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Therapeutic Effects of Dehydroepiandrosterone Metabolites in Diabetes Mutant Mice (C57BL/KsJ-*db*/*db*)*

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ABSTRACT. Dehydroepiandrosterone (DHEA) fed at 0.4% in the diet is known to exert strong antihyperglycemic effects in C57BL/KsJ genetically diabetic (*db*/*db*) mice. Three of the major metabolic products of DHEA; DHEA sulfate, α-hydroxyetiocholanolone (α-ET), and β-hydroxyetiocholanolone (β-ET) when fed at 0.1% in the diet, and one putative product, 17β-estradiol, when fed at 0.005% also prevented the development of severe diabetes while having little effect on the amount of food eaten or the rate of weight gain. When suboptimal doses (5–20 μg/week) of estradiol were injected in combination with diets containing either α-ET or β-ET, marked potentiating effect was noted, normalization of the hyperglycemia being produced with as little as 0.025% of β-ET and 0.05% of α-ET. The ability of the etiocholanolones to maintain islet integrity and prevent the development of most diabetes symptoms suggests that these metabolites are not merely inactive end products of steroid metabolism, but are physiological effectors in their own right. (*Endocrinology* **115:** 239–243, 1984)

DEHYDROEPIANDROSTERONE (DHEA) and its sulfate derivative are major adrenal secretory products in humans, yet their biological functions remain unclear. Beneficial effects of DHEA fed in the diet to rodents have been reported with respect to tumor incidence (1) autoimmune diseases (2), obesity (3, 4), and diabetes (5, 6). The sulfate derivative of DHEA and possibly the free steroid can be converted to androgens in peripheral tissues (7) and to estrogen in the placenta (8), and may provide a major source of sex steroid synthesis in castrate and aged individuals. However, any recognized biological role for DHEA or DHEA-sulfate in normal individuals remains to be established (9).

The diabetic syndrome in C57BL/KsJ-*db*/*db* (diabetes) mice is characterized by obesity, chronic hyperglycemia, relative insulinopenia coupled with severe insulin resistance, extensive β-cell necrosis, and islet atrophy (10). Our studies using DHEA in treating this genetic form of diabetes have shown that the compound had hypoglycemic activity only when fed. When injected or incorporated into the diet using agar as a matrix, the expected hypoglycemic effects were not obtained (6). These data suggested that DHEA must be converted to some metabolite or metabolites, possibly within the gut lumen or gut cells in order to become effective. DHEA is recognized to be metabolized to four isomeric excretory products; two, androsterone (A) and epiandrosterone (EA) in which the double bond at the 5-position is reduced in the trans (5α) configuration and two, 3β-hydroxyetiocholanolone (β-ET) and 3α-hydroxyetiocholanolone (α-ET), in which the double bond is reduced in the cis (5β) configuration (9). DHEA and DHEA sulfate have both been shown to be precursors of estrogen (11, 12). We previously reported that estrogen is very potent in stabilizing and decreasing the severity of the diabetes in CBA/LtJ-*db*/*db* male mice (13) and we proposed that the effectiveness of the DHEA treatment may be related to an increased production of estrogens (6). These present studies compare the beneficial effects of DHEA with those produced with the major DHEA metabolites, DHEA sulfate, β-ET, and α-ET.

Materials and Methods

Animals

Diabetes (*db*/*db*) and normal (+/+) control male mice of the C57BL/KsJ strain were produced in our research colony. All mice were maintained three per cage in controlled environmental (23 C) and photoperiod (12-h light/day, lights on 0600 h) conditions. In each study, weanling mice were divided into groups of five to eight mutants, one group fed chow alone (Old Guildford diet 96) and the other groups fed powdered chow into

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which DHEA or its metabolites had been incorporated. The powdered diets were fed in food cups that were filled every second day. For studies involving food consumption, the powdered chow containing DHEA was repelleted in a hydraulic press (5000 pounds/sq. in.) and weighed amounts of these pellets were fed each day. Daily food consumption was determined by weighing the amount left after each 24-h period. All steroids were fed in the diet except E_2, which was either fed or injected sc in 50 μl volumes of peanut oil. β-ET, α-ET, and DHEA were obtained from Progenics Inc. (New York, NY). All other steroids were obtained from Sigma Chemical Co. (St. Louis, MO).

Analytical procedures

Mice were weighed weekly at the time of bleeding for determination of the blood sugar concentration. Plasma immunoreactive insulin (IRI) concentrations were undertaken periodically during the treatment period and at the time of termination of each experiment. After killing, the pancreas was removed, weighed, and half was fixed in Bouin's solution for subsequent histological study and morphometric analysis using an Optomax IV Image Analyzer System (Optomax, Hollis, NH). Hydrated 5-μm sections were stained with aldehyde fuchsin to detect β-cells. Islets for measurement were selected at random until data from 8–15 individual islets were accumulated per mouse. These were combined to give an average value for islet area and percent granulated β-cells. The other half of the pancreas was homogenized in acid-ethanol (1.5 ml concentrated HCl in 70% ethanol) to determine pancreatic insulin content. Blood glucose, IRI concentrations, and glucose tolerance tests were carried out as previously described (13). Data are expressed as mean ± SEM from groups of mice in the various treatment regimens. Statistical comparisons were calculated using Students' t test. Differences were considered significant at $P < 0.01$.

Results

Normal C57BL/KsJ littermates (+/?) treated with DHEA or metabolites at various concentrations in the diet and combined with E_2 (10 μg twice per week) caused no signs of toxicity, as indicated by normal activity and food consumption (3–4 g food/day·animal). Blood sugar concentrations and rate of weight gain were slightly decreased with diets containing 0.4% DHEA. Plasma and pancreatic insulin concentrations remained within the normal range and were not significantly different with any treatment regimens (Table 1). Morphological analysis typically revealed three to five small, well granulated islets in each section of the pancreases from treated normal mice. The size, distribution, and extent of β-cell granulation (70–85%) were not effected by any dietary treatment in normal mice (Table 1).

The rate of weight gain and blood sugar concentrations in mutants treated for 18 weeks with α-ET (0.1%), β-ET (0.1%), or DHEA (0.4%) are compared with those of

TABLE 1. Effects of treatment with DHEA and DHEA metabolites in normal C57BL/KsJ mice

Treatment	E_2 (10 μg 2 times/ week)	Plasma IRI (μU/ml)	Pancreatic insulin (U/g)	Granulated β-cells (%)
Chow alone	−	42.4 ± 0.95	3.11 ± 0.31	79.2 ± 1.1
Chow alone	+	56.7 ± 4.9	3.39 ± 0.79	74.1 ± 3.8
Chow + DHEA (0.4%)	−	31.4 ± 1.7	3.51 ± 0.28	70.3 ± 1.9
Chow + β-ET (0.05%)	+	48.2 ± 5.9	2.60 ± 0.40	74.7 ± 2.1
Chow + α-ET (0.05%)	+	28 ± 8.3	2.14 ± 0.17	84.1 ± 2.5

Littermate normal control C57BL/KsJ (+/?) mice were treated for 16 weeks. Values represent average ± SEM from three to six individual mice obtained at time of killing. No significant differences were seen in any parameter compared to those fed chow alone.

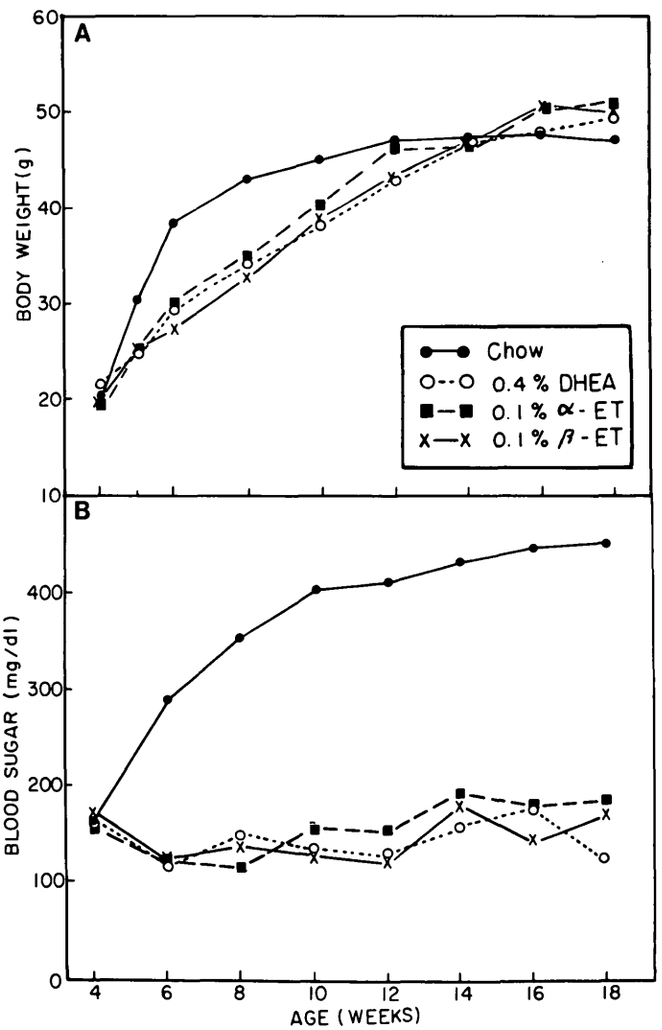

FIG. 1. A, Effect of dietary treatment on weight with age in C57BL/KsJ diabetes *db/db* mutants. B, Effect of dietary treatment on blood sugar concentrations with age. Data are based on five or six male mice per treatment. Values obtained from all treatment groups were significantly different from those values obtained from mutants fed chow alone.

mutants maintained on chow alone (Fig. 1). Obesity remained a consistent feature of all treated mutants. All treatments slowed the initial rate of weight gain when compared with chow-fed mutants (Fig. 1A). After 16 weeks all treated mice were still gaining weight whereas those fed chow alone were beginning the terminal decline in body weight (Fig. 1A). Mutants maintained on all diets remained obese and hyperphagic; food consumption (6.0–7.5 g/day) was similar to that seen for mutants fed chow alone. Both α-ET and β-ET fed at 0.1% were equally as effective as 0.4% DHEA with respect to preventing the development of hyperglycemia (Fig. 1B). The other two trans (5α) isomers, EA and A (Table 2), fed at the 0.1% concentration were not nearly as effective as the etiocholanolones in reducing most manifestations of diabetes, although some improvement was seen as compared to chow-fed mutants with respect to both lowered blood sugar concentrations and increased plasma insulin concentrations. The percent of granulated β-cells remained low in mutants fed A or EA and not significantly different from mutants fed chow, whereas granulated β-cells were significantly increased in mutants fed 0.1% of either of the etiocholanalones (Table 2). DHEA fed at 0.1% was not as effective as either of the etiocholanolones (0.1%) as judged by the significantly elevated average blood sugar concentrations ($P < 0.01$) observed after 16 weeks of treatment (Table 2).

The feeding of 0.1% DHEA sulfate in the diet to C57BL/KsJ diabetes mutants (Table 2) was equally as effective as 0.4% DHEA (Fig. 1B) with respect to lowering blood sugar and maintaining high plasma and pancreatic insulin concentrations. When E_2 was fed in the diet (0.005%) to male mutants (Table 2) it prevented the hyperglycemia and islet atrophy, maintained pancreatic IRI concentrations, and increased the percentage of granulated β-cells (Table 2). The E_2-fed mutants showed a reduced weight gain, but maintained an obese body composition. E_2 injected sc (50 μg twice per week) also had a marked beneficial effect on all parameters, similar to that seen when E_2 was fed in the diet (Table 2). In contrast, injections of β-ET in propylene glycol (2 mg/day, sc), or DHEA sulfate in water (2 mg/day, ip), for a period of 4 weeks, was without any beneficial effect suggesting that these metabolites like DHEA itself, require some biotransformation to become fully effective. The beneficial effects of any of the treatments with respect to reducing blood sugar concentrations were usually reflected in elevations in the pancreatic insulin content and the percentage of granulated β-cells (Table 2). Increased numbers of larger islets with increased, but still below normal, numbers of granulated β-cells were consistent findings in mutants treated with 0.4% DHEA, 0.1% DHEA sulfate, or 0.1% of either of the etiocholanolones. Islet granulation was normal in *db/db* mice fed the E_2-containing diet and the islets showed an almost normal size distribution (data not shown). Islet granulation was also normal in the *db/db* mice receiving E_2 by injection, but islet areas were also increased. The difference in islet size was also reflected by a 10-fold difference in plasma insulin (Table 2). No signs of islet atrophy were ever observed with these treatments, whereas mutant mice treated with 0.1% DHEA, A, or EA exhibited some atrophy with almost no improvement in β-cell granulation.

Since α-ET, β-ET, and E_2 all showed similar beneficial effects to those seen with DHEA, studies were initiated to assess any further beneficial effects of combined treatments of feeding each etiocholanolone to mutants injected with amounts of E_2 that were suboptimally therapeutic. E_2 injected alone at the reduced dose of 5 or 10 μg twice weekly (Table 3) was only slightly beneficial in contrast to the marked beneficial effects of the higher dose of injected E_2 (Table 2). β-ET alone, fed in the diet at 0.025%, had a slight (but not significant) beneficial

TABLE 2. Effects of DHEA and its metabolites on the diabetes syndrome in C57BL/KsJ-*db/db* mice

Diet	Blood sugar (mg/dl)	Plasma IRI (μU/ml)	Pancreatic insulin (U/g)	Granulated β-cells (%)
Chow	443 ± 30	100 ± 29	0.61 ± 0.16	4.05 ± 1.08
DHEA (0.1%)	234 ± 36	1158 ± 178[a]	4.60 ± 0.73[a]	8.20 ± 1.50
DHEA sulfate (0.1%)	121 ± 15[a]	388 ± 25[a]	4.12 ± 0.43[a]	52.8 ± 6.99[a]
β-ET (0.1%)	148 ± 20[a]	3141 ± 394[a]	13.5 ± 0.55[a]	15.3 ± 6.34
α-ET (0.1%)	180 ± 23[a]	251 ± 34	5.57 ± 0.72[a]	27.6 ± 4.70[a]
EA (0.1%)	296 ± 25	1517 ± 205[a]	2.60 ± 0.67[a]	6.94 ± 2.28
A (0.1%)	336 ± 47	328 ± 72[a]	1.93 ± 0.18[a]	8.60 ± 2.69
E_2 (0.005%)	123 ± 14[a]	78.1 ± 19	5.44 ± 0.16[a]	73.7 ± 6.30[a]
E_2 (injected) (50 μg 2 times/week)	132 ± 21[a]	793 ± 98[a]	6.38 ± 0.31[a]	60.5 ± 6.78[a]

Mutant male mice were studied for 16–20 weeks after weaning at which time they were killed. Values represent average values ± SEM of from four to eight individual mice obtained at time of killing.

[a] Significantly different from values from mutants fed chow alone. $P < 0.01$.

TABLE 3. Effect of combined treatment with estrogen and etiocholanolones on diabetes *db/db* mice

Diet	E_2 (10 μg 2 times/week)	Blood sugar (mg/dl)	BW (g)	Plasma IRI (μU/ml)	Pancreatic insulin (U/g)	Granulated β-cells (%)
Chow alone	−	454 ± 34	49.5 ± 2.1[a]	205 ± 3.1	1.06 ± 0.29	6.8 ± 1.7
Chow alone	+	338 ± 13[b]	67.2 ± 0.8[b]	351 ± 83	1.19 ± 0.20	8.8 ± 1.5
Chow + β-ET (0.05%)	+	114 ± 11[b]	58.0 ± 1.3[b]	563 ± 18[b]	8.70 ± 0.82[b]	64.8 ± 7.0[b]
Chow + α-ET (0.05%)	+	137 ± 7.8[b]	59.9 ± 0.96[b]	527 ± 113[b]	2.69 ± 0.81[b]	26.5 ± 6.7[b,c]
Chow + β-ET (0.025%)	+ (5 μg 2 times/week)	121 ± 7.9[b]	55.1 ± 1.4[b]	199 ± 52	5.65 ± 0.21[b]	73.0 ± 1.4[b]
Chow alone	+	281 ± 17[b]	65.9 ± 2.0[b]	286 ± 18	2.29 ± 0.15[b]	7.5 ± 2.5
Chow + β-ET (0.025%)	−	332 ± 9.5[b]	46.4 ± 1.2[a]	156 ± 11	0.57 ± 0.09	5.9 ± 1.0
Chow + β-ET (0.025%)	+	156 ± 7.7[b]	60.7 ± 1.0[b]	447 ± 73[b]	4.18 ± 0.54[b]	69.4 ± 2.6[b]

Figures represent mean ± SEM from groups of five to eight male BL/Ks diabetes (*db*) mice. The values presented were obtained at killing after 16 weeks of each treatment.

[a] Maximal weight was obtained after 12 weeks of treatment. Body weight always decreases in untreated mutants in the terminal stages of the diabetes.

[b] Statistically different from values obtained from mutants fed chow alone. $P < 0.01$.

[c] Statistically different from values from mutants fed β-ET and E_2. $P < 0.01$.

effect on blood sugar concentrations but was without any significant effect with regard to all other parameters studied (Table 3). In contrast, treatment with β-ET at a concentration of 0.025% in the diet in combination with E_2 injections (10 μg twice weekly) was more effective with respect to producing normoglycemia, maintaining plasma and pancreatic insulin concentrations and preventing islet atrophy (Table 3) than either low dose of E_2 alone or β-ET at 0.1% (Table 2). The combined treatment with either 0.05% or 0.025% of β-ET and E_2 (10 μg two times/week) was sufficient to produce maximal beneficial effects on all parameters. However, combined α-ET-E_2 treatment while being effective with respect to maintaining normoglycemic control and preventing islet atrophy, was only able to increase the percent of granulated β-cells to about 50% of that seen with the other regimens (Table 3). In addition, these islets were larger and looked similar to the hyperfunctional islets typically seen in untreated diabetes mutants between 8–12 weeks of age. A further reduction of the amount of E_2 injected to 5 μg (twice per week) combined with dietary β-ET (0.025%) retained full effectiveness with respect to circumvention of hyperglycemia and islet destruction (Table 3). The consumption of food in mutants on all steroid-supplemented diets, either singly or in combined β-ET-E_2 regimens remained high (7.5 g/day) and typical of chow-fed mutants. Pancreases from all mutants treated with β-ET and E_2 in various combinations were consistently characterized by a more normal spectrum (in terms of size and number) of well granulated islets.

Discussion

Three major metabolites of DHEA (α-ET, β-ET, and DHEA sulfate), as well as E_2, were all effective in preventing severe diabetes in C57BL/KsJ-*db/db* mice when fed at concentrations considerably lower than that of the effective dose of DHEA. These findings suggest that some, if not all of the beneficial effects of DHEA may be mediated by its conversion to these metabolites. That DHEA and the ETs are not effective when injected suggests that any metabolic conversion is initiated in the digestive system. The enhanced effects of DHEA sulfate, as compared to DHEA, may relate to the unique solubility properties of this conjugate (being relatively water soluble), thereby permitting access to specific domains in cells not accessible to the free steroid. A similar sulfation of the active metabolites of DHEA could provide the differential solubility properties required for their maximal effect. The solubility, as well as the effectiveness of each steroid, may be a function of the free to conjugate ratio in each tissue which, in turn, may be controlled by the concentration and location of various sulfokinases and sulfatases.

When suboptimal doses of E_2 (10 and 20 μg/week) were combined with diets containing nontherapeutic concentrations of either β- or α-ET (0.05%), a marked potentiation of the effects of these metabolites was produced. Whether E_2 potentiates the action of β- and α-ET or whether the ETs potentiate the therapeutic action of estrogen remains to be established and is under further study. α-ET, like DHEA, was considered to be an inert end product whose sole fate was conjugation and excretion until it was shown that in its free (unconjugated) state it had highly potent pyrogenic effects when injected im in men, less in women, and none in other species (14). The spectrum of biological significance for etiocholanolone was extended to include the regulation of porphyrin and heme synthesis in hepatic and erythroid cells (15) when it was shown that β-ET as well as other nonpyro-

genic 5-β saturated steroids induced porphyrin synthesis. Thus the possibility was raised that a class of steroid metabolites (of which etiocholanolone is the prototype) might possess novel and potent pharmacologic properties, certain of which may have clinical relevance (16).

Alasandro *et al.* (17) found decreased DHEA, A, and etiocholanolone, but increased amounts of a major cortisol metabolite, α-cortol, in urinary profiles of diabetic patients. Since both DHEA and cortisol arise from a common precursor, pregnenolone, it would be expected that a decrease in one would permit an increase in the other. Hypercorticism is a prominent feature of both the obese (*ob*) and diabetes (*db*) mutations which occur after the start of obesity and is thought to be secondary to the diabetes-obesity syndrome (18, 19). The feeding of DHEA may restore a more near normal hormonal milieu in these mutants by providing sufficient adrenal androgens to offset some of the effects of excessive glucocorticoid production.

Another view of hormonal balance would consider steroid precursors as potential prehormones which have little or no biological potency themselves, but are converted peripherally to more active compounds which can then contribute significantly to the overall biological effects (20). DHEA sulfate has been shown to be a better precursor of E_2 than either the DHEA or testosterone (11, 12). Consequently the relative potency of DHEA and its sulfate in the diet may relate to their conversion to estrogen but could potentiate the action of the increased concentration of the other metabolites (α-ET or β-ET).

In summary, the beneficial effects of the DHEA metabolites with respect to the various diabetes parameters studied were similar to those seen with DHEA, but were elicited with smaller doses. The marked increase in beneficial effects seen when nontherapeutic doses of both ETs were combined with nontherapeutic doses of E_2 suggest that this interaction may be exploited to produce optimal diabetes therapy at reduced concentrations of metabolites low enough to preclude estrogen side effects. The action of β-ET and α-ET in sustaining plasma insulin concentrations and reducing blood sugar concentrations in C57BL/KsJ diabetes (*db/db*) mutants suggests that both compounds have physiological significance and are not just metabolic end products of sterol metabolism.

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References

1. Schwartz AG 1979 Inhibition of spontaneous breast cancer formation in female C3H (A^{vy}/a) mice by long term treatment with dehydroepiandrosterone. Cancer Res 39:1129
2. Tannen RH, Schwartz AG 1982 Reduced weight gain and delay of Coombs positive hemolytic anemia in NZB mice treated with dehydroepiandrosterone (DHEA). Fed Proc 41:463 (Abstract)
3. Yen TT, Allan A, Pearson DV, Action JM, Greenberg MM 1977 Prevention of obesity in A^{vy}/a mice by dehydroepiandrosterone. Lipids 12:409
4. Cleary MP, Zisk, J 1983 Effect of dehydroepiandrosterone (DHEA) in adult Zucker rats. Fed Proc 42:536 (Aabstract)
5. Coleman DL, Leiter EH, Schwizer, RW 1982 Therapeutic effects of dehydroepiandrosterone (DHEA) in diabetes mice. Diabetes 31:830
6. Coleman DL, Schwizer R, Leiter EH 1984 Effect of genetic background on the therapeutic effects of dehydroepiandrosterone (DHEA) in diabetes-obesity mutants in mice. Diabetes 33:26
7. Lebeau MC, Baulieu EF 1973 On the significance of the metabolism of steroid hormone conjugates. In: Freshman WH (ed) Metabolic Conjugation and Metabolic Hydrolysis. Academic Press, New York, vol 3:151
8. Baulieu EE, Dray F 1963 Conversion of ^{3}H-dehydroepiandrosterone (3α-hydroxy-Δ^5-androsterone-17-one) sulfate to ^{3}H-estrogens in normal pregnant women. J Clin Endocrinol Metab 23:1298
9. Vande Wiele R, Sieberman S 1960 The metabolism of dehydroisoandrosterone. In: Pincus G, Vollmer E (eds) Biological Activities of Steroid Hormones in Relation to Cancer. Academic Press, New York, p 93
10. Coleman DL, Hummel KP 1967 Studies with the mutation, diabetes, in the mouse. Diabetologia 3:238
11. Harper MJK 1969 Estrogenic effects of dehydroepiandrosterone and its sulfate in rats. Endocrinology 84:229
12. Morato T, Lemus AE, Gual C 1965 Efficiency of dehydroepiandrosterone sulfate as an estrogen precursor. Steroids [Suppl I] 5:59
13. Leiter EH 1981 The influence of genetic background on the expression of mutations at the diabetes locus in the mouse. IV. Male lethal syndrome in CBA/Lt mice. Diabetes 30:1035
14. Kappas A, Palmer H 1965 Thermogenic properties of steroids. In: Dorfman R (ed) Methods in Hormone Research. Academic Press, New York, vol IV:1
15. Granick S, Kappas A 1967 Steroid control of porphyrin and heme biosynthesis: a new biological function of steroid hormone metabolites. Proc Natl Acad Sci USA 57:1463
16. Wolff SM, Kimball HR, Perry S, Root R, Kappas A 1967 The biological properties of etiocholanolone. Ann Intern Med 67:1268
17. Alasandro M, Wiesler D, Rhodes G, Novotny M 1982 Quantitative alteration of steroid urinary profiles associated with diabetes mellitus. Clin Chim Acta 126:243
18. Dubuc P, Mobley PW, Mahler RJ 1975 Elevated glucocorticoids in obese-hyperglycemic mice. Horm Metab Res 7;102
19. Coleman DL, Burkhart DL 1978 Plasma corticosterone concentrations in diabetic (*db*) mice. Diabetologia 13:25
20. Baird D, Horton R, Longcope C, Tait JF 1968 Steroid prehormones. Perspect Biol Med 11:384