Serum Concentrations of Δ^5-3β-Hydroxysteroids in Type 2 Diabetes Mellitus

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We examined the serum concentrations of Δ^5-3β-hydroxysteroids, pregnenolone (Preg), 17-hydroxypregnenolone (17-OH-Preg), dehydroepiandrosterone (DHEA), androstenediol (ADIOL) and their sulfates in 30 well controlled (Group I: HbA1c<7.0%) and 15 poorly controlled (Group II: HbA1c>7.1%) type 2 diabetic patients, and 30 normal controls. These patients were treated with diet therapy or anti-diabetic agent. The distribution of gender and age of the subjects were matched between the groups. The serum levels of sulfo-conjugated and unconjugated steroids described above were measured by GC-MS and enzyme immunoassay (EIA), respectively. The serum levels of the entire sulfo-conjugated steroid measured in this study were significantly lower in Groups I and II than in controls. On the other hand, Preg levels in both Groups I and II were significantly higher than those in controls, whereas the serum levels of the downstream unconjugated steroids were not different from controls. To investigate the effect of sulfonylurea (SU) on the serum levels of steroids, the serum concentrations of steroids between the patients who were treated with diet therapy and SU agent were compared in Group I. No significant differences were observed between both groups. These results suggest that (1) since increased Preg levels did not cause any changes in the downstream Δ^5-3β-hydroxysteroid levels, the metabolic pathway of Δ^4-3-ketosteroids may be accelerated in type 2 diabetes; (2) serum steroid levels were not affected by SU treatment; (3) sulfo-conjugated steroid catabolism was altered in type 2 diabetes; (4) the decreased sulfo-conjugated steroids especially ADIOLS may contribute to the alteration of sex steroid levels and onset or exacerbate infectious diseases in diabetes.

Key words androstenediol; dehydroepiandrosterone; diabetes mellitus; enzyme immunoassay (EIA); GC-MS

Androstenediol (5-androsten-3β,17β-diol, ADIOL) was first isolated from adrenal vein blood by Hirschmann *et al.*[1] For many years, research on ADIOL and its sulfate (androstenediol 3-sulfate, ADIOLS), which are synthesized from dehydroepiandrosterone (DHEA), mostly in the peripheral tissues such as skin, has focused on their role as an intermediate of sex steroid hormone biosynthesis. However, recent studies showed that these steroids have direct hormonal actions.[2—5] ADIOLS could be the most significant source of estrogen activity especially in tissues rich in steroid sulfatase. ADIOL was also suggested to augment the response of the immune system or to counteract or modulate the immunosuppressive effects of glucocorticoids such as cortisol.[6—8] Although, DHEA also bears such immuno-modulative effects, ADIOL was at least 100-fold more effective than DHEA against Coxsackie virus infection.[9,10] Therefore, defects in the ADIOL and/or DHEA metabolism might deteriorate the sex hormone balance and immune response in humans.

On the other hand, several studies have shown that the low serum levels of DHEA(S) are associated with diabetes.[11—14] The patients with type 2 diabetes mellitus frequently suffer from obesity and hypercholesterolemia. It was suggested that DHEA(S) have some protective effect against diabetes mellitus, cardiovascular disease, obesity, hypercholesterolemia, cancer and Alzheimer's disease in humans and experimental animals[15—19] Thus, a decrease in the serum levels of DHEA(S) was suggested to involve exacerbation and development of diabetes and its related diseases.

Many studies and clinical trials have been carried out to elucidate the mechanism of DHEA(S) on anti-diabetic effects. However, there has been no study on serum ADIOL(S) in patients with diabetes mellitus. In addition, it was reported that a high glycemic state, such as in patients with type 2 diabetes mellitus, is associated with alteration of the serum testosterone and estradiol levels,[20,21] and that the poorly controlled blood glucose levels may lead to infectious disease.[22] Taking these findings and biological effects of ADIOL including ADIOLS as described above together, it would be interesting to know whether ADIOL(S) metabolism would be altered in patients with diabetes. In light of these considerations, we investigated the serum concentrations of ADIOL, DHEA and their precursors, Preg and 17-OH-Preg, and their sulfo-conjugates in patients with type 2 diabetes mellitus.

MATERIALS AND METHODS

Patients This study was comprised of 45 type 2 diabetic patients, who were classified into Group I and Group II, and 30 normal healthy controls. Group I (n=30) consisted of patients who were well controlled (HbA1c<7.0%) by diet (n=14) or by oral anti-diabetic agents (those were 13 of sulfonylurea, SU; 2 of SU and biguanide, BG; 1 of SU, BG and α-glucosidase inhibitor, GI). The patients defined as Group II (n=15) were poorly controlled (HbA1c>7.1%) group even with the use of oral anti-diabetic agents (those were 7 of SU, 4 of SU and BG, 3 of SU and GI, and 1 of SU, BG and GI). Their medication was managed by diet only or with oral anti-diabetic agents and patients under insulin therapy were excluded from this study. Venipuncture was performed from 8:30 to 11:00 in the morning and informed consent was ob-

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Table 1. Clinical Characteristics of the Patients with Well and Poorly Controlled Type 2 Diabetes (Groups I and II) and Normal Healthy Controls

Patients and subjects	Controls	Group I	Group II
Number of examined	30	30	15
Age (years)	46.0±5.0	48.0±2.5	45.0±6.1
Male/Female[a)]	15/15	15/15	7/8
HbA1c (%)		6.1±0.4 (5.7−6.9)	7.7±0.2 (7.4−10.5)
BMI (kg/m^2)		24.8±2.1	25.7± 2.5
Total cholesterol (mmol/l)		5.57±0.56	5.57±0.36
Triglyceride (mmol/l)		1.20±0.28	1.51±0.23
HDL-cholesterol (mmol/l)		1.24±0.08	1.30±0.12

Values are indicated as median±quartile deviation. *a*) Number of males and females. HbA1c, hemoglobin A_{1c}; BMI, body mass index; HDL, high density lipoprotein.

tained from all patients. Gender and age were matched between the groups. Body mass index (BMI) and the serum levels of total cholesterol, triglyceride and high density lipoprotein (HDL)-cholesterol were not significantly different from Groups I and II (Table 1).

Materials Preg, 17-OH-Preg, DHEA, ADIOL and their 3-monosulfates were purchased from Steraloids Inc. (Newport, RI, U.S.A.). Oasis® Max cartridge (size: 1 ml/30 mg) was from Waters Corporation (Milford, MA, U.S.A.). Antisera raised in rabbits against Preg-3-hemisuccinate-bovine serum albumin (BSA), 17-OH-Preg-3-hemisuccinate-BSA, DHEA-7-*O*-(carboxymethyl)oxime-BSA and ADIOL-7-*O*-(carboxymethyl)oxime-BSA were purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan) and Biogenesis Ltd. (Poole, England). Enzyme labeled antigens were prepared according to the previous methods.[23,24)] Costar E.I.A./R.I.A. 8 Well Strip (flat bottom) from Corning Inc. (Corning, NY, U.S.A.) was used for the assay plate. The IgG fraction of goat anti-rabbit IgG (whole molecule) was from Seikagaku Kogyo, Co., Ltd. (Tokyo, Japan). Arylsulfatase (EC 3.1.6.1, from *Helix pomatia*) was obtained from Roche Diagnostics GmbH (Mannheim, Germany). *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other reagents and solvents were of analytical grade.

Calibrator and Sample Preparation To measure the serum steroid concentrations, the calibrator for each analyte were prepared by adding the authentic steroid to steroid free-serum.[25)] To dissociate steroids from serum steroid binding proteins, 2 ml of 0.6% glutamic acid aqueous solution was added to 0.2 ml of the serum sample or calibrator and then the mixture was heated at 60 °C for 10 min. After cooling the solution to the room temperature, it was applied to the solid-phase extraction column, Oasis® Max cartridge, which was pretreated with 1 ml of methanol and 1 ml of water. The cartridge was washed with 2.6 ml of water and the unconjugated steroids were eluted with 4 ml of 72% methanol containing 0.3 M acetic acid. After washing the cartridge with 1 ml of methanol and 4 ml of 72% ethanol containing 0.3 M acetic acid, the sulfo-conjugated steroids were eluted with 2 ml of acetonitoril : methanol : water=(60 : 12 : 28) containing 0.3 M sodium chloride. The resultant unconjugated and sulfo-conjugated fractions were evaporated *in vacuo*.

Assays for Unconjugated Steroids The unconjugated steroid fraction obtained above was applied to HPLC system for the further purification. The eluate corresponding to each steroid fraction was collected. After evaporation of the solvent, concentrations of the unconjugated steroids were measured by enzyme immunoassay (EIA). Detailed HPLC conditions for unconjugated steroids and the EIA procedure for ADIOL were described elsewhere.[23)] EIA for 17-OH-Preg, DHEA and Preg were performed according to the same procedure for ADIOL,[23)] except that the immobilized second antibody to micro-titer plates were used for B/F separation. The measurable ranges of ADIOL, DHEA, 17-OH-Preg and Preg were 1.38—517 nmol/l (0.4—150 ng/ml), 0.13—521 nmol/l (0.04—150 ng/ml), 0.05—45.2 nmol/l (0.02—15 ng/ml) and 0.02—23.7 nmol/l (0.01—7.5 ng/ml), respectively. Overall recovery of these assays exceeded 82%. The intra- and inter-assay coefficient of variations (C.V.s) of these assays were 3.1—6.6% and 4.7—15.6%, respectively.

Measurement of Steroid Sulfates Because serum levels of sulfo-conjugated steroids, ADIOLS, DHEAS, 17-OH-PregS and PregS, are 100—500 times higher than those of unconjugated steroids, simultaneous measurement of the sulfo-conjugated steroids was performed by GC-MS. The sulfo-conjugated fraction were hydrolyzed with arylsulfatase and was applied to HPLC under the same conditions described above for purification.[23)] After HPLC separation, each eluate correspond to each steroid were combined into a tube. ADIOL diacetate in methanol was added to the tube (20 ng/sample) as an internal standard. The solvent was evaporated under vacuum to dryness. After tri-methylsilylation of the steroids with BSTFA, samples were applied to GC-MS. Selected ion monitoring was carried out at *m*/*z* 360 $[M]^+$ for DHEA, *m*/*z* 433 $[M-43]^+$ for 17-OH-Preg, *m*/*z* 388 $[M]^+$ for Preg and *m*/*z* 314 $[M-60]^+$ for ADIOL diacetate. The detailed methods for quantitation of the steroid sulfates including enzymatic hydrolysis, HPLC and GC-MS conditions, and derivatization were described previously.[26)] The resulting values were expressed as the mono-sulfate concentration in serum. The sensitivity of the GC-MS for ADIOL, DHEA, 17-OH-Preg and Preg was 8 nmol/l. The intra-assay C.V.s for DHEAS, 17-OH-PregS and PregS were less than 3.0%, and the inter-assay C.V.s for these steroids were less than 8.3%. The recovery of DHEAS, 17-OH-PregS and PregS exceeded 84.9%.

Statistical Analysis Differences between two groups were statistically analyzed using the Mann–Whitney *U* test.

RESULTS

The Serum Levels of Unconjugated and Sulfo-conjugated Δ^5-3β-Hydroxysteroids in Patients with Type 2 Diabetes under Treatment with Diet Therapy or Anti-diabetic Agents The serum concentrations of unconjugated

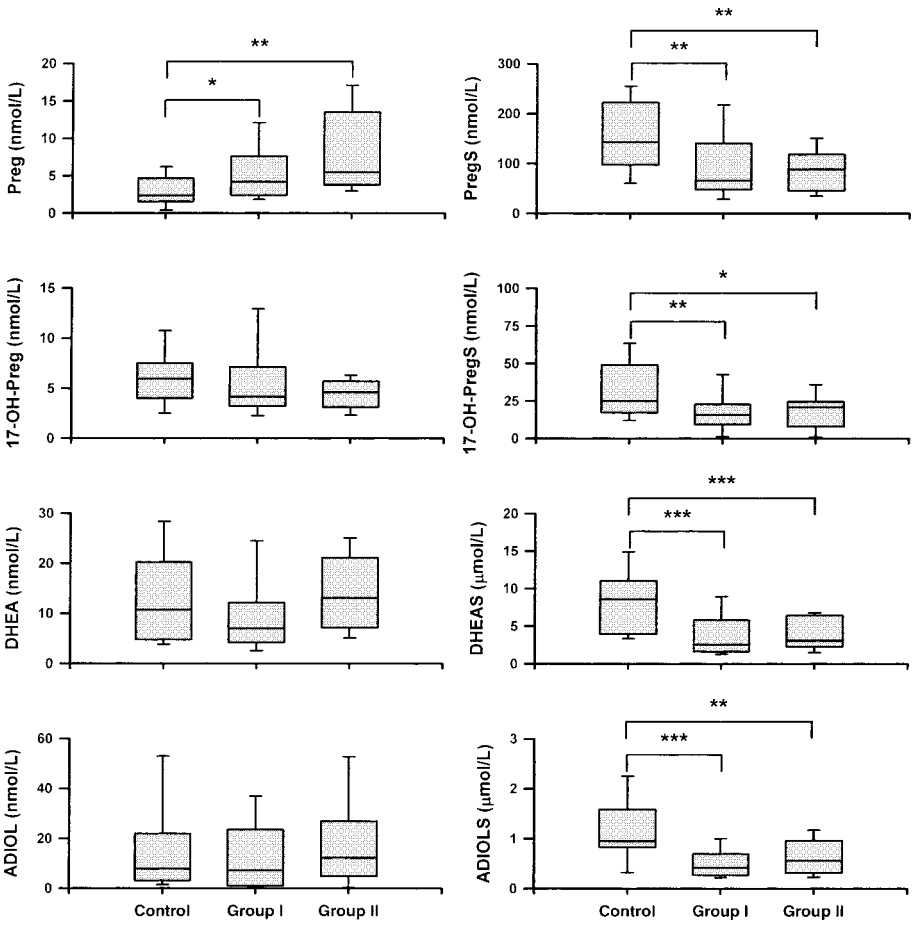

Fig. 1. Serum Levels of Preg(S), 17-OH-Preg(S), DHEA(S) and ADIOL(S) in Patients with Type 2 Diabetes Mellitus

Groups I and II consisted of the well and poorly controlled patients whose HbA1c levels were less than 7% and greater than 7.1%, respectively. A line within the box marks the median, and a box represents quartile deviation. Whiskers above and below the box indicate the 90th and 10th percentiles. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* normal controls.

and sulfo-conjugated Preg, 17-OH-Preg, DHEA and ADIOL were measured in 30 well controlled (Group I), 15 poorly controlled (Group II) type 2 diabetes patients, and age- and sex-matched normal healthy controls (Table 1).

The levels of sulfo-conjugated steroids, PregS, 17-OH-PregS, DHEAS and ADIOLS, in Groups I and II were significantly lower than those in controls ($p<0.05$—0.001, Fig. 1), but no significant differences were found between those in Groups I and II. On the other hand, the levels of unconjugated steroids, 17-OH-Preg, DHEA and ADIOL, in diabetic patients were not different from those in controls except for the significantly higher levels of Preg in Groups I ($p<0.05$) and II ($p<0.01$) than controls (Fig. 1).

The Effects of Sulfonylurea (SU) Agent on Serum Δ^5-3β-Hydroxysteroid Concentrations Since some patients were receiving oral anti-diabetic agents in this study, it was suggested the agents might affect the serum steroid levels. We examined the serum steroid concentrations between the patients that received diet therapy ($n=14$) and SU treatment ($n=13$). As a result, there were no significant differences for the entire steroid level between the diet and SU treatment (Table 2).

Table 2. Comparison of the Serum Concentrations and Clinical Characteristics between the Patients Who Were Being on a Diet and Receiving SU Agent in Group I

Patients	Diet only	SU only
Numbers	14	13
Age (years)	47±1	50±2
Male/Female[a)]	7/7	6/7
Preg (nmol/l)	3.06±2.34	4.9±2.70
17-OH-Preg (nmol/l)	3.60±3.71	4.45±0.80
DHEA (nmol/l)	5.95±4.95	6.88±3.39
ADIOL (nmol/l)	3.10±7.99	8.60±10.1
PregS (nmol/l)	89.7±41.6	58.1±0.50
17-OH-PregS (nmol/l)	17.5±9.35	13.8±6.89
DHEAS (μmol/l)	2.40±2.66	2.68±1.20
ADIOLS (μmol/l)	0.41±0.21	0.41±0.16
HbA1c (%)	6.2±0.3	5.9±0.2
BMI (kg/m^2)	26.4±2.6	24.0±2.4
HDL-cholesterol (mmol/l)	1.19±0.05	1.30±0.05
Triglyceride (mmol/l)	1.33±0.36	1.12±0.21
Total cholesterol (mmol/l)	6.19±0.26	5.05±0.41[b)]

Values are indicated as median±quartile deviation. *a*) Numbers of males and females. *b*) $p<0.01$ *vs.* diet only. HbA1c, hemoglobin A_{1c}; BMI, body mass index; HDL, high density lipoprotein.

DISCUSSION

Although the exact mechanisms for the anti-diabetic effect of DHEA(S) are unclear, changes in the serum levels of Δ^5-3β-hydroxysteroid, DHEA and/or DHEAS, were reported in patients with diabetes mellitus.[11,13,27)] In addition, it was also

reported that the metabolism of Δ^4-3-ketosteroids, such as cortisol and testosterone is impaired in diabetic patients.[21,28] The metabolic pathway of Δ^5-3β-hydroxysteroid including the sulfo-conjugated form is Preg(S)→17-OH-Preg(S)→DHEA(S)→ADIOL(S) and that of Δ^4-3-ketosteroid is Preg→progesterone→17-OH-progesterone→cortisol, or Preg→progesterone→corticosterone→aldosterone. Thus, Preg and PregS are a common source for these steroid metabolic pathways. Any defects or alterations in the upstream of the steroid metabolic pathway might possibly affect the downstream of the metabolism. However, it appears that little attention has been paid to the changes in the up- or downstream metabolites of DHEA. Therefore, we examined the changes in the serum levels of Δ^5-3β-hydroxysteroid, Preg, 17-OH-Preg, DHEA and ADIOL, including their sulfo-conjugates in patients with type 2 diabetes mellitus. To our knowledge, this is the first study on the changes in the serum levels of these steroids, except DHEA or DHEAS, in diabetic patients.

As shown in Fig. 1, the serum levels of Preg in Groups I and II were significantly higher than controls. However, no changes in the levels of the downstream steroids, 17-OH-Preg, DHEA and ADIOL, were observed in either Group compared with those of controls. We also found a significant decrease in the serum levels of PregS, 17-OH-PregS, DHEAS and ADIOLS in Groups I and II compared with those of controls, although these levels did not differ from Groups I and II. Despite the increase in the upstream steroid, Preg, the downstream steroids were unchanged. One explanation could be that the conversion of Preg from cholesterol might be activated and the metabolic pathway from Preg to cortisol, Δ^4-3-ketosteroid pathway, might be predominant in diabetic conditions. These hypothesis are supported by the observations of higher serum levels of ACTH and cortisol in type 2 diabetes mellitus than normal controls.[12,21,29] Therefore, the elevated levels of Preg in Groups I and II might be to increase cortisol production although we did not measure the serum levels of ACTH and cortisol. Despite the patients receiving oral anti-diabetic agents or diet therapy in this study, the serum concentrations of these unconjugated and sulfo-conjugated steroids were not different between patients receiving SU and diet therapy in Group I (Table 2). These results suggest that SU is unlikely to affect the serum levels of Preg and the downstream Δ^5-3β-hydroxysteroids measured in the present study.

Ueshiba *et al.* reported that DHEA(S) levels were not different from those of controls after 6-month treatment with diet only or with SU in type 2 diabetes mellitus.[12] Although our results on DHEA levels were similar to theirs, the DHEAS levels were significantly lower than controls in the present study. Conflicting results in the DHEAS levels were also reported in untreated diabetic patients. Andersson *et al.*[28] and Zietz *et al.*[21] reported no changes in the DHEAS levels between the patients with non-insulin-dependent diabetes mellitus (NIDDM) and normal controls. On the other hand, decreased serum levels of DHEAS in NIDDM or type 2 diabetes were reported by Yamauchi *et al.*[30] and Ueshiba *et al.*[12] It was reported that hyperglycemia may reduce serum DHEA and/or DHEAS levels.[30] Other studies showed that hyperinsulinemia may cause low serum levels of these steroids rather than hyperglycemia.[13] At present the exact factors affecting the serum levels of DHEA or DHEAS are unknown.

ADIOL is derived from DHEA *in vivo*, particularly in the skin and the brain.[31—36] Padgett *et al.*[6] reported that ADIOL functions to augment the response of the immune system or to counteract or modulate the immunosuppressive effects of corticosteroids such as cortisol. Moreover, ADIOL was reported to be a more biologically active metabolite than DHEA. For example, ADIOL was at least 100-fold more effective in up-regulating systemic resistance against Coxackie virus infection.[9,10] Although contribution of ADIOL and/or ADIOLS on the pathogenesis of diabetes mellitus has not been elucidated, it was reported that the response of serum ADIOL to ACTH stimulation was greater in obese euandrogenic healthy females than thin ones.[37] Thus, we investigated the serum levels of ADIOL(S) in patients with type 2 diabetes mellitus who were treated with diet therapy or anti-diabetic agents such as SU. Despite the difference in the control of serum glucose between patients with low levels of HbA1c (6.1±0.4%, mean±S.D., Group I) and high levels of HbA1c (7.7±0.2%, Group II), no significant difference in the serum concentration of ADIOL in Groups I and II was observed. The ADIOL levels in Groups I and II were also not significantly different from those of controls. Although the changes in the serum levels of ADIOL in untreated type 2 diabetes mellitus are unknown at present, similar results may also be obtained in untreated diabetic patients as above, because there were no direct effects of SU on the changes in the serum levels of ADIOL as shown in Table 2.

Regarding ADIOLS, a significant decrease was observed in Groups I and II compared with controls. These results were similar to those obtained in other sulfo-conjugated steroids. It can be excluded the hypothesis that hyperglycemia may cause low levels of ADIOLS because decreased levels of ADIOLS were also seen in well controlled type 2 diabetes (Group I).

Since steroid sulfatase presents in the various tissues of human, ADIOL and/or DHEA could be librated easily from their sulfo-conjugated steroids. The marked decrease in ADIOLS and/or DHEAS found in diabetic patients might involve with the pathology of type 2 diabetes *via* diminishing the potency of ADIOL and DHEA in peripheral tissues. As mentioned above, ADIOL and DHEA were suggested to have immunoregulatory activity and to enhance resistance to infection in animal experiments.[38,39] It was also demonstrated that these hormones may down-regulate the Th2 immune response[40] and augment the Th1 immune reaction.[19,41] Further, ADIOL and DHEA are known to be the important source of sex steroid hormones. It appears that diabetic patients are generally prone to infectious diseases,[22,42,43] and it has been reported that alterations in the sex steroid hormone levels was found in diabetic patients.[20,21] Therefore, a marked decrease of ADIOLS, DHEAS and their sulfo-conjugated precursor steroid hormones in the patients with type 2 diabetes might contribute to the onset or exacerbate infectious diseases, and alterations in sex steroid hormone levels.

Further studies should be continued to clarify the mechanism of modulation of these Δ^5-3β-hydroxysteroid metabolisms in patients with type 2 diabetes mellitus.

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REFERENCES

1) Hirschmann H., Courcy C., Levy R. P., Miller K. L., *J. Biol. Chem.*, **235**, PC48—49 (1960).
2) Markiewicz L., Gurpide E., *Am. J. Obstet. Gynecol.*, **159**, 500—504 (1988).
3) Adams J. B., Archibald L., Clarke C., *Cancer Res.*, **38**, 4036—4040 (1978).
4) Santen R., *Cancer Surv.*, **5**, 597—616 (1986).
5) Thijssen J. H., Blankenstein M. A., *Eur. J. Cancer Clin. Oncol.*, **5**, 1953—1959 (1989).
6) Padgett D. A., Loria R. M., Sheridan J. F., *J. Neuroimmunol.*, **78**, 203—211 (1997).
7) Whitnall M. H., Elliott T. B., Landauer M. R., Jackson W. E. 3rd, Wilhelmsen C. L., McKinney L., Kumar K. S., Srinivasan V., Ledney G. D., Seed T. M., *Exp. Biol. Med.*, **226**, 625—627 (2001).
8) Loria R. M., Conrad D. H., Huff T., Carter H., Ben-Nathan D., *Ann. N. Y. Acad. Sci.*, **917**, 860—867 (2000).
9) Loria R. M., Padgett D. A., *Ann. N. Y. Acad. Sci.*, **685**, 293—295 (1993).
10) Loria R. M., Padgett D. A., Huynh P. N., *J. Endocrinol.*, **150** (Suppl.), S209—S220 (1996).
11) Couch R.M., *Acta Endocrinol.*, **127**, 115—117 (1992).
12) Ueshiba H., Shimizu Y., Hiroi N., Yakushiji F., Shimojo M., Tsuboi K., Miyachi Y., *Eur. J. Endocrinol.*, **146**, 375—380 (2002).
13) Yamaguchi Y., Tanaka S., Yamakawa T., Kimura M., Ukawa K., Yamada Y., Ishihara M., Sekihara H., *Clin. Endocrinol.*, **49**, 377—383 (1998).
14) Yamauchi A., Takei I., Kasuga A., Kitamura Y., Ohashi N., Nakano S., Takayama S., Nakamoto S., Katsukawa F., Saruta T., *Eur. J. Endocrinol.*, **135**, 101—104 (1996).
15) Barrett-Connor E., Khaw K. T., Yen S. S., *N. Engl. J. Med.*, **315**, 1519—1524 (1986).
16) Gordon G. B., Bush D. E., Weisman H. F., *J. Clin. Invest.*, **82**, 712—720 (1988).
17) Schwartz A. G., Perantoni A., *Cancer Res.*, **35**, 2482—2487 (1975).
18) Mohan P. F., *Lancet*, **2**, 1048—1049 (1989).
19) Daynes R. A., Dudley D. J., Araneo B. A., *Eur. J. Immunol.*, **20**, 793—802 (1990).
20) Nyholm H., Djursing H., Hagen C., Agner T., Bennett P., Svenstrup B., *J. Clin. Endocrinol. Metab.*, **69**, 946—949 (1989).
21) Zietz B., Cuk A., Hugl S., Buttner R., Straub R. H., Bauer B., Daffner P., Scholmerich J., Palitzsch K., *Eur. J. Intern. Med.*, **11**, 322—328 (2000).
22) Bell D. S., *Endocr. Pract.*, **6**, 272—276 (2000).
23) Tagawa N., Tamanaka J., Fujinami A., Kiguchi T., Naito T., Takano T., Amino N., Kobayashi Y., *Clin. Chim. Acta*, **296**, 193—201 (2000).
24) Tagawa N., Tamanaka J., Fujinami A., Kobayashi Y., Takano T., Fukata S., Kuma K., Tada H., Amino N., *Clin. Chem.*, **46**, 523—528 (2000).
25) Heyns W., Baelen H., Moor P. D., *Clin. Chim. Acta*, **18**, 361—370 (1967).
26) Tagawa N., Kusuda S., Kobayashi Y., *Biol. Pharm. Bull.*, **22**, 1262—1265 (1999).
27) Barrett-Connor E., Ferrara A., *J. Clin. Endocrinol. Metab.*, **81**, 59—64 (1996).
28) Andersson B., Marin P., Lissner L., Vermeulen A., Bjorntorp P., *Diabetes Care*, **17**, 405—411 (1994).
29) Hashimoto K., Nishioka T., Takao T., Numata Y., *Endocr. J.*, **40**, 705—709 (1993).
30) Yamauchi A., Takei I., Nakamoto S., Ohashi N., Kitamura Y., Tokui M., Nakano S., Takayama S., Kasuga A., Katsukawa F., Saruta T., *Endocr. J.*, **43**, 285—290 (1996).
31) Akwa Y., Morfin R. F., Robel P., Baulieu E. E., *Biochem. J.*, **288**, 959—964 (1992).
32) Gallegos A. J., Berliner D. L., *J. Clin. Endocrinol. Metab.*, **27**, 1214—1218 (1967).
33) Berliner D. L., Pasqualini J. R., Gallegos A. J., *J. Invest. Dermatol.*, **50**, 220—224 (1968).
34) Faredin I., Fazekas A. G., Toth I., Kokai K., Julesz M., *J. Invest. Dermatol.*, **52**, 357—361 (1969).
35) Mathur C., Prasad V. V., Raju V. S., Welch M., Lieberman S., *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 85—88 (1993).
36) Toth I., Faredin I., *Acta Med. Hung.*, **40**, 139—145 (1983).
37) Azziz R., Potter H. D., Bradley E. L. Jr., Boots L. R., *Fertil. Steril.*, **62**, 321—326 (1994).
38) Ben-Nathan D., Padgett D. A., Loria R. M., *J. Med. Microbiol.*, **48**, 425—431 (1999).
39) Whitnall M. H., Elliott T. B., Harding R. A., Inal C. E., Landauer M. R., Wilhelmsen C. L., McKinney L., Miner V. L., Jackson W. E., Loria R. M., Ledney G. D., Seed T. M., *Int. J. Immunopharmacol.*, **22**, 1—14 (2000).
40) Padgett D. A., Sheridan J. F., Loria R. M., *Ann. N. Y. Acad. Sci.*, **774**, 323—325 (1995).
41) Daigle J., Carr D. J., *J. Immunol.*, **160**, 3060—3066 (1998).
42) Grant-Theule D. A., *Periodontal. Abstr.*, **44**, 69—77 (1996).
43) Joshi N., Caputo G. M., Weitekamp M. R., Karchmer A. W., *N. Engl. J. Med.*, **341**, 1906—1912 (1999).