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The Relationship Between Red Blood Cell Na/K-ATPase Activities and Diabetic Complications in Patients with Type 2 Diabetes Mellitus

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The development of complications does not depend entirely on diabetes duration and control. Red-blood-cell Na/K-ATPase plays a central role in the regulation of intra- and extracellular cation homeostasis. Alteration of this transport enzyme is thought to be linked to several complications of diabetes mellitus. The aim of this study was to find out any association between diabetic complications and red-blood-cell Na/K-ATPase activities in type 2 diabetes mellitus. Sixty-seven patients and 25 controls were enrolled in the study. Patients were evaluated for retinopathy, neuropathy, and nephropathy. The membrane Na/K-ATPase activities were measured. The studies were done twice with and without ouabain. The results of the calculations are written as micromol Pi/mg protein/h.

The duration of diabetes and enzyme levels were negatively correlated ($r = -0.38$, $p = 0.001$). Na/K-ATPase enzyme activity was significantly lower in the diabetic patients than the control group ($p < 0.0001$). In neuropathic patients the activity was also significantly lower ($p < 0.0001$). The enzyme activities of the people with retinopathy were significantly lower than the ones without retinopathy ($p < 0.001$). The enzymatic activities did not differ among the degrees of nephropathy. The results indicate that erythrocyte Na/K-ATPase enzyme activities are decreased in type 2 diabetes and the decrement of the enzyme is correlated with the diabetes duration.

Key Words: Na/K-ATPase; diabetic complications; neuropathy; retinopathy; nephropathy.

Introduction

The degenerative complications that occur in type 2 diabetes are mainly related with the duration of disease and the quality of control. However, improving glycemic control does not necessarily mean that it will result in improved health outcomes *(1)*. The decreases in Na/K adenosine triphosphatase (ATPase) activity observed in several tissues of diabetic patients play role in the development of long-term complications *(2–4)*. The erythrocyte Na/K-ATPase enzyme activity reflects the enzyme activities of the other tissues and can be obtained more easily. For some authors, lowered erythrocyte membrane Na/K-ATPase activity may be a predictor of future complications *(5)*.

The relationship between diabetic neuropathy and erythrocyte Na/K-ATPase enzyme activities are well defined. High glucose with suppressed Na^+/K^+ pump activity might induce an increase of Ca influx through either Ca channels or reverse Na/Ca exchange, possibly leading to the elevation of Ca-activated voltage-dependent K channels. Both a decrease in inward Na current and an increase in K conductance may result in decreased nerve conduction *(6)*. The association between enzyme activities and nephropathy and retinopathy were also reported *(7,8)*. However much of the current data about the Na/K-ATPase enzyme activities belong to the type 1 diabetic cases and experimental animal studies *(2,9,10)*. The mechanisms of type 1 and type 2 diabetes are entirely different. First of all, type 1 diabetics are younger than the type 2 diabetics, and age is reported to influence the Na/K-ATPase levels *(11)*. It is not clear whether erythrocyte Na/K-ATPase enzyme activities are diminished in the type 2 diabetic patients. Also the relationship between the diabetic complications and Na/K-ATPase activities are not well defined in type 2 diabetes. In their series, Mimura et al. reported that there is a slight decrement of Na/K-ATPase levels in type 2 diabetes with microalbuminuria *(12)*, while De La Tour et al. reported that the enzyme activities are not different from controls in type 2 diabetes *(13)*.

Thus the aim of the study was to evaluate the erythrocyte Na/K-ATPase activities in patients with type 2 diabetes and find out any association between the enzyme activities and diabetic complications. A carefully selected group of patients and age and sex matched controls were enrolled in the study and the association between diabetic complications, metabolic control parameters and enzyme activities were investigated.

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Table 1
Demographic, Clinical, and Biochemical Characteristics of the Study Group and Control Subjects

	Patients (n = 67)			
	Neuropathic (n = 30)	Nonneuropathic (n = 37)	Total (n = 67)	Controls (n = 25)
Gender[1]	18/12	24/13	42/25	12/13
Age[2]	59.8 ± 8.7	55.1 ± 9.8	57.2 ± 9.6	53.6 ± 13.8
Diabetes duration[2]	15.6 ± 6.8[a]	7.6 ± 6.4[b]	11.2 ± 7.6	—
Family history of diabetes[3]	18/12	20/17	38/29	0/25
Smoking habits[4]	9/21	5/32	14/53	8/17
Treatment protocol[5]	11/19/0	11/26/2	45/20/2	—
Retinopathy[6]	3/10/17[c]	26/10/1[d]	29/21/17	—
Nephropathy[6]	18/7/5	24/10/3	42/17/8	—
Daily caloric intake[7]	1707 ±101	1705 ± 88	1706 ± 94	2012 ± 93
BMI[8]	26.6 ± 3.3	27.7 ± 3.6	27.2 ± 3.5	25.4 ± 3.5
Fasting glucose[9]	229 ± 83.3	205.8 ± 67.5	216.5 ± 75.3	87.3 ± 8.6
HbA1c [10]	10.1 ± 2.0	9.3 ± 2.1	9.6 ± 2.1	5.8 ± 0.4
Mean corpuscular volume[11]	86.8 ± 3.8	88.2 ± 3.9	87.6 ± 3.9	88.5 ± 3.3
Total cholesterol[9]	216.7 ± 50.1	217.0 ± 44.2	216.8 ± 46.6	209.4 ± 37.5
LDL-Cholesterol[9]	136 ± 40.5	133.2 ± 39	134.5 ± 39.4	132.7 ± 31.5
HDL-Cholesterol[9]	43.6 ± 5.9	44.5 ± 6.1	44.1 ± 5.9	44.2 ± 4.5
Triglyceride[9]	197.5 ± 150.4	182.2 ± 92.3	189.1 ± 121.2	159.2 ± 62.2

The units: 1 = F/M, 2 = year, 3 = present/absent, 4 = smoker/nonsmoker, 5 = insulin/OAD/diet, 6 = 0/1 /2, 7 = Kcal/d, 8 = kg/m^2, 9 = mg/dL, 10 = %, 11 = fL.

Mann–Whitney U test: *a* vs *b*, $p < 0.05$; *c* vs *d*, $p < 0.05$.

Results

There was no significant difference in the demographic of the study group and the control group (Table 1). The serum glucose levels, HbA1c levels, family history of diabetes, gender profile (18/12, 24/13) were not different between the neuropathic and nonneuropathic individuals. In the neuropathic group, diabetes duration was longer ($p < 0.05$). Nephropathy incidence was similar between the two groups, while retinopathy was higher in the neuropathic group ($p < 0.001$). The peroneal nerve conduction rates were significantly slower in patients with neuropathy (35 ± 5 and 42 ± 3 m/s, $p < 0.05$).

In the neuropathy group, Na/K-ATPase enzyme activity was significantly lower (2.09 ± 2.54) than the nonneuropathic patients (3.67 ± 1.54) ($p < 0.0001$). Both groups had lower enzyme activities than the control subjects (9.23 ± 2.54) ($p < 0.0001$). The enzyme activities of the people with retinopathy were significantly lower than the ones without retinopathy ($p < 0.001$). The enzymatic activities did not differ between the individuals having background retinopathy and the ones without retinopathy. There was no difference between the enzyme activities of the patients with nephropathy and the ones without nephropathy. Also the enzymatic activities did not differ among the degrees of nephropathy. The enzyme activity was higher in the healthy female controls (10.78 ± 3.26) than the male controls (8.43 ± 2.56) ($p < 0.01$). In the study group the enzyme activity was similar in both sexes (Table 2).

Table 2
The Na/K-ATPase Activities of the Patient Subgroups and the Controls

Na/K-ATPase Activities (micromole Pi/mg protein/hour)	Patients (n = 67)	Controls (n = 25)
Neuropathy		
Present	2.09 ± 0.72[a]	
Absent	3.67 ± 1.54[b]	9.23 ± 2.54[e]
Gender		
Male	2.92 ± 1.47	8.43 ± 2.56[f]
Female	2.99 ± 1.48	10.79 ± 3.26[g]
Treatment		
Insulin	2.70 ± 0.90	
OAD	3.05 ± 1.57	
Smoking		
Yes	2.98 ± 1.73	9.27 ± 2.35
No	2.96 ± 1.40	9.21 ± 2.15
Family history		
Present	2.88 ± 1.45	
Absent	3.07 ± 1.50	
Retinopathy		
No	3.50 ± 1.62[c]	
Background	3.00 ± 1.35	
Proliferative	2.05 ± 0.79[d]	
Nephropathy		
No	3.04 ± 1.69	
Microalbuminuria	2.84 ± 1.16	
Macroalbuminuria	2.83 ± 0.64	

Mann–Whitney U test: $p < 0.0001$ for *a* vs *b*, *a* vs *e*, *b* vs *e*; $p < 0.001$ for *c* vs *d*; $p < 0.01$ for *f* vs *g*.

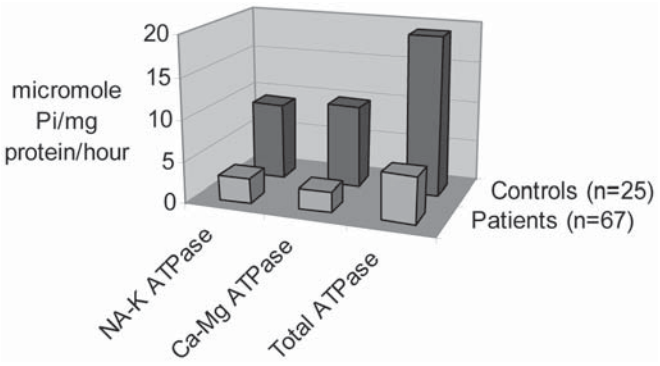

Fig. 1. The Na/K-ATPase, Ca/Mg-ATPase, and total ATPase activities of the patients and controls.

The Na/K-ATPase activities were not different between the ouabain (OAD) and insulin subgroups of the patients. The activities of OAD and insulin treatment groups were also compared in the neuropathic and nonneuropathic subgroups. There was no difference in either of the subgroups.

In the study group there was a negative correlation between diabetes duration and enzyme activity ($r = -0.38$, $p = 0.001$). There was a negative correlation between age and enzyme activity in the control group ($r = -0.49$, $p = 0.01$). No correlation was established between the enzyme activity and BMI, HbA1c, serum glucose, LDL cholesterol, HDL cholesterol, or triglycerides in the patients' group. Also there was no correlation between the enzyme activities and the biochemical parameters in the control group. Smoking was not related with the enzyme activity in either group. According to the chi square test, the Na/K-ATPase levels were not affected by smoking, family history of diabetes, and the number of subgroups.

According to the results of the ouabain study, Ca/Mg-ATPase activity was not different between the neuropathic and nonneuropathic patients. The Na/K-ATPase, Ca/Mg-ATPase and total ATPase activities of the patients (2.96 ± 1.46, 2.56 ± 1.96, 5.53 ± 2.27, respectively) were lower than the controls' parameters (9.24 ± 2.54, 9.89 ± 2.68, 19.13 ± 7.35, respectively) ($p < 0.0001$) (Fig. 1).

Discussion

Red blood cell Na/K-ATPase plays a central role in the regulation of intra- and extracellular cation homeostasis. Alteration of this transport enzyme is thought to be linked to several complications of diabetes mellitus: hypertension, nephropathy, peripheral neuropathy, and microangiopathy *(2–4,7,8).* Among the metabolic mechanisms of diabetic complications are the increase of the polyol pathway, linoleic acid metabolism abnormalities, decrement of carnitine level, the increase of protein glycation, nerve growth factor abnormalities, and high production of oxygen free radicals *(2,4).* These factors could account for nerve membrane phospholipid pattern disorder and a decrease of Na/K-ATPase activity. The evidence indicates that both the Na/K-ATPase and Ca-pumping ATPase of type 2 diabetics' membranes are less functional than the enzymes in normal erythrocytes *(14).* In the present study, both enzymes' activities were found to be lower in diabetic group than the controls. As the patients were in the same ethnic group with equal gender distributions and without a family history of neuropathy, the low enzyme levels were not the result of patient selection biases *(15–17).*

Regarding the diabetic complications, the Na/K-ATPase activities were significantly lower in retinopathy and neuropathy groups, although there was no difference in nephropathy. Migdalis et al. reported that Ca/Mg-ATPase enzyme might be involved in diabetic complications *(18).* However, the results of the present study did not support this observation. The Ca/Mg-ATPase activities were not different in the neuropathy, nephropathy and retinopathy groups of the patients. Thus it is conceivable to say that the decrement of the total ATPase activity was the result of the alteration of the Na/K-ATPase enzyme activity. Also the results did not support the data indicating the decrement of erythrocyte Na/K-ATPase activity in microalbuminuria *(7).* However, the number of the patients was not sufficient to estimate that there was actually a lack of association. As the aim was primarily to investigate the alterations in diabetic neuropathy, the number of the patients in the subgroups of retinopathy and nephropathy was not high. This point is a handicap of the study. Thus, the smaller number of patient subgroups may be the cause of the lack of association between the Na/K-ATPase activities and microalbuminuria.

In case of retinopathy, however, the Na/K-ATPase levels were significantly lower. With elevated levels of glucose, some of the glucose is shifted into the polyol pathway in which aldose reductase converts glucose to sorbitol that acts osmotically to shift fluid into cells. Aldose reductase requires NADPH. Reduction of the NADPH decreases the amount of nitric oxide synthase so the levels of nitric oxide are decreased, diminishing blood flow and retinal ischemia *(19).* Decreased NADPH also reduces the amount of myo-inositol. Lower levels of myo-inositol result in decreased Na/K-ATPase activity, which causes decreased retinal photoreceptor function *(8).* It is therefore conceivable to find lower enzyme levels in both background and proliferative retinopathy.

Infusion of insulin is said to restore the enzyme activity in red blood cells, and recent arguments have been developed for a similar role of C-peptide *(20).* However, according to the results, the enzyme activities do not differ between the insulin and OAD treatments. Moreover, Na/K-ATPase activity was not dependent on the degree of diabetic control, nor was it correlated with either fasting blood glucose or HbA1c. According to current data the primary factor for diabetic complications is poor metabolic control *(2,21).* So it would be conceivable to find an association between

the metabolic control parameters and low Na/K-ATPase enzyme levels. However, some studies indicate that diabetes-induced Na/K-ATPase dysfunction could be implicated in the pathogenesis of human diabetic neuropathy and is not related with good metabolic control *(2,22).* Raccah et al. indicated that the development of diabetic complications does not entirely depend on diabetes duration and control, and low enzyme activities could be implicated in the susceptibility to diabetic complications *(23).* However, in the present study, the design is not suitable to say that the low Na/K-ATPase activity precedes the diabetic complications. Further prospective studies must be done to find the right answer.

The duration of diabetes and the Na/K-ATPase activity were negatively correlated in the study group ($r = -0.38$, $p = 0.001$). In the control group there was a negative correlation between the ages of subjects and enzyme activities ($r = -0.49$, $p = 0.01$). The relation between aging and Na/K-ATPase enzyme activity is well defined *(24,25).* The oxidative destruction of membrane lipids, decrement of cytosolic gylcolytic enzymes, and low intracellular K^+ concentration contributes to altered enzyme activity in the aging process *(26).* Thus it is reasonable to find low enzyme levels in aging individuals. However, in the patient group there was no correlation between age and enzyme levels. As there is a significant difference between the Na/K-ATPase levels of the study and control groups, the reason for the low enzyme levels of the patient group is due to factors other than aging. It was conceivable to think that poor glycemic control is related with erythrocyte Na/K-ATPase levels. However, in our study HbA1c levels were not correlated with Na/K-ATPase levels. To our knowledge the relationship betweeen poor glycemic control and decreased Na/K-ATPase levels have not been reported so far. The longer the duration of diabetes, the higher the incidence of complications. That may explain the association of the diabetes duration and low enzyme activities of the study population.

The gender differences of erythrocyte Na/K-ATPase levels were of interest. Although there was no difference in the study group, the female control subjects had significantly higher levels than the males. Former data indicate that the Na/K-ATPase levels can differ due to the sexual differences *(27,28).* The distinctions in erythrocyte Na/K-ATPase levels, was attributed to the hormonal differences of both sexes *(27).* However, the differences in Na/K-ATPase levels were noted in only control subjects. There are no data that account for this finding. It may be speculated that the erythrocyte Na/K-ATPase levels of women are more susceptible to decrements in diabetic complications.

Another point to be stressed is that deductions about the neurons, glomerules, and retinal cells were done from the erythrocyte Na/K-ATPase levels. Although all studies of this type were done from erythrocytes, it must be remembered that erythrocyte Na/K-ATPase activity may not represent Na/K-ATPase activity in all parts of the body *(29).*

In conclusion, the results of the present study indicate that erythrocyte Na/K-ATPase activities are significantly reduced in patients with type 2 diabetes. The enzymes in neuropathy and retinopathy are also lower than the patients without these complications. There is a significant correlation between diabetes duration and erythrocyte Na/K-ATPase activities which may be attributed to the time dependent increment of diabetic complications.

Material and Methods

Patients

One hundred type 2 diabetic subjects (62 female, 38 male) were evaluated by the outpatient clinics of Gulhane School of Medicine Departments of Internal Medicine and Endocrinology. A standard questionnaire was used to rule out other neuromuscular diseases, exposure to neurotoxins and family history of neuropathy. As the ethnic differences influence the enzyme levels *(15–17)* all the patients were carefully selected to have the similar ethnic features of Asia Minor *(30).* The patients were the residents of Ankara, the capital city of Turkey, although they were born in different regions of Turkey. Their actual language was Turkish and they and their grandparents had no history of immigration to Turkey. The patients were informed about the study and their written consent was taken.

To prevent confounding variables that might influence Na/K-ATPase levels other than diabetes, some subjects were eliminated. Twenty subjects were eliminated due to hypertension (13 female, 7 male), six subjects (3 male, 3 female) with congestive heart failure, two subjects (2 female) with nodular goiter taking L-tyroxine, one subject (male) with alcohol abuse, four subjects (2 male, 2 female) with lumbar and cervical discopathy who were not clearly discriminated from diabetic neuropathy.

The final group consisted of 42 female (age 30–70, mean 57.9 ± 9,0) and 25 male (age 33–70, mean 56.1 ± 10.5) totally 67 patients. The patients were all type 2 diabetic, normotensive, with normal thyroid and parathyroid functions. None of the patients had a family history of neuropathy and none had any hematological diseases; 45 patients (28 female, 17 male) were taking oral antidiabetic drugs, while 20 patients (13 female, 7male) were in insulin treatment. Two patients (1 male, 1 female) were not taking any medicine for diabetes at the time of study.

Control Subjects

Age and body mass index matched 13 healthy males (ages 35–70) and 12 healthy females (ages 35–71) were enrolled as the control group. The subjects were normotensive, without diabetes, and taking no medication. The control subjects also had the Anatolian ethnic profile. The physical examinations and laboratory tests were all normal.

Diagnosis of Neuropathy and Other Clinical Conditions

Peripheral neuropathy was diagnosed according to the criteria of DCCT *(21).* The patients having the two of the following three criteria were investigated:

1. Abnormal motor and sensorial system findings.
2. Symptoms of peripheral neuropathy.
3. Abolished deep tendon reflexes.

Motor transmission rate was calculated by the standard method of EMG. Retinopathy was assessed in three stages as 0 (no), 1 (background retinopathy), 2 (prolipherative retinopathy with neovascularisations, macular edema, additional complications).

Nephropathy was assessed in three levels according to the proteinuria states: 0 (no), 1 [urinary albumin excretion (UAE) = 30–300 mg/d]), 2 (macroalbuminuria, UAE > 300 mg/d or creatinine >1.5 mg/dL).

Reagents

KCl (Sigma, P-4504, USA), $MgCl_2$ (Sigma, M-9272, USA), NaCl (Sigma, S-7653, USA), Tris hydrochloride (Sigma, T-1503), trichloroactic acid (TCA) (Sigma), Na_2ATP (Sigma, A-3284, USA), ouabain (Sigma, O-3125, USA). All reagents were freshly prepared at the time of the examinations.

Extraction of Erythrocyte Membranes

After 12 h of fasting, 10 cm^3 venous sample from each subject were taken and put into the citrated tubes. In patients under insulin treatment, samples were taken without injection of the last two insulin doses. In the oral antidiabetic group, samples were taken after the discontinuation of the drug for 2 d. The study was performed immediately after the fresh venous specimens were taken. For erythrocyte membrane extraction, high-speed centrifugation was performed (IEC-Centra MP4R, USA). Hb-free ghost membranes were obtained according to the method of Dodge et al. *(31).* Samples were centrifuged in 5500*g* for 6 min. After the separation of buffy coat, the rest of the samples were washed twice with isotonic NaCl. After each washing, centrifugation was done and the supernatant was extracted. Final cells were lysed by the 5 mmol/L, ice-cold, pH 8.0, hypotonic phosphated buffer and centrifigutaed in 10°C for 40 min with 16000*g*. The extracted ghost membranes were kept at –60ºC until the time of analysis.

Calculation of the Amount of Erythrocyte Membrane Proteins

Membrane fractions were precipitated with 10% TCA and then 0.05% deoxycolic acid was added. Bovine serum albumin was used during the procedures. The mean protein content of the erythrocyte membrane suspensions was defined as mg protein/mL.

Calculation of Na/K-ATPase Activity

The membrane Na/K-ATPase activity was measured by the method of Kitao and Hattori *(32).* The pH of buffer solution was 7.7 and contained 40 mmol/L Tris hydrochloride, 14 mmol/L KCl, 140 mmol/L $MgCl_2$, and 5 mmol/L NaCl. The membranes were incubated in 37°C (0.1 mL membrane contains 0.2–0.35 mg protein). ATPase reaction was started by the addition of 3 mmol/L Na_2ATP and 20 min later ended with 1 mL of 15% TCA. After 5 min of centrifuging at 6000*g*, supernatant was extracted and 3 mL 0.1 *N* Na acetate, 0.4 mL molybdate/H_2SO_4 mixture (1:1), and finally 0.4 mL of 1% ascorbic acid were added; 20 min later 800 nm spectrophotometric readings were done (UV-2100 S, Shimadzu, Japan).

The studies were performed in duplicate, with and without ouabain. The difference between the amounts of inorganic phosphates in two calculations was defined as the Na/K-ATPase activity. The results of the calculations were written as micromole Pi/mg protein/h.

Statistics

The results were written as mean ± standard deviation. The homogeneity of the variances were calculated with the Levene test, the differences between the two groups were analyzed with Mann–Whitney U test, and the differences between the means of all the groups were analyzed with ANOVA test. Levels of $p < 0.05$ were expressed as significant. The correlations between enzyme activities and ages, serum glucose, LDL cholesterol, HDL cholesterol, triglycerides, HbA1c levels, and body mass indexes were analyzed in both patient and control groups. The correlation of diabetes duration with the above parameters were analyzed in the patients' group. Pearson's test was used for the correlation analysis. Chi square test was used in order to find out whether Na/K-ATPase levels were related to diabetes duration, smoking, family history of diabetes, and the number of subgroups.

References

1. UK Prospective Diabetes Study (UKPDS) Group. (1998). *Lancet* **12,352(9131),** 837–853.
2. Raccah, D., Fabreguettes, C., Azulay, J. P., and Vague, P. (1996). *Diabetes Care* **19(6),** 564–568.
3. Scarpini, E., Bianchi, R., Moggio, M., Sciacco, M., Fiori, M. G., and Sciacco, G. (1993). *J. Neuro. Sci.* **120,** 159–167.
4. Jannot, M. F., Raccah, D., Dufayet de la, T. D., Coste, T., Gouvernet, J., and Vague, P. (1999). *Diabetes Metab.* **25(1),** 35–42.
5. Raccah, D., Gallice, P., Pouget, J., and Vague, P. (1992). *Diabetes Metab.* **18(3),** 236–241.
6. Takigawa, T., Yasuda, H., Terada, M., et al. (2000). *Neuroreport* **3(11),** 2547–2551.
7. Mimura, M., Makino, H., Kanatsuka, A., Asai, T., and Yoshida, S. (1994). *Horm. Metab. Res.* **26(1),** 33–38.
8. Pulido, J. S. (1999). *Ophthalmology Clinics of North America* **12,** 563–571.

9. Totan, A. R. and Greabu, M. (2002). *Acta Pol. Pharm.* **59(4),** 307–311.
10. Raccah, D., Lamotte-Jannot, M. F., Issautier, T., and Vague, P. (1994). *Diabetes Metab.* **20(3),** 271–274.
11. Bozzo, C., Goria, M., Marengo, C., Marena, S., Veglia, F., and Pagano, G. (1990). *Metabolism* **39,** 808–814.
12. Mimura, M., Makino, H., Kanatsuka, A., Asai, T., and Yoshida, S. (1994). *Horm. Metab. Res.* **26(1),** 33–38.
13. De La Tour, D., Raccah, D., Jannot, M. F., Coste, T., Rougerie, C., and Vague, P. (1998). *Diabetologia* **41,** 1080–1084.
14. Okegbile, E. O., Odusan, O., and Adeola, O. (1997). *Biosci. Rep*. **17(5),** 499–506.
15. Pirart, J. (1977). *Diabetes Metab.* **3,** 97–107, 173–182, 245–256.
16. Beutler, E., Kuhl, W., and Secks, P. (1983). *N. Engl. J. Med.* **309,** 756–760.
17. Lasker, N., Hopp, L., Grossman, S., Bamfoth, R., and Aviv, A. (1985). *J. Clin. Invest.* **75,** 1813–1820.
18. Migdalis, I. N., Xenos, K., Chairopoulos, K., Varvarigos, N., Leontiades, E., and Karmaniolas, K. (2000). *Diabetes Res. Clin. Pract*. **49(2–3),** 113–118.
19. Costantino, L., Rastelli, G., Vianello, P., Cignarella, G., and Barlocco, D. (1999). *Med. Res. Rev.* **19(1),** 3–23.
20. Djemli-Shipkolye, A., Gallice, P., Coste, T., et al. (2000). *Metabolism* **49(7),** 868–872.
21. The Diabetes Control and Complications Trial Research Group. (1995). *Ann. Int. Med.* **122,** 561–568.
22. Jannot, M. F., Raccah, D., De La Tour, D. D., Coste, T., and Vague, P. (2002). *Metabolism* **51(3),** 284–291.
23. Raccah, D., Dadoun, F., Coste, T., and Vague, P. (1996). *Horm. Metab. Res.* **28(3),** 128–132.
24. Gambert, S. R. and Duthie, E. H. (1983). *J. Gerontol.* **38(1),** 23–25.
25. Mazzanti, L., Rabini, R. A., Salvolini, E., et al. (1997). *Metabolism* **46(1),** 59–61.
26. Mazzanti, L., Faloia, E., Rabini, R. A., et al. (1992). *Clin. Biochem.* **25,** 41–46.
27. Persky, V., Ostrow, D., Langenberg, P., Ruby, E., Bresolin, L., and Stamler, J. (1990). *J. Hypertens.* **8(2),** 121–128.
28. Smith, J. B., Wade, M. B., Fineberg, N. S., and Weinberger, M. H. (1988). *Hypertension* **12,** 251–258.
29. Tsong, T. Y. and Chang, C. H. (2003). *Physica A* **321,** 124–138.
30. Arnaiz, V. A., Karin, M., Bendikuze, N., et al. (2001). *Tissue Antigens* **57(4),** 308–317.
31. Dodge, J. T., Mitchell, C., and Hanahan, D. J. (1963). *Arch. Biochem. Biophys.* **100,** 119–130.
32. Kitao, T. and Hattori, K. (1983). *Experientia* **39(12),** 1362–1364.