Uncarboxylated Osteocalcin Stimulates 25-Hydroxy Vitamin D Production in Leydig Cell Line Through a GPRC6a-Dependent Pathway

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Recent studies disclosed a cross talk between testis and bone. By the action of LH, Leydig cells are able to modulate bone metabolism through testosterone and insulin-like factor 3. Moreover, LH modulates the Leydig expression of CYP2R1, the key enzyme involved in vitamin D (Vit D) 25-hydroxylation. However, pathways regulating CYP2R1 expression have been poorly investigated. The cross talk from the bone to the testis of the vitamin D 25-hydroxylase CYP2R1 involves osteocalcin (OC), which is produced by the osteoblasts and stimulates the production of testosterone by the Leydig cells through its putative receptor GPRC6A, a cation-sensing G-protein-coupled receptor. The aim of this study was to investigate the possible action of OC on CYP2R1 expression and 25-hydroxy Vit D (25-OH Vit D) production in a mouse Leydig cell line (MA-10). After confirmation of the expression of GPRC6A by MA-10, we found that stimulation with either human chorionic gonadotropin or uncarboxylated-OC (ucOC) increases CYP2R1 protein expression in a dose-dependent manner and, in turn, increases the release of 25-OH Vit D in culture medium. This effect was abolished by receptor blockade with, respectively, anti-LH receptor and anti-GPRC6A antibodies. Moreover, both agonists converged to phosphorylation of Erk1/2 by a likely differential action on second messengers. Human chorionic gonadotropin induced slow “tonic” increase of intracellular calcium and accumulation of cAMP, whereas ucOC mainly induced phasic increase of cell calcium. Supporting these findings, we found that serum ucOC positively correlated with 25-OH Vit D levels in 40 overweight male patients and 21 controls. Altogether, our results suggest that OC contributes with LH to 25-OH Vit D production by Leydig cells. (Endocrinology 155: 4266–4274, 2014)
whose major known endocrine role is the regulation of the transabdominal phase of testicular descent by action on the gubernacula (5). Recent evidence from our group demonstrated that the disruption of INSL3 pathway in both humans and animal model is associated with low bone mass phenotype and osteoporosis caused by functional alterations in the osteoblasts causing little bone formation, little mineralizing surface, and ultimately, a negative balance between bone formation and bone resorption (6, 7). The third modulation pathway involves the 25-hydroxylation of vitamin D (Vit D) (8). Vit D is a master regulatory factor of bone mineralization and calcium homeostasis, and it requires a 2-step activation process through sequential hydroxylation at positions 25 and 1 (9). Data from human and animal models clearly showed that CYP2R1 (microsomal vitamin D 25-hydroxylase) is the major enzyme involved in Vit D 25-hydroxylation (10–12), it is highly represented in the Leydig cells of the testis, and its expression is under the control of LH (13). According to this model, clinical conditions with testicular damage are frequently associated with reduced 25-hydroxy Vit D (25-OH Vit D) serum levels together with increased serum PTH and increased risk of osteopenia and osteoporosis (8, 13).

On the other hand, osteocalcin (OC), a small protein secreted by bone-forming osteoblast, showed ability to modulate Leydig function, such as the production of T (14). In this regard, posttranslational γ-carboxylation of glutamate residues of OC seems to strongly influence its biological activity, because only uncarboxylated-OC (ucOC) lacking of γ-carboxylation of glutamate residues at position 17, 21, and 23 displayed modulating activity on the Leydig cells (14, 15). The receptor that mediates the activity of OC is probably the G protein-coupled receptor GPRC6A, highly expressed in Leydig cells as well (14, 16). Interestingly, Pi et al (17) found that GPRC6A-deficient mice had a number of bone/metabolic derangements that were likely related to the observed impaired steriodogenesis. In fact, GPRC6A−/− mice showed defective mineralization of bone and impaired osteoblast function, glucose intolerance, and metabolic syndrome together with reduced levels of T (17). Although no significant reduction of 1,25-di-hydroxy Vit D levels were found, the authors did not investigate any possible involvement of 25-hydroxylation of Vit D in this complex phenotype.

In order to better understand the hormonal pathways related to GPRC6A in Leydig cells, in this study, we investigated the modulation of both CYP2R1 expression and 25-OH Vit D production by OC in MA-10 cell line, a well established in vitro model of Leydig cells. Moreover, we verified the existence of this hormonal pathway in vivo by evaluating the relationship between circulating levels of ucOC and 25-OH Vit D in a cohort of obese men and age-matched controls.

### Materials and Methods

#### OC decarboxylation

Decarboxylation of γ-carboxyglutamamic residues of human (h)OC was performed (following an established protocol) as reported elsewhere with minor modifications (18). Briefly, an aliquot of about 60 µg of human carboxylated-OC (cxOC) was freeze dried, resuspended in 200 µL of 50mM HCl in a glass vial, and lyophilized 1 more time through repeated cycles of nitrogen and vacuum. The vial was then placed in a vacuum oven at 110°C for 24 hours. After incubation, the lyophilizate was resuspended in 0.1% trifluoro-acetic acid-H2O and analyzed by reverse phase chromatography on a C18 analytical column with a linear gradient of CH3CN-CN-0.078% trifluoro-acetic acid from 20% to 60% in 30 minutes. Mass spectrometry analysis of the eluted species confirmed the decarboxylation of all amino acid residues γ-glutamatic acid present in human carboxylated osteocalcin and showed a degree of purity of the sample greater than that of the starting product (Supplemental Table 1).

#### Cell culture

The clonal strain of mouse MA-10 Leydig cell line was generously provided by Mario Ascoli from the University of Iowa College of Medicine and was handled as described previously (19). Briefly, cells were seeded on 0.1% gelatin-coated plastic-ware and maintained in DMEM/F12 medium (pH 7.7) (GIBCO-Invitrogen), supplemented with 20mM HEPES, 15% horse serum, and 50-µg/mL gentamicin.

For stimulation experiments, 60% confluence cells were starved in serum-free medium for 24 hours then exposed for 48 hours to human chorionic gonadotropin (hCG) (Gonasi, IBSA Farmaceutici Italia Srl), human cxOC (Bachem), ucOC at various concentrations in serum-free medium with the addition of cholecalciferol (Sigma-Aldrich), and the 25-OH Vit D precursor, at the concentration of 10 mg/mL.

In receptor blockade experiments, before the application of the stimulus with hCG or OC, cells were incubated for 30 minutes at 37°C with saturating concentration of rabbit anti-hLH receptor (50 µg/mL; Millipore Corp) or rabbit anti-hGPRC6A (20 µg/mL, H-300; Santa Cruz Biotechnology, Inc) and then stimulated as described above. Specificity of antibody blockade was verified by coinoculation with isotype-rabbit IgG (20 µg/mL; Santa Cruz Biotechnology, Inc). Scraping-harvested cells and supernatants were then stored at −80°C for further analysis. Experiments were performed 3 times in triplicate.

#### Immunofluorescence

MA-10 cells seeded onto glass slides (BD Biosciences) were fixed with 4% paraformaldehyde/PBS solution for 15 minutes at room temperature. When necessary, cells were permeabilized with 1% Triton X-100/PBS solution for 10 minutes at room temperature. Subsequently, samples were saturated with 5%BSA/5% normal donkey serum in PBS for 30 minutes and then incubated overnight at 4°C with rabbit anti-hGPRC6A (2 µg/mL; Santa Cruz Biotechnology, Inc) or rabbit anti-hINSL3 (2.5 µg/mL; Phoenix Pharmaceuticals Burlingame). In negative control, primary antibodies were omitted. The day after, primary immunoreaction was detected by incubation with biotin-conjugated secondary antibody followed by Streptavidin-Texas Red (both 1:200; Santa Cruz Biotechnology, Inc). Finally, cells were
counterstained with diamidino-phenylindole, mounted with antifade buffer, and analyzed with video-confocal (VICO) fluorescence microscope (Nikon). Results are representative of 3 independent experiments.

**Western blotting**

Protein extraction was performed by physical procedure (freeze-thaw cycles in liquid nitrogen followed by a 37°C water bath) into lysis buffer (Bio-Rad) containing protease inhibitor. Total protein content was assessed by determination of optical density at 280 nm with Nanodrop ND-1000 Spectrophotometer (Thermo Fisher). Samples were denatured with sodium dodecyl sulphate and 2-β-mercaptoethanol, boiled for 10 minutes, and then fractionated using SDS-PAGE gel (Bio-Rad). After blotting onto Hybond ECL Nitrocellulose Membrane (PerkinElmer) and blocking with 5% nonfat milk in 0.1% PBS-Tween 20 (Bio-Rad), blots were incubated overnight at 4°C with the goat anti-hCYP2R1 antibody (sc-48985; Santa Cruz Biotechnology, Inc) and visualized using enhanced chemiluminescence reagent (PerkinElmer) with the Chemidoc XRS System (Bio-Rad). Quantification of band densities was performed as described above. Results are representative of 3 independent experiments in triplicate.

**Intracellular calcium concentration measurement**

For intracellular calcium measurement, MA-10 cells were grown on round Petri dishes and underwent overnight starvation before experimentation. Cells were then loaded with fluorescent calcium indicator Fluo-4 (Molecular Probes, OR, USA) at the final concentration of 10μM for 30 minutes at 37°C in serum-free medium added with 2-ml Pluronic Acid (Invitrogen) and 250mM Sulfipyrazonyzine (Sigma-Aldrich) as improved dye loading reagents. Fluorescence was monitored with an Olympus IX 81 inverted microscope by recording and emission signal at 516 nm at 3-second intervals exciting at 494 nm. Cellular vitality was evaluated adding 200nM Ca ionophore (A23187; Fluka). Experiments were performed 3 times in triplicate.

**Intracellular CAMP concentration measurement and Erk1/2 phosphorylation**

MA-10 cells were cultured as described above in 6-well plates at the cell density of 10⁵ cells per well grown 48 hours followed by overnight incubation serum-free medium. Starved cells were then treated with ucOC, cxOC, or hCG for 20 minutes at 37°C as previously described. A total of 100nM forskolin was employed as positive control for CAMP quantification. In receptor blockade conditions, before stimulation with agonists, cells were incubated for 30 minutes at 37°C with saturating concentration of rabbit anti-hLH1 receptor or rabbit anti-hGPRC6A as described above. A 6-well plate of cells was cultured for each stimulating condition: 3 wells were devoted to CAMP quantification, by lysing cells with 0.5-ml 0.1N HCl, and 3 wells were devoted to protein extraction as already described. CAMP levels were measured by using a cAMP enzyme immunoassay kit (Cayman Chemical) under the manufacturer’s protocol. Erk1/2 phosphorylation was evaluated by the use of anti-ERK1/2 and antiphospho-ERK1/2 rabbit primary antibodies from Cell Signaling Technology. Quantification of band densities was performed as described above. Results are representative of 3 independent experiments in triplicate.

**Study subjects**

We recruited 40 consecutive male patients featured by body mass index (BMI) ≥ 25 kg/m² (mean age ± SD, 42.0 ± 12.6 y) between August 1, 2006 and June 30, 2009, referred to the Center for the Study and the Integrated Treatment of Obesity of the University of Padova, and 21 age-matched male healthy controls (mean age ± SD, 39.3 ± 10.3 y; 18.5 ≤ BMI ≤ 24.9 kg/m²). Nobody had hepatic or renal failure. All subjects were free from drugs known to influence bone and calcium metabolism (ie, steroids, Vit D and Vit K, calcitonin, biphosphonates, and thiazolidinediones). All patients underwent physical examination, including anthropometric measurements and blood pressure measurement, and a venous blood sample was collected after overnight fasting and stored at −80°C after plasma separation. All subjects gave an informed consent to the study, which have been approved by the local Ethical Committee. The investigation was conformed to the principles of the Declaration of Helsinki.

**Hormone assays**

Total T and 17-β-estradiol (E2) were evaluated by commercial electrochemiluminescence immunoassay methods (Elecsys 2010; Roche Diagnostics). 25-OH Vit D was determined with direct, competitive chemiluminescent immunoassay (LIAISON 25 OH Vitamin D TOTAL Assay; DiaSorin, Inc). All determinations were performed according to manufacturer’s instructions. cxOC and ucOC were measured in blood serum and culture supernatants by ELISA according to the manufacturer’s instruction (Takara). All determinations were performed in duplicate. Total OC (t-OC) was estimated as the sum of cxOC and ucOC. The portion of ucOC within the total circulating pool of OC was defined as the ratio between ucOC and t-OC (ratio).

In the analysis of culture supernatants, in order to better express the release of 25-OH Vit D and T in relationship with cellularity of each sample, concentration was normalized for total protein concentration of cell pellets.

**Statistical analysis**

SPSS 13.0 (IBM Corp) was used to perform statistical analysis. Differences among protein band densities and hormone release in each experimental condition were evaluated by ANOVA test and Bonferroni correction. Comparisons between serum parameters were made using Mann-Whitney U test. Relationships between continuous variables were assessed using nonparametric Spearman’s ρ correlation test. Multiple stepwise regression analysis was performed to determine the associations between serum 25-OH Vit D and OC, BMI, T, steroid hormone-binding globulin (SHBG), and E2 concentration after adjusting for potential confounders. The significance level was set to P = .05. Variables are given as mean ± SEM.
Expression of GPRC6A in MA-10 cell line

The molecular phenotype of MA-10 cell line was confirmed by the expression of INSL3 by immunofluorescence (Supplemental Figure 2). The expression of GPRC6A by MA-10 cells was evaluated by Western blot analysis, which revealed a specific band of the expected molecular mass of about 100 kDa (Figure 1A). A further confirmation was obtained by immunofluorescence on fixed cultured cells that showed signal for GPRC6A mainly on the cell surface (Figure 1B).

Modulation of CYP2R1 expression by OC

The ability of OC to modulate CYP2R1 protein expression was assessed by Western blot analysis (Figure 2). hCG was employed as a reference control (13) and modulated CYP2R1 expression in a dose-dependent way. Both fully cxOC and ucOC were employed to stimulate MA-10 for 48 hours at a concentration ranging from 1 to 10 ng/mL. Compared with the untreated control, cxOC induced a slight increase in CYP2R1 expression only at the highest concentration of 10 ng/mL. On the contrary, ucOC stimulated the expression of CYP2R1 even at 1 ng/mL, with a progressively increasing effect at the highest concentrations of 3 and 10 ng/mL.

Figure 1. A, Western blot analysis of GPRC6A expression in MA-10 mouse Leydig cell line. In control lane (CTRL), primary antibody was omitted. B, Immunofluorescence analysis of GPRC6A (red) expression in MA-10 cell line. Cells were counterstained with DAPI (blue). In the negative control (CTRL), primary antibody was omitted.

Figure 2. Western blot assessment of CYP2R1 protein expression in MA-10 cell line after stimulation with hCG or cxOC or ucOC at different concentrations for 48 hours. Data are reported as the ratio between the band density of CYP2R1 with the corresponding band density of β-actin as internal control. Results are the mean of the independent experiments. *, P < .05.
25-OH Vit D production by MA-10 cells stimulated with OC

In order to evaluate whether there was a correspondence between CYP2R1 expression and Vit D hydroxylation or not, we assessed the release of 25-OH Vit D in culture supernatants after 48 hours of hormonal stimulation (Figure 3A). Only ucOC increased the release 25-OH Vit D in cultured medium at all concentrations tested, as was the reference control hCG. The overall responsiveness of the in vitro model was assessed by measuring T released in culture medium (Figure 3B). Both hCG, at the concentration of 10 and 50 ng/mL, and ucOC were able to increase T release in culture medium. This effect was not evidenced after simulation with fully cxOC.

The next experimental session was performed to assess whether 25-OH Vit D production by Leydig MA-10 cells induced by ucOC stimulation was the result of downstream LH-dependent pathway or was actually only GPRC6A dependent (Figure 4). The production of 25-OH Vit D under hCG stimulation was strongly reduced after blockade of hCG receptor by saturation with specific polyclonal antibody. This effect was only partially reversed by stimulation with ucOC. On the other hand, specific blockade of GPRC6A by polyclonal antibody blunted 25-OH Vit D production induced by ucOC stimulation but was almost completely reversed by stimulation with hCG (Figure 4A). The dosage of T in culture supernatant showed a very similar behavior, suggesting that hCG/LH promotes Vit D 25-hydroxylation in an OC-independent manner (Figure 4B). The overall blockade pattern was not significantly affected by coinoculation with isotype-rabbit IgG, confirming the specificity of blocking antibodies (Supplemental Figure 3).

Profiling of OC signaling pathway in MA-10 cells

In order to evaluate the signaling pathway associated to OC stimulation in MA-10 cells, the involvement of main secondary messengers was investigated.

An evaluation of intracellular calcium trafficking was performed by the use of the specific intracellular probe Fluo-4 (Figure 5A). The overall cell responsiveness was tested by the use of the calcium ionophore A23187 that provoked a very fast and transient increase of intracellular calcium concentration. Interestingly, ucOC, used at the most active concentration of 10 ng/mL, induced a phasic calcium increase with a peak intensity within the first 240 seconds from the application of stimulus. This effect was not significantly blunted by the blockade with anti-LH receptor antibody but was almost abolished by saturating anti-GPRC6A antibody. Conversely, 10-ng/mL hCG induced a progressive slow increase of intracellular calcium without reaching a peak event. Such a tonic calcium increase was not perturbed by blockade with saturating anti-GPRC6A antibody but was blunted by LH receptor blockade.

Subsequently, the quantification of intracellular cAMP levels was performed (Figure 5B). Cell viability and responsiveness was tested with 100nM forskolin. As expected, stimulation of LH receptor signaling with hCG induced a significant increase of intracellular cAMP concentration, which was blunted only by LH receptor blockade. On the contrary both ucOC and cxOC were not able to induce any variation of cAMP levels.

Figure 3. Evaluation of the release of 25-OH Vit D (A) and T (B) from MA-10 cell line after stimulation with hCG or cxOC or ucOC at different concentrations for 48 hours. Data are normalized as pmol of substance per mg of DNA of the corresponding cell pellet. Results are the mean of the independent experiments. *, P < .05.
Finally, involvement of downstream Erk1/2 phosphorylation was assessed (Figure 5C). Both ucOC and hCG were able to induce a significant increase of phospho-Erk1/2 levels with no effect on total Erk1/2. Such a phenomenon was blunted by blockade of, respectively, GPRC6A and LH receptor. cxOC did not induce any significant variation of either phospho-Erk1/2 or total Erk1/2.

In vivo correlation between OC and 25-OH Vit D

The hormonal characteristics of subjects, according to different BMIs, are summarized in Table 1. As expected, overweight/obese patients showed significantly lower total T, SHBG, 25-OH Vit D, and ucOC and higher levels of E2 compared with controls, in agreement with previously published studies (20, 21). The prevalence of glucose intolerance (defined as fasting glucose between 110 and 125 mg/dL) and diabetes (defined as fasting glucose > 126 mg/dL) was significantly higher in group of overweight/obese patients as well.

In the whole cohort, serum levels of 25-OH Vit D negatively correlated with BMI ($\rho = -0.351; P = .003$), cxOC ($\rho = -0.268; P = .018$), and E2 ($\rho = -0.234; P = .035$). On the contrary, positive correlation was observed with total T ($\rho = 0.365; P = .002$), SHBG ($\rho = 0.251; P = .025$), ucOC ($\rho = 0.215; P = .048$), and the ucOC/t-OC ratio ($\rho = 0.325; P = .005$). Furthermore, multiple stepwise regression analysis was performed to determine the associations between serum levels of 25-OH-Vit D and OC, BMI, T, SHBG, E2, glucose intolerance, and diabetes after adjusting for potential confounders. As shown in Table 2, total T levels and ucOC/t-OC ratio showed to be independent predictors of serum 25-OH Vit D (total T: $\beta = 0.329$, $t = 2.802; P = .007$; and ucOC/t-OC ratio: $\beta = 0.284$, $t = 2.413; P = .019$).

Discussion

In this study, we confirmed the role of Leydig cells in Vit D 25-hydroxylation and provided novel evidence of the possible modulation of Vit D 25-hydroxylation by OC.

Recently, the importance of the testis in 25-OH Vit D production received increasing support (8, 13, 22–24). In fact, inactivating mutations of the CYP2R1 gene in human models have been primary associated with 25-OH Vit D deficiency and altered bone status, ascribing to CYP2R1 a key role in Vit D 25-hydroxylation (21). Moreover, tests, and especially the Leydig cells, has the highest CYP2R1 expression both at transcriptional and translational levels (23, 24). Finally, recent studies from our group evidenced that clinical conditions affecting testis function may lead to severe impairment of bone metabolism through reduced expression of CYP2R1 and therefore reduction of 25-OH Vit D serum levels (8). Furthermore, we suggested that CYP2R1 expression in Leydig cells is under LH control, as demonstrated also by normalization of 25-OH Vit D levels after 3 months of treatment with hGH in patients with hypogonadotropic hypogonadal (13). Here, we completed the missing piece of the puzzle showing that Leydig cells are able to basally secrete 25-OH Vit D and that its
production is further stimulated by hCG in a dose-dependent manner.

The role of bone as an endocrine organ through the secretion of OC is currently under debate. OC undergoes to different degrees of \( \gamma \)-carboxylation of its glutamate residues at positions 17, 21, and 23, and it has been shown that the uncarboxylated form achieves an overall gain in bioactivity (15). Indeed, OC is thought to mediate a number of metabolic and hormonal effects acting on its putative receptor GPRC6A (16), which is expressed in several tissues with very few exceptions (25). Accordingly, low levels of circulating OC have been associated with altered metabolic/hormonal parameters subtending incoming pathological condition in human models. Díaz-López et al (26), in a prospective study on 153 newly diagnosed diabetic subjects and 306 matched controls, observed that OC levels were inversely and significantly associated with Homeostasis Model Assessment index in cases of incident diabetes, and with fasting glucose concentrations in control subjects, independently from other relevant confounders. In addition, the odds ratios for diabetes incidence in the lowest vs the highest tertile of cxOC and ucOC were 2.03 and 1.88, respectively. Furthermore, the association between OC and total T in the general population emerged from a population-based epidemiological Study of Health in Pomerania (27). Multivariable analysis on 1338 men confirmed the positive association between OC and total T even after adjustment for age, BMI, and other confounding factors. Here, we show that OC, in particular ucOC, stimulates 25-hydroxylation of Vit D in Leydig cells through a direct effect on the expression of the main actor in the 25-hydroxylase activity of Vit D, the CYP2R1 protein. This observation prompted us also to investigate on the interdependency of LH and OC pathways on 25-OH Vit D production by means of experiments in which specific receptor blockade was performed. When
GPRC6A was blocked by specific antibody, 25-OH Vit D production by ucOC was abolished. This effect was not observed when cells were stimulated with ucOC in course of LH receptor blockade with specific antibody. In the reversed situation, 25-OH Vit D production by hCG stimulation was also abrogated only by LH receptor blockade. Interestingly, our results somehow resemble those reported in a recent study by Oury et al (28), aimed to distinguish between LH-dependent and OC-dependent regulation of male fertility and performed in an in vivo murine model. In that study, on one hand, treatment of 6-week-old OCN−/− or GPRC6A−/− male mice with hCG for 1 month induced a normalization of testes weight, seminal parameters, and T circulating levels. On the other hand, the fact that daily injections of ucOC in 6-week-old LH-deficient male mice did not normalize circulating T levels led the authors to suggest that LH promotes T biosynthesis in an OC-independent manner. Together with the results of the present study, it seems that both LH and OC contribute to 25-OH Vit D production by Leydig cells through 2 independent pathways. In fact, data on cell signaling showed that, despite stimulation with either hCG or ucOC converged to phosphorylation of Erk1/2, the 2 agonists acted differentially on second messengers. In particular, hCG induced slow “tonic” increase of intracellular calcium together with accumulation of cAMP, in agreement with the finding that steroidogenesis is mediated by LH-through cAMP-mediated increase of intracellular calcium from internal stores (ie, endoplasmic reticulum) (29). On the other side, ucOC mainly induces phasic increase of intracellular calcium but seemed to had no effect on intracellular cAMP levels. To this regard, as reported for other mammalian cell models (30), we could speculate that GPCR6A might couple with the Gq protein to activate phospholipase Cγ, increasing intracellular inositol trisphosphate and subsequently intracellular calcium. Confirming this hypothesis, the primary involvement of Gq protein for GPRC6A responses has also been demonstrated by Jacobsen et al (31).

The recent availability of data from epidemiological studies provides a likely link between OC and 25-OH Vit D status in human models. In fact, in a study performed on 86 male subjects stratified for BMI, Migliaccio et al (21) observed that reduced levels of OC were associated with both low levels of 25-OH Vit D and T as soon as the BMI increases. Accordingly, here, we show that in a cohort of 61 male subjects with different BMIs, both serum levels of ucOC and the ucOC/t-OC ratio positively and significantly correlate with 25-OH Vit D levels, supporting the direct modulating effect of OC on Vit D hydroxylation in the testis. Although it is generally assumed that a decrease in serum 25-OH Vit D in obese subjects is mainly due to direct sequestration of Vit D in lipid drops of adipose tissue (32), a multiple stepwise regression analysis performed to determine the associations between serum 25-OH-Vit D and OC after adjusting for potential confound-

### Table 1. Biochemical and Hormonal Characteristics of the Patients According to Different BMI

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Values are expressed as means ± SD. The significance level was set to P = .05. Significant P values are in bold.

### Table 2. Regression Analysis

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Total T and ucOC/t-OC ratio are an independent variable from serum 25-OH Vit D. The significance level was set to P = .05. Significant P values are in bold.
ers showed that ucOC/t-OC ratio is independent predictors of serum 25-OH Vit D. Together with the low number of human subjects enrolled, the main limitation of this study is that results were shown in a cell line, and future experiments will be required to demonstrate their physiologic significance in vivo.

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