibitors or the aromatase inhibitor both inhibited T enhancement of GSIS. In conclusion, male and female human islets convert T into DHT and E2 via the intracrine activities of SRD5A1 and aromatase. This process is necessary for T enhancement of GSIS.

Accumulated evidence suggests that the gonadal steroid testosterone (T) is necessary for proper glucose-stimulated insulin secretion (GSIS) in men and promotes insulin hypersecretion and β -cell dysfunction in women with androgen excess (1–4). Accordingly, the active metabolite

of T, dihydrotestosterone (DHT), enhances GSIS in cultured islets from male human donors (3). Male mice lacking the androgen receptor (AR) selectively in β -cells (β ARKO) exhibit impaired GSIS, leading to glucose intolerance, and develop diabetes (3). In addition, exposure of cultured islets from female human donors to DHT promotes insulin hypersecretion (4). In a female mouse model of chronic androgen excess, DHT promotes hyperinsulinemia associated with secondary pancreatic β -cell dysfunction via action on AR in β -cells (4).

In healthy men and hyperandrogenic women, T is the main circulating gonadal androgen. T is a weak androgen and a prohormone that undergoes local conversion in target tissues to either DHT via action of one of the 5α -reductase (5α -R) isoforms (5) or 17β -estradiol (E2) via action of the enzyme aromatase to activate AR or estrogen receptors (ERs), respectively (5,6). Notably, activation of ERs by E2 in male and female human β -cells enhances insulin synthesis, GSIS, and promotes survival from multiple metabolic injuries (7–11). Therefore, circulating T could have a clinically relevant impact on β -cell function in healthy men and hyperandrogenic women via conversion to DHT and/or E2 within pancreatic islets. However, the extent to which 5α -R isoforms and the aromatase are present in human islets from both sexes and able to convert T to DHT and E2 to directly affect β -cell function is unknown.

In this study, we have used pancreas sections and cultured islets from male and female human donors to study the expression of the three 5α -R isoforms and the

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aromatase, quantify the conversion of T to DHT and E2, and assess the functional significance of intracrine conversion of T in pancreatic islets on GSIS.

RESEARCH DESIGN AND METHODS

Immunohistochemistry

Human pancreas sections were obtained from the Network for Pancreas Organ Donors with Diabetes (nPOD). Sections went through deparaffinization and antigen retrieval, followed by incubation with primary antibodies. Insulin (1:100; Abcam) staining from pancreas sections was performed as described (9). For steroidogenic enzyme staining, sections were incubated in primary antibody, anti-aromatase (1:50; Novus Biologicals), anti-SRD5A1 (1:50; Abcam), anti-SRD5A2 (1:50; Santa Cruz Biotechnology), and anti-SRD5A3 (1:50; Abcam) and then incubated in the goat anti-rabbit secondary antibody (1:300). Images were taken using a Nikon A1 confocal microscope.

Human Islet Steroid Conversion Assays

Human islets were obtained from the Integrated Islet Distribution Program (see Supplementary Table 1 for donor information) and recovered overnight in complete medium: RPMI 1640 (Gibco) supplemented with 10% charcoal-stripped FBS and penicillin/streptomycin (100 units/mL, 100 μ g/mL). Islets were treated with T (100 nmol/L) (Sigma-Aldrich), the 5 α -R inhibitors finasteride (100 nmol/L) (Sigma-Aldrich) and dutasteride (100 nmol/L) (Sigma-Aldrich), the aromatase inhibitor anastrozole (100 nmol/L) (Sigma-Aldrich), or vehicle (ethanol and DMSO). Other control conditions included culture medium without FBS, complete medium with finasteride and dutasteride, or with anastrozole. Culture medium and islets were harvested for further analysis after a 24-h incubation period. For normalization of the steroid concentrations to total protein content of the pancreatic islet incubations, islet cells were lysed in 2 $\times$ lysis buffer (Cell Signaling Technology) supplemented with 1 mmol/L phenylmethylsulfonyl fluoride, 0.1 mol/L dithiothreitol, and protease inhibitor mix (Roche). Protein content of the lysate was quantified in the supernatant using the Pierce 660nm Protein Assay (Thermo Fisher Scientific).

Steroid Quantification by Ultra-High-Performance Liquid Chromatography–Tandem Mass Spectrometry

Mass spectrometry–based analysis of steroids was performed with islets from eight donors (four male and four female). For each donor, islet incubations were performed in technical triplicates. For the measurement of androgens, 500 μ L of culture medium or external standard mix were combined with an internal standard mixture and extracted by liquid-liquid extraction with *tert*-butyl methyl ether (Acros Organics). For the measurement of E2, 200 μ L of sample or external standard was diluted with 150 μ L of deionized water and mixed with the internal standard. Samples were extracted by supported liquid extraction (Biotage) with methyl *tert*-butyl ether (Thermo

Fisher Scientific). Chromatographic separation and steroid quantification were performed using an ACQUITY ultra-performance liquid chromatography system (Waters Corporation) coupled to a Xevo TQ-XS triple-quadrupole mass spectrometer (Waters Corporation). Mass-to-charge transitions monitored in multiple-reaction monitoring used for quantification are summarized in Supplementary Table 2. Peak area ratios of analyte and internal standard, 1/*x* weighting, and linear least square regression were used to produce the standard curves for quantification. Limits of quantifications were 0.24 nmol/L for T, 2.8 nmol/L for androstenedione (A4), 0.24 nmol/L for 5 α -DHT, 0.8 nmol/L for 5 α -androstenedione (Adione), 0.8 nmol/L for androsterone, and 10 pmol/L for E2. The limit of detection for E2 was 5 pmol/L. Additional details on ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) method are provided in the Supplementary Methods.

Measurement of Insulin Secretion in Static Incubation

Human islets were handpicked under a dissection microscope and treated with finasteride (100 nmol/L) (Sigma-Aldrich), dutasteride (100 nmol/L) (Sigma-Aldrich), anastrozole (100 nmol/L) (Sigma-Aldrich), or vehicle for 6 h prior to adding steroids. T (10 nmol/L) (Sigma-Aldrich), DHT (10 nmol/L) (Steraloids Inc.), E2 (10 nmol/L) (Steraloids Inc.), or vehicle were then added at 2.8 mmol/L and then 16.7 mmol/L glucose for 40 min sequentially. Insulin release from islets was measured with Human Insulin ELISA kit (Millipore Sigma) as described (9). See Supplementary Table 1 for donor information.

Statistics

Statistical analyses were performed with GraphPad Prism. When results showed a Gaussian distribution, one-way ANOVA (with Bonferroni post hoc test) was performed. Results were expressed as the mean $\pm$ SEM, and $P < 0.05$ was considered to be significant. Significance was expressed as follows: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Data and Resource Availability

Data supporting the results reported in the article will be shared upon request. Resource reported in the article will be shared upon request.

RESULTS

Expression of 5 α -R and Aromatase Enzymes in Human Islets

We examined the expression of the aromatase enzyme, CYP19A1, a member of the cytochrome P450 superfamily of enzymes, and 5 α -R isoforms in pancreas sections from male and female human donors without diabetes. Three isoforms of 5 α -R exist: 5 α -R type 1 (SRD5A1), 5 α -R type 2 (SRD5A2), and 5 α -R type 3 (SRD5A3) (12). SRD5A1 showed expression in the cytoplasm of β -cells in male and female islets without expression in α cells or in adjacent exocrine cells (Fig. 1). We did not observe reliable

expression of SRD5A2 or SRD5A3 in either male or female islet cells (Supplementary Fig. 1). The aromatase was expressed in the cytoplasm of β -cells and possibly in other non- α/β islet cells and with minimal exocrine location in both male and female human islets (Fig. 1). The expression of SRD5A1 and CYP19A1 in human β -cells was confirmed using publicly available data sets of RNA sequencing from whole pancreas, bulk islets, and FACS-purified β -cells (Supplementary Fig. 1). Interestingly, at the mRNA level, SRD5A1 expression was higher in females than in males.

Human Islets Can Metabolize Testosterone Into Active Steroids

We treated cultured islets from male and female human donors with T and quantified the conversion of T to androgenic

and estrogenic metabolites by UHPLC-MS/MS. Figure 2A illustrates the possible T conversion pathways: T can be converted by 5 α -reduction to DHT and by 17 β -hydroxysteroid dehydrogenase (17 β -HSD) activity to A4. A4 is further converted to its downstream metabolites, 5 α -androstenedione (Adione) and androsterone, by sequential 5 α -R and 3 α -HSD activities. T can also be aromatized to E2.

After treatment of cultured male and female human islets with T, we detected and quantified DHT in the culture supernatant (Fig. 2B and C). DHT was not detected when islets were cotreated with the potent 5 α -R inhibitors finasteride and dutasteride (12) (Fig. 2B and C). However, DHT was detected when islets were cotreated with the selective aromatase inhibitor anastrozole (6,13) (Fig. 2B

Figure 1—Expression of 5 α -R type 1 (SRD5A1) and aromatase in human islets. Immunohistochemical staining of SRD5A1 (red), aromatase (Ar; red), insulin (green), and glucagon (blue) in pancreas sections from male and female human donors without diabetes. Representative images are shown.

Figure 2—Human islets convert T to 5α-DHT. **A**: Schematic presentation of the enzymatic steps involved in sex steroid metabolism. Conversion of T (100 nmol/L) to DHT in male (**B**) and female (**C**) human islets is blocked by treatment with 5α-R inhibitors finasteride (100 nmol/L) and dutasteride (100 nmol/L), but unaffected by treatment with aromatase (Ar) inhibitor (anastrozole; 100 nmol/L). T conversion to A4, followed by further conversion to 5α-androstenedione (Adione) and androstosterone (An) in male (**D**) and female (**E**) islets, is blocked by treatment with 5α-R inhibitors, but unaffected by Ar inhibitor treatment. Steroid concentrations were quantified by LC-MS/MS and normalized to total protein of the islet lysate. The mean ± SEM and scatter plot of technical triplicates for each donor (four men and four women) are shown. Samples with steroids concentrations less than the lower limit of quantitation are represented as 0.

and **C**). This demonstrates that human islets of both sexes can convert T to DHT via 5α-R activity.

In addition, in the culture supernatants of T-treated male and female islets, we detected the presence of A4, consistent with 17β-HSD activity in these islets. Notably, male and female islets treated with T also produced Adione and androstosterone (Fig. 2D and E), and these metabolites were not detected when islets were treated with 5α-R inhibitors but were still detected when islets were treated with the aromatase inhibitor (Fig. 2D and E). This demonstrates that human islets of both sexes can convert A4 into Adione via 5α-R activity. In addition, we observed the formation of androstosterone, a downstream metabolite of Adione.

Despite immunohistochemical and transcriptomic evidence of aromatase expression in male and female human islets (Fig. 1) and β-cells (Supplementary Fig. 2), we did not detect E2 in the culture media of T-treated female islets. However, we detected E2 in the media of T-treated islets from two of four male donors, irrespective of absence or presence of 5α-R inhibitors (Fig. 3). For one of them (male 4, Fig. 3), E2 was detectable in all incubations with T and T plus 5α-R inhibitors. In five of those six incubations, E2 concentrations could be accurately quantified and ranged from 12 to 32 pmol/L. For the other male islet donor (male 1, Fig. 3) we could detect E2 concentrations below the limit of quantification but clearly above the limit of detection in one of the three technical replicates incubated with T and in all three replicates incubated with T and 5α-R inhibitors (Fig. 3). Notably, E2 was not detected in either donor when islets were coincubated with T and aromatase inhibitor (Fig. 3).

Figure 3—Male human islets convert T to E2. Conversion of T (100 nmol/L) to E2 in two male donors is blocked by treatment with aromatase inhibitor (anastrozole, 100 nmol/L) but retained following treatment with 5 α -R inhibitors (finasteride and dutasteride, 100 nmol/L). **A**: Heat map showing quantifiable, detectable, and nondetectable E2 concentrations measured by LC-MS/MS in each replicate ($n = 3$) for each male islet donor ($n = 4$). **B**: Chromatogram of the LC-MS/MS runs for all treatments and technical replicates of the two male donor islets with detectable or quantifiable E2. The arrows show the location of the E2 peak.

Inhibition of SRD5A1 and Aromatase Activities Prevent T Amplification of GSIS

Having observed that male and female human islets express SRD5A1 and aromatase and convert T to DHT and in males also to E2, we next examined the physiological relevance of intracrine T metabolism to male and female human islet function in an experiment assessing GSIS in

static incubation. At 16.7 mmol/L glucose, male and female human islets exposed to T showed increased GSIS to an extent similar to those exposed to DHT compared with those exposed to vehicle only (Fig. 4A and C). Notably, exposure of T-treated male and female islets to the 5 α -R inhibitors finasteride and dutasteride blocked the effect of T in amplifying GSIS (Fig. 4A and C). Consistent with the

expression of SRD5A1 in β -cells, exposure of T-treated islets to finasteride alone (SRD5A2 and 3 inhibitor) had no effect on T enhancement of GSIS compared with vehicle. In contrast, in the presence of dutasteride alone (SRD5A1, 2, and 3 inhibitor), the ability of T to amplify GSIS compared with vehicle was no longer significant (Supplementary Fig. 3). Additionally, at 16.7 mmol/L glucose, male human islets exposed to E2 showed increased GSIS to an extent

similar to those exposed to T compared with those exposed to vehicle only (Fig. 4B). Importantly, despite the lack of E2 detection in the T-treated islets, but consistent with the presence of aromatase in human islets, as shown above (Fig. 1 and Supplementary Fig. 2), the aromatase inhibitor anastrozole blocked the ability of T to amplify GSIS (Fig. 4B). Similar results were obtained in islets from female human donors (Fig. 4C and D). Together, these data

Figure 4—Inhibition of SRD5A1 and aromatase prevents T-induced amplification of GSIS. GSIS measured in static incubation in male human islets treated with vehicle, T (10 nmol/L), 5 α -R inhibitors (5 α -RI) (finasteride and dutasteride, 100 nmol/L), and DHT (10 nmol/L) (A); male human islets treated with vehicle (V), T, ArI inhibitor (ArI) (anastrozole, 100 nmol/L), and E2 (10 nmol/L) (B); female human islets treated with vehicle, T, 5 α -R inhibitors and DHT (C); and female human islets treated with vehicle, T, ArI, and E2 (D). The mean $\pm$ SEM and scatter plot of technical triplicates for each donor (four men and three women) are shown. * P < 0.05, ** P < 0.01, *** P < 0.001.

demonstrate that T acutely amplifies GSIS in male and female human islets and that the insulinotropic effect of T requires 5 α reduction to DHT and aromatization to E2.

DISCUSSION

Original studies by Walsh et al. (14) in male subjects with undervirilization led to the discovery that T requires conversion to DHT by SRD5A2 for masculinization of the male external genitalia (15). Beyond masculinization, however, T also exhibits important metabolic actions, including effects on insulin secretion (2,16,17).

We show that human islets from both sexes can convert T to DHT via the enzyme SRD5A1 expressed in β -cells. SRD5A2 is involved in sexual development and is primarily localized to classical androgen target tissues, whereas SRD5A1 is expressed in skin and other extragenital androgen target tissues (5). Our finding that treatment with 5 α -R inhibitors abolishes T-induced increase in GSIS demonstrates that in β -cells, T acts primarily as a prohormone, requiring conversion to DHT by SRD5A1 to exert its actions. Therefore, in healthy men, circulating T provides a precursor for intracrine activation to DHT in β -cells to stimulate insulin production (3). Notably, 5 α -R inhibitors are used to treat benign prostatic hyperplasia, a disease affecting ~50% of older men. Men with benign prostatic hyperplasia exposed to the 5 α -R inhibitors finasteride and dutasteride exhibit an increased risk of developing new-onset type 2 diabetes compared with men receiving the α -blocker tamsulosin (18). Taken together, these data suggest that 5 α -R inhibitors, by blocking T conversion to DHT in β -cells, promote β -cell dysfunction, thus predisposing to new-onset type 2 diabetes.

In women with androgen excess, various degrees of β -cell dysfunction have been described (2,19–21), and circulating T concentrations are closely linked to risk of type 2 diabetes in women (22,23). In female mice with androgen excess, chronic AR activation in β -cells promotes insulin hypersecretion and β -cell dysfunction (4). Our finding that T is converted to DHT in female human islets suggests that intracrine androgen activation also plays an important role in mediating these adverse effects of T on β -cell function in women.

Despite evidence of aromatase expression in male and female β -cells, E2 was not detected in the culture media of T-treated female islets, although we were able to detect it in the supernatant of two out of four T-treated male islet cultures. It is conceivable that the islets of the two other donors formed low levels of E2, but the resulting E2 concentrations were below the limit of detection of our E2 assay (10 pmol/L). Alternatively, following an intracrine principle, E2 locally produced in the β -cell could directly and efficiently interact with ERs in the same β -cells followed by inactivation in the same cells (24). Most importantly, and consistent with efficient conversion of T into E2 in β -cells in both sexes, T enhancement of GSIS from cultured male and female islets was blunted after incubation with the aromatase inhibitor anastrozole.

Consistent with islet conversion of T to E2, anastrozole abolished E2 production by the male islets with detectable E2 production at baseline. Taken together, these findings show that T also requires aromatization to E2 to enhance GSIS. Surprisingly, 5 α -R and aromatase inhibitors similarly abolish the ability of T to enhance GSIS, suggesting that both DHT and E2 signaling pathways are necessary to this effect. E2 and DHT activate multiple signaling pathways in β -cell metabolism (3,4,7–11), which may act synergistically to produce the optimal effect of T on GSIS in males and female β -cells (25).

In conclusion, using highly selective and specific tandem mass spectrometry assays, we show for the first time that human pancreatic islets can locally activate androgens and estrogens from circulating T and that this activity is localized to β -cells. We show that these local steroid metabolic pathways drive GSIS, thereby establishing an intracrine mode of sex steroid action in β -cells.

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Author Contributions. W.X. designed and performed experiments of immunohistochemistry (IHC) and GSIS, analyzed the data, prepared the figures, and wrote the manuscript. L.S. performed steroid conversion experiments, including androgen profiling by UHPLC-MS/MS, analyzed the data, and edited the manuscript. M.M.F.Q. and P.M.D.S. performed experiments of GSIS in human islets. Y.Z. performed experiments of IHC from pancreas sections. J.H. and B.G.K. performed E2 measurements by UHPLC-MS/MS. H.W. performed experiments and analysis of IHC images from pancreas sections, prepared Fig. 1, and edited the manuscript. W.A. designed and provided interpretation of the steroid conversion experiments and edited the manuscript. F.M.-J. designed the study, analyzed the data, and wrote and revised the manuscript. F.M.-J. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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