CLINICAL STUDY

Oral low-dose testosterone administration induces whole-body protein anabolism in postmenopausal women: a novel liver-targeted therapy

Vita Birzniece^{1,2,3}, Margot A Umpleby⁴, Anne Poljak⁵, David J Handelsman⁶ and Ken K Y Ho^{1,2,7}

¹Department of Endocrinology, Garvan Institute of Medical Research, St Vincent's Hospital, Sydney, New South Wales, Australia, ²University of New South Wales, Sydney, New South Wales, Australia, ³School of Medicine, University of Western Sydney, Sydney, New South Wales, Australia,

⁴Diabetes and Metabolic Medicine, Faculty of Health and Medical Sciences, University of Surrey, Surrey, UK, ⁵Bioanalytical Mass Spectrometry Facility and School of Medical Sciences, University of New South Wales, Sydney, New South Wales, Australia, ⁶ANZAC Research Institute, Concord Hospital, University of Sydney, Sydney, New South Wales, Australia and ⁷Princess Alexandra Hospital, Brisbane, Queensland, Australia

(Correspondence should be addressed to K K Y Ho at Princess Alexandra Hospital; Email: k.ho@uq.edu.au)

Abstract

Objective: In hypopituitary men, oral delivery of unesterified testosterone in doses that result in a solely hepatic androgen effect enhances protein anabolism during GH treatment. In this study, we aimed to determine whether liver-targeted androgen supplementation induces protein anabolism in GH-replete normal women.

Design: Eight healthy postmenopausal women received 2-week treatment with oral testosterone at a dose of 40 mg/day (crystalline testosterone USP). This dose increases portal concentrations of testosterone, exerting androgenic effects on the liver without a spillover into the systemic circulation.

Outcome measures: The outcome measures were whole-body leucine turnover, from which leucine rate of appearance (LRA, an index of protein breakdown) and leucine oxidation (Lox, a measure of irreversible protein loss) were estimated, energy expenditure and substrate utilization. We measured the concentration of liver transaminases as well as of testosterone, SHBG and IGF1.

Results: Testosterone treatment significantly reduced LRA by $7.1 \pm 2.5\%$ and Lox by $14.6 \pm 4.5\%$ ($P < 0.05$). The concentration of liver transaminases did not change significantly, while that of serum SHBG fell within the normal range by $16.8 \pm 4.0\%$ and that of IGF1 increased by $18.4 \pm 7.7\%$ ($P < 0.05$). The concentration of peripheral testosterone increased from 0.4 ± 0.1 to 1.1 ± 0.2 nmol/l ($P < 0.05$), without exceeding the upper normal limit. There was no change in energy expenditure and fat and carbohydrate utilization.

Conclusions: Hepatic exposure to unesterified testosterone by oral delivery stimulates protein anabolism by reducing protein breakdown and oxidation without inducing systemic androgen excess in women. We conclude that a small oral dose of unesterified testosterone holds promise as a simple novel treatment of protein catabolism and muscle wasting.

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Introduction

Testosterone is a potent anabolic agent that increases muscle mass and physical performance (1, 2, 3). A reduction in the concentration of testosterone is linked to the development of sarcopenia and frailty in men (4). It has emerged as a potential therapeutic agent for muscle loss associated with ageing and systemic disease. A meta-analysis of 11 randomized controlled trials has provided strong evidence that testosterone supplementation increases muscle strength in elderly men (5). Bhasin *et al.* (1) have demonstrated that the increase in muscle mass and strength from testosterone supplementation is dose dependent in men. The low levels of circulating testosterone in women are also associated with a reduction in muscle mass, which can

be reversed by testosterone supplementation (6, 7). The exploitation of systemic testosterone supplementation has been limited by systemic side effects, in both men and women (1, 8, 9, 10, 11, 12, 13). These include gynaecomastia, sleep apnoea, mood changes, polycythemia and acceleration of prostatic disease in elderly men and virilization in women.

Testosterone and GH exert independent and additive regulatory effects on protein metabolism (14, 15, 16, 17). The impact of testosterone may be related to the initial levels of circulating testosterone. It is significantly anabolic in hypogonadal men (18), whereas this is not observed always in healthy elderly men (19). Importantly, both testosterone and GH are required for a maximal anabolic effect (16, 17, 19). We have recently investigated whether the site of GH and

testosterone interaction during protein metabolism is hepatic or extrahepatic. We compared the impact of testosterone administered via the oral and transdermal routes on whole-body protein metabolism in hypopituitary men during GH replacement therapy (17). We employed a 40 mg daily oral dose of unesterified testosterone that increases portal concentrations of testosterone without inducing a pharmacological hepatic response and without a spillover into the systemic circulation (20). The effects were compared with those of transdermal delivery that elevated the concentration of testosterone in peripheral blood to the normal range. The protein anabolic impact of testosterone supplementation was similar during systemic and solely hepatic administrations, indicating the liver as the primary site of interaction between these two hormones (17). Therefore, in the GH-sufficient state, oral delivery that achieves solely hepatic exposure to testosterone via the portal vein may induce a whole-body anabolic effect without systemic androgenic effects.

Our previous studies involved hypogonadal men in whom oral testosterone significantly stimulated protein anabolism (17). In the present study, we determined whether the delivery of a similar dose of oral testosterone induces a significant protein anabolic effect in healthy postmenopausal women.

Subjects and methods

Subjects

Eight healthy postmenopausal women were recruited from the community through an advertisement. The mean age of the participants was 64.2 ± 1.6 years and mean BMI was 26.8 ± 1.7 kg/m². The study participants were in good health, and all had normal haematological test results and renal and hepatic function. Exclusion criteria included hypothalamic or pituitary disorders, diabetes mellitus and chronic renal or hepatic illnesses. The subjects were not taking hormone replacement therapy or any medications known to interfere with the endocrine system. All the participants were instructed to follow their usual diet and physical activity as well as to continue their usual medications or supplements throughout the study period.

The Human Research Ethics Committee of St Vincent's Hospital approved the study. The study was conducted in accordance with the principles of the Declaration of Helsinki. All participants gave written informed consent. The study was registered with the Australian and New Zealand Clinical Trials Registry (ACTRN12605000482662).

Study design

This was an open-label study. The participants were studied at baseline and after 2 weeks of testosterone

administration at a dose of 40 mg/day orally. Testosterone was prepared by Fresh Therapeutics (Sydney, NSW, Australia) as capsules filled with crystalline testosterone USP without excipients. The daily 40 mg dose of crystalline testosterone was divided into three doses taken every 8 h to achieve steady hepatic exposure. The dose was based on our previous work on hypogonadal men in whom 40 mg/day of oral crystalline testosterone was the highest dose that did not increase systemic levels of testosterone and did not reduce the blood levels of SHBG, indicative of an absence of pharmacological androgen effects on the liver (20). No significant increase in the concentration of testosterone occurred in peripheral blood, indicative of the absence of a systemic spillover (20).

We investigated the impact in postmenopausal women of oral testosterone delivery on i) the concentrations of testosterone, SHBG and markers of hepatic function in peripheral blood; ii) resting energy expenditure and fat oxidation; and iii) whole-body leucine turnover from which the rates of leucine appearance (L_{Ra}, an index of protein breakdown), leucine oxidation (Lox, an index of oxidative loss of protein) and leucine incorporation into protein (LIP, an index of protein synthesis).

The participants were studied after an overnight fast in the early morning in the Clinical Research Facility, Garvan Institute of Medical Research. Studies were undertaken at baseline and at the end of the treatment period. Blood samples for the measurement of the concentrations of testosterone and SHBG and liver function were collected around 0900 h before the start of the leucine turnover study. At each visit, study blood samples were collected and placed on ice; plasma and serum were separated and stored at -80°C until analysis.

Methods

Protein turnover Whole-body protein metabolism was measured using the leucine turnover technique over a 3-h period. The method is based on the principle of steady-state kinetics in which the rate of appearance of a substrate equals its rate of disposal. For leucine, there are two pathways of disposal: oxidation and re-incorporation into protein. The fractional partitioning between these two pathways of disposal is determined from the fraction of infused isotope that appears in breath. The L_{Ra} and Lox were calculated as described previously (21). α -Ketoisocaproic acid (KIC) is formed when leucine undergoes transamination and is used as a surrogate marker of leucine as it more accurately reflects the intracellular environment (22).

After an overnight fast, a 0.104 mg/kg priming dose of NaH¹³CO₃ was administered to the participants, followed by a primed constant 3-h infusion of 1-[¹³C]leucine (prime 0.5 mg/kg, infusion 0.5 mg/kg per h), as described

previously (23, 24). $\text{NaH}^{13}\text{CO}_3$ and $1\text{-}[^{13}\text{C}]\text{leucine}$ were obtained from Cambridge Isotope Laboratories, Woburn, MA, USA. On each visit, blood and breath samples were collected before (-10 and 0 min) and during (140 , 160 and 180 min) leucine infusion. Blood was placed on ice and plasma was separated and stored at -80°C . KIC was extracted from plasma as described by Nissen *et al.* (25). Plasma KIC enrichment with ^{13}C was measured by gas chromatography–mass spectrometry (MSD 5971A, model 5890, Hewlett-Packard Co., Palo Alto, CA, USA). CO_2 enrichment with ^{13}C in breath samples was measured at the University of Surrey, United Kingdom, on a Delta Plus XP isotope ratio mass spectrometer fitted with a Gas Bench II inlet system (Thermo Fisher Scientific, Hemel Hempstead, UK). The CV for LRA and Lox in our study based on seven subjects studied on a second occasion after a median of 34 days were 3.5 and 6.1% respectively (26).

Indirect calorimetry Whole-body energy expenditure and substrate oxidation were measured by indirect calorimetry. This involved using an open-circuit ventilated hood system (ParvoMedics, Sandy, UT, USA), calibrated against standard gases before each study. The participants rested on a bed for at least 30 min. A clear plastic hood was placed over their head for a 20-min period. The measurements were taken during two 20-min periods and averaged.

Assays All samples for each individual were measured in the same assay run for each analyte. The concentration of serum IGF1 was measured using RIA after acid ethanol extraction as described previously (18, 24, 27). The CV for IGF1 were 8.3% at 14.7 nmol/l and 7.4% at 28.6 nmol/l. The concentration of serum SHBG was measured by RIA using a commercial assay kit (Immulite 2000, Siemens Medical Solution Diagnostics, Los Angeles, CA, USA). In postmenopausal women, the normal range for SHBG concentration is $21\text{--}149$ nmol/l (RPAH, Australia). Testosterone, 17β -oestradiol (E_2), and DHT and its 3α -diol and 3β -diol metabolites were measured using LC–MS/MS, as described previously (28). The limit of detection for testosterone was 0.04 nmol/l, for E_2 3.4 pmol/l and for DHT 0.01 nmol/l. In cases where the level of E_2 was below the detection limit, the value was imputed as 0. The inter-assay CV for testosterone was 3.9–6.5%, for E_2 4.8–8.6% and for DHT 6.7–13.4%. The CV for SHBG at 5.3 and 86.2 nmol/l were 5.0 and 7.5% respectively.

Statistical analysis

Treatment effects were assessed using the paired *t*-tests. The results are expressed as means with S.E.M., and a *P* value of <0.05 was considered significant. Statistical

analysis was undertaken using the statistical software package SPSS statistics v20 (IBM corporation).

Results

The clinical, hormonal and metabolic indices obtained at baseline and during testosterone treatment are given in Table 1. Oral testosterone administration increased the levels of circulating testosterone from 0.4 ± 0.1 to 1.1 ± 0.2 nmol/l ($P < 0.05$; Fig. 1). The mean level of DHT along with that of its 3α -diol and 3β -diol metabolites (data not shown) did not change significantly during testosterone administration. The level of E_2 was below the detection limit in most of the samples and did not change during testosterone administration. The mean level of SHBG decreased slightly but significantly during oral testosterone treatment by 8.8 ± 2.5 nmol/l ($P < 0.01$). All the changes in hormone levels occurred within the normal range for women of reproductive age.

Oral testosterone treatment significantly increased the levels of IGF1 by $18.4 \pm 7.7\%$ ($P < 0.05$). The change in the levels of IGF1 did not significantly correlate with any of the parameters measured. Neither serum urea, creatinine and albumin levels nor ALT, AST and GGT levels changed significantly with oral testosterone administration.

Energy expenditure and substrate metabolism

In the post-absorptive state, oral testosterone administration did not significantly affect resting energy expenditure and carbohydrate and fatty acid oxidation in healthy postmenopausal women.

Table 1 Clinical, hormonal and metabolic measures at baseline and during oral testosterone treatment. Data are presented as means $\pm$ S.E.M.

	Baseline	Testosterone	<i>P</i> value
Weight (kg)	69.5 ± 4.5	69.6 ± 4.6	0.75
Testosterone (nmol/l)	0.4 ± 0.1	1.1 ± 0.2	<0.05
SHBG (nmol/l)	47.7 ± 8.1	38.9 ± 6.4	<0.01
DHT (nmol/l)	0.3 ± 0.1	0.4 ± 0.1	0.59
Oestradiol (pmol/l)	1.5 ± 1.5	6.5 ± 3.6	0.11
IGF1 (nmol/l)	12.6 ± 1.1	14.8 ± 1.3	<0.05
REE (kcal/day)	1182 ± 57.7	1105 ± 40.8	0.11
Fox (mg/min)	51.3 ± 5.4	44.3 ± 4.5	0.10
Cox (mg/min)	43.5 ± 9.0	47.0 ± 11.4	0.67
Urea (mmol/l)	5.2 ± 0.5	5.3 ± 0.5	0.90
Creatinine ($\mu\text{mol/l}$)	65.1 ± 3.0	65.7 ± 3.7	0.75
Albumin (g/l)	39.9 ± 0.6	41.1 ± 0.5	0.13
ALT (IU/l)	16.1 ± 2.0	17.6 ± 1.7	0.51
AST (IU/l)	21.9 ± 0.7	21.9 ± 1.7	1.00
GGT (IU/l)	22.5 ± 5.5	18.2 ± 3.7	0.06

REE, resting energy expenditure; Fox, fat oxidation; Cox, carbohydrate oxidation.

Figure 1 Circulating testosterone levels at baseline and at the end of the 2-week treatment period with oral unesterified testosterone (40 mg/day). Dotted line represents the upper normal limit of circulating testosterone levels measured by LC–MS/MS in women (36, 37, 38). Data are expressed as means $\pm$ S.E.M.

Protein metabolism

Oral testosterone administration significantly reduced the LRA, a measure of protein degradation, by $7.1 \pm 2.5\%$ compared with baseline ($P < 0.05$; Fig. 2A). Net Lox, a measure of irreversible loss of protein, was significantly reduced by $14.7 \pm 4.5\%$ ($P < 0.05$; Fig. 2B).

Next, we next analysed the data to determine whether the intervention induced a significant reduction in leucine oxidized as a proportion of LRA, representing an inverse measure of protein synthesis. This proportion was reduced by testosterone treatment, by $8.1 \pm 4.0\%$, with the difference approaching significance ($P = 0.07$).

Discussion

We studied for the first time whether hepatic exposure to a low dosage of testosterone by oral delivery increases whole-body protein anabolism in healthy women. Oral testosterone administration resulted in a significant reduction in the rate of leucine appearance, an index of protein breakdown, and the rate of Lox, an index of irreversible loss of protein. The concentration of serum SHBG decreased, while those of testosterone and IGF1 increased, all within the physiological range. There was no significant change in energy expenditure and fat and carbohydrate utilization. The concentrations of circulating liver transaminases were unaffected by testosterone treatment. Thus, physiological androgen exposure of the liver to orally administered unesterified testosterone induced net protein anabolism by reducing protein breakdown and oxidation without inducing peripheral androgen excess in women.

The liver is a major site of protein metabolism and a principal site where testosterone interacts with GH to promote anabolism (17). The mechanism for this is unknown, but it may involve nitrogen recycling, which

is regulated by the liver. It has been demonstrated that in humans a reduction in urea synthesis is accompanied by an increase in amino acid uptake in muscle (29). Hepatic urea formation is suppressed by GH administration in healthy men and in GH-deficient patients, providing at least partly the mechanism for the anabolic effect of GH (30, 31, 32). We propose that the reduction in oxidative metabolism of protein estimated from whole-body leucine turnover in this study represents the reduction in urea synthesis, partitioning nitrogen away from disposal. testosterone reduced Lox; therefore, reduction in hepatic urea production may be a determining factor underlying the whole-body anabolic effect. This is supported by the observation that testosterone supplementation reduces blood urea levels (33). In this study, we did not observe a significant decrease in the concentration of urea; however, this may be due to the small sample size. Taken together, we propose that the liver is a critical site for the regulation of whole-body protein metabolism and that hepatic exposure to testosterone may shift the partitioning of nitrogen towards protein synthesis, which merits further investigation.

To gauge the significance of changes in the indices of whole-body leucine metabolism, we compared the magnitude of change with those observed during androgen deprivation and replacement. In a study of normal men in whom hypogonadism was induced by a GnRH antagonist, protein synthesis decreased by $\sim 13\%$

Figure 2 Effects of testosterone on whole-body protein turnover. (A) Leucine rate of appearance (LRA), a measure of protein breakdown, and (B) net leucine oxidation (Lox), a measure of irreversible loss of protein. Data are expressed as means $\pm$ S.E.M.

and was accompanied by a reduction in fat-free mass of 2 kg (34). In a study of hypopituitary men, testosterone replacement at standard doses by i.m. injection reduced Lox by 28%, when compared with GH replacement, which reduced it by 15% (18). In our recent study of GH-replaced hypopituitary men, oral testosterone administration reduced Lox by 16% (17). In the present study, the reduction in protein oxidation in postmenopausal women by oral testosterone of ~15% is of similar magnitude. As oxidation represents the irreversible loss of protein, a reduction equates the net anabolism of protein. We have previously observed that a reduction in protein oxidation of 14% was accompanied by a 2.2 kg gain of lean body mass (LBM) after 12 weeks of GH replacement (26). Considering that leucine represents 8% of whole-body protein or 590 $\mu\text{mol/g}$ protein, the reduction in protein oxidation $\sim 5.7 \pm 1.6$ mg/min. If this reduction in protein loss is maintained over the long term, oral testosterone supplementation theoretically translates into a gain of 3 kg protein mass over 1 year.

Testosterone treatment is usually delivered parenterally by injection of testosterone esters or transdermal gel or patch delivery of unesterified testosterone and not by the oral route apart from oral testosterone undecanoate. When testosterone or its esters are administered orally, they are rapidly metabolized in the gut before reaching the liver via the portal circulation. Testosterone undecanoate is absorbed mainly through the lymphatic system with low bioavailability. Thus, a substantial fraction bypasses the liver, elevating the levels of testosterone in peripheral blood. Large doses (over 250 mg vs endogenous production rate of 0.25 mg/day in women) are required to overcome hepatic metabolism to achieve physiological levels in peripheral blood required for androgen replacement therapy (35). Our testosterone formulation does not bypass the liver and hepatic exposure is achieved without peripheral androgen excess, thereby potentially extending anabolic testosterone therapy to women in whom systemic effects of testosterone are undesirable.

We used an oral testosterone dose that was similar to that used in an earlier study in hypogonadal men, in whom the levels of circulating testosterone did not increase after administration of a 40 mg daily dose orally (17, 20). In the present study carried out in postmenopausal women, we observed a small but significant increase in the levels of circulating testosterone, reaching 1.1 ± 0.2 nmol/l. Studies assessing the levels of testosterone in women of reproductive age by LC-MS/MS have reported the levels of circulating testosterone to be in the range of 0.1–2.8 nmol/l, with median levels of 0.9 nmol/l (36, 37, 38). Thus, this low-dose oral regimen did not cause androgen excess in peripheral blood. There was a small but significant reduction in the levels of SHBG within the normal range for postmenopausal women, consistent with a local hepatic effect of mildly elevated levels of testosterone in

the portal circulation. The significance of these changes in androgen indices is unknown. Testosterone replacement in women with low levels of androgen has been studied, showing benefits on mood, sexual function, body composition and general health (7, 39). Thus, it is conceivable that the modest increase in the levels of circulating testosterone may provide some benefits to postmenopausal women during therapy with this oral formulation of low-dose unesterified testosterone.

The reduction in Lox in postmenopausal women by oral testosterone delivery was accompanied by a small increase in the mean levels of circulating testosterone of 0.65 nmol/l. In hypogonadal men, systemic testosterone replacement that elevated the levels of circulating testosterone by more than 15 nmol/l did not exert an additional protein anabolic effect over and above that achieved by oral testosterone administration with solely hepatic exposure (17). Thus, the liver plays a crucial role in the modulation of protein turnover, and the small increase in the concentration of testosterone in peripheral blood in this study is unlikely to have reduced leucine turnover and oxidation rates by 15%. This study provides evidence that sole hepatic exposure to testosterone can be developed as a novel, safe and cost-effective treatment for frailty in both men and women.

We observed an 18% increase in the levels of IGF1, which may have contributed to the anabolic effect of the liver-targeted testosterone administration. This finding is in agreement with those of our study in hypopituitary men, in whom during GH replacement administration of oral testosterone significantly increased the levels of circulating IGF1 by 16% (17). In oophorectomized women, systemic testosterone administration results in a significant increase in the levels of IGF1, at the same time reducing the secretion of GH (40). This observation suggests that there is a direct effect of testosterone on IGF1 production, which is not centrally mediated. The liver is the principal source of circulating IGF1; however, there is also a substantial local production in other tissues, such as muscle. In muscle, testosterone locally potentiates the effect of GH on muscle *IGF1* gene expression (41, 42). However, this mechanism is probably not responsible in this study as the increase in the levels of circulating testosterone was minimal. Therefore, we speculate that orally administered testosterone may potentiate GH receptor signalling in the liver, in this way increasing hepatic IGF1 production. To our knowledge, studies investigating the direct effects of testosterone on hepatic IGF1 production have not been published. Thus, there is emerging evidence that the liver may be a central site of interaction between testosterone and GH signalling.

This study has some limitations. A weakness is the small sample size and that it was not blinded or placebo controlled. Nevertheless, this study is a proof of principle that in healthy women small doses of liver-targeted

testosterone administration significantly reduce protein loss after only 2 weeks of administration. The results are unlikely to occur by chance since changes in the components of protein turnover (LRA – 7.1% and Lox – 14.7%) exceed those of the reproducibility of the method (CV for LRA and Lox 3.5 and 6.1% respectively).

In summary, LRA and oxidative loss were reduced by testosterone administration in healthy postmenopausal women. The levels of circulating testosterone stayed within the normal range for postmenopausal women. We conclude that oral testosterone administration of a physiological dose stimulates protein anabolism by reducing protein breakdown and oxidation without inducing peripheral androgen excess in women. Testosterone administration via the oral route may open new treatment strategies for frail elderly men and women.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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