Impaired NA^+,K^+ATPase Activity in Red Blood Cells in Euthyroid Women Treated With Levothyroxine After Total Thyroidectomy for Graves' Disease

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In patients suffering from hyperthyroidism dependent on Graves' disease, a reduction in Na^+,K^+ATPase activity has been demonstrated in red blood cells (RBCs), as well as an inverse correlation between this enzymatic action and free triiodothyronine (FT_3) levels. The restoration of normal FT_3 values also brings about a normalization of Na^+,K^+ATPase activity in erythrocytes. These results have made it possible to hypothesize that the thyroid hormones control Na^+,K^+ATPase activity and that this control is manifested by means of variations in the number of ouabain-binding sites. For this reason, the measurement of the activity of the Na/K pump can be considered as a further indicator of the peripheral effects of thyroid hormones. With a view to assess the relation between the course of treated hyperthyroidism and Na^+,K^+ATPase activity during antithyroid therapy and after surgical thyroidectomy followed by replacement therapy, we studied 24 patients affected by Graves' disease (group Graves [GG]). They were compared with 24 female Graves' patients who underwent total thyroidectomy for nontoxic and diffuse nodular goiter (NDNG) (group control [GC]) and with 24 normal healthy women (group normal [GN]). When Graves' hyperthyroidism was diagnosed, the Na^+,K^+ATPase activity in RBCs was impaired in all GG patients. Thionamide treatment restored the normal activity of the Na/K pump, accompanied by normalization of the number of ouabain-binding sites. One hundred eighty days after thyroidectomy, in conditions of clinical and biochemical euthyroidism due to replacement therapy with levothyroxine, the activity of Na^+,K^+ATPase in RBCs was once again reduced in GG, while appearing normal in GC and GN (1.77 ± 0.16 mmol Pi $h^{-1} \cdot L^{-1}$ RBCs v 2.09 ± 0.26 v 2.09 ± 0.24, $P < .05$). Different instrumental or biochemical parameters, such as glycemia, serum lipids, ions, serum alkaline phosphatase (AlPh), serum creatine phosphokinase (CPK), blood pressure, and heart rate, were evaluated and appeared normalized in GG and GC 180 days after surgery. We conclude that (1) in patients suffering from Graves' disease, subjected to total thyroidectomy followed by levothyroxine replacement therapy, there is a reduction in the activity of the Na^+,K^+ATPase on erythrocytes 6 months after the surgical approach; and (2) a similar alteration is not observed in patients subjected to thyroidectomy for NDNG. These findings allow the formulation of the hypothesis that (1) treatment with levothyroxine for 180 days after thyroidectomy in GG is not long enough to restore the normality of all the peripheral indicators of action of the thyroid hormones; and (2) levothyroxine replacement therapy is unable to guarantee euthyroidism in all the tissues in GG (eg, during hematopoiesis in the bone marrow).

IN HYPERTHYROID PATIENTS, the activity of Na^+,K^+ATPase and the number of ouabain-binding sites in red blood cells (RBCs) are impaired[1-7] and may be normalized by restoring a normal level of serum free triiodothyronine (FT_3) using thionamide therapy.[6] Although the mechanism by which hyperthyroidism mediates the disappearance of pump units in RBCs remains unclear,[8] it is likely that, since RBCs lack a nucleus and cannot synthesize new proteins, the recovery of enzyme activity during hyperthyroidism is not possible. It has been suggested that it may be considered a direct consequence of the increased FT_3, which causes a breakdown of many membrane compounds (ie, phospholipids and proteins) and consequently causes an alteration in sodium influx and calcium uptake.[6,8] This degradation may occur in circulation during the aging of RBCs or during maturation in bone marrow.[1,3] On the contrary, in hypothyroid patients, the activity of Na^+,K^+ATPase in RBCs has been described both as normal or defective.[3,7] Moreover, the measurement of Na^+,K^+ATPase activity has been considered as a marker of relapse in patients with Graves' disease who are under going medical therapy.[9] In light of these statements, the measurement of the activity of the Na/K pump can be considered as a further indicator of the peripheral effects of thyroid hormones.[6,8,9]

In patients who underwent total thyroidectomy or in hypothyroid subjects, replacement therapy with levothyroxine is able to provide normalization of FT_3, free thyroxine (FT_4) and thyrotropin (TSH). Other biochemical and clinical parameters, such as lipids, enzymes, and ECG, became normal during replacement therapy, but there is no information about the activity of Na^+,K^+ATPase in Graves' patients after total thyroidectomy while receiving levothyroxine.

The purpose of this study was to verify whether, in patients affected by Graves' hyperthyroidism subjected to total thyroidectomy, the activity of Na^+,K^+ATPase in RBCs is normalized during euthyroidism due to levothyroxine replacement therapy.

PATIENTS AND METHODS

The study protocol was approved by the ethical committee of our hospital, and informed, written consent was obtained from all subjects.

We screened 96 consecutive hyperthyroid female patients attending the Endocrinology Department of our hospital (mean age, 30 ± 2.4 years). All were clinically hyperthyroid and affected by Graves' disease as confirmed by a plasma thyroid hormone test, antibody determination, thyroid scans, and clinical grounds. After the diagnosis of hyperthyroidism, all patients started antithyroid therapy using methimazole at a dosage of 15 to 20 mg/d. Antithyroid treatment was progressively reduced when patients became clinically and biochemically euthyroid, and the subjects were kept euthyroid under treatment by adjusting the dose of methimazole.[10,11] No β-blockers, iodine, or corticosteroids were used. In 18 patients, methimazole was discontinued, but 12 patients developed a relapse of hyperthyroidism that required the reinstitution of treatment. Following the course of Graves' disease, 24 patients were

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considered for total thyroidectomy; it was established in 12 cases because the patients experienced relapse of hyperthyroidism after discontinuing methimazole treatment, in eight cases because of unhealthy thyroid-associated ophthalmopathy, and in four cases because of rapid increase in goiter size.[11] The patients who underwent thyroidectomy were enrolled onto the study (group Graves [GG]). As suggested from the description given by the patients or from medical information received by their home-care physician, the time that lapsed between the onset of symptoms caused by thyrotoxicosis and the moment when Graves' disease was diagnosed in GG was 13 ± 7 months (range, 1 to 25); the time between the diagnosis of Graves' disease and the surgical approach was 16 ± 6 months (range, 8 to 30). GG were compared with 24 women enrolled as follows: 30 affected by nontoxic diffuse and nodular goiter (NDNG) were screened; in light of their clinical and instrumental features (particularly as space-occupying lesions), they were candidates for surgical thyroidectomy; if histologic evaluation performed after surgery was consistent with the diagnosis of NDNG, they were enrolled onto the study (two women were excluded because a histologic examination showed occult papillary carcinoma, and four because of histologic evidence of follicular adenoma); finally, the group was composed of 24 female patients (mean age, 31 ± 1.9 years; group control [GC]); they never used suppressive therapy with levothyroxine.

A second control group was composed of 24 female normal volunteers in good health (mean age, 29 ± 3.1 years; group normal [GN]) who were clinically and biochemically euthyroid, as determined by three tests over 180 days.

Total thyroidectomy was followed in GG and GC patients by the start of replacement therapy with levothyroxine using a dosage of 1.2 μg/kg/d; no calcium or vitamin D was used. During the course of the study, none of the subjects manifested other diseases or took any other drug. Considering both the estimated premorbid and final body mass index (BMI, kg/m^2), none were obese (BMI < 25); furthermore, there was no history of familial hypertension or diabetes mellitus.

In all GG and GC patients, thyroid hormone serum concentrations TSH, glycemia, serum lipids, ions, serum alkaline phosphatase (AlPh), serum creatine phosphokinase (CPK), blood pressure, heart rate, activity of Na^+,K^+ATPase, and the number of ouabain-binding sites in RBCs were examined at the time of the diagnosis of Graves' disease or NDNG (time 0), 7 days before surgery (during persistent euthyroidism due to thionamide treatment), and 180 days after surgery in a euthyroid state due to levothyroxine replacement therapy. The interval of 180 days was considered necessary and sufficient to obtain a condition of euthyroidism and a new population of mature erythrocytes. The state of thyroid function was tested once more in all patients 30 and 60 days after surgery with the aim to confirm the state of biochemical euthyroidism (data not shown).

Plasma FT_4 (normal values in our laboratory, 6.0 to 14.0 pg/mL) and FT_3 (normal values, 2.6 to 5.6 pg/mL) were determinated by radioimmunoassay using a commercial kit from Diagnostic Products (Milan, Italy). Plasma TSH (normal values, 0.3 to 4.0 UI/mL) was determinated by IRMA using commercial kits (Ares-Serono, Milan, Italy). 3-H ouabain (activity, 22 Ci/mmol [814 GBq/mmol]) was purchased from Amersham International, Amersham, UK; ouabain and adenosine triphosphatase (ATP) were purchased from Sigma Chemical (St Louis, MO).

Erythrocyte Studies

All subjects were studied after an overnight fast. Twenty milliliters of venous blood was collected from an antecubital vein for erythrocyte studies. Plasma was separated from heparinized blood by centrifugation at 1,000 × *g* for 20 minutes and further centrifuged at 3,000 × *g* for 10 minutes to eliminate remaining white blood cells and platelets.

Na^+,K^+ATPase activity. Na^+,K^+ATPase activity was determined as previously described.[6] Briefly, packed erythrocytes were hemolyzed by freezing and thawing three times in a dry ice/methanol mixture. An aliquot of the hemolysate was incubated in duplicate in a buffer solution at final concentrations of ATP 2 mmol/L, NaCl 100 mmol/L, $MgCl_2$ 3 mmol/L, KCl 25 mmol/L, and EGTA 1 mmol/L. The mixtures were incubated for 2 hours and the reaction was stopped by the addition of trichloroacetic acid (0.6 mol/L); the phosphate in the supernatant was determinated by the method of Daly and Ertinghausen[12] on a centrifugal analyzer (Cobas-Bio, Milan, Italy). ATPase activity in RBCs was calculated as micromoles of phosphate liberated per hour per liter of RBCs. Na^+,K^+ATPase activity is expressed by the difference between total and ouabain-insensitive ATPase activities.[6,13,14]

Ouabain-binding sites. Packed erythrocytes were washed three times with excess ice-cold $MgCl_2$ solution (110 mmol/L, 290 mOsm/kg) and resuspended at a hematocrit of 20%. A 0.5-mL quantity of this suspension was incubated with 3-H ouabain (7.4×10^{-9} mol/L), together with different concentrations of nonradioactive ouabain ranging from 9.25×10^{-10} to 1.85×10^{-7} mol/L. To determine the nonspecific binding, cells were incubated in the presence of excess of ouabain (10^{-2} mol/L). Following incubation at 37°C for 4 hours, an aliquot (0.1 mL) of the cells was washed four times with ice-cold $MgCl_2$ and the bound ouabain was extracted three times with perchloric acid (1.7 mol/L) and counted in an aliquot scintillation counter with

Table 1. Clinical Data of Subjects Studied

Variable	GG (n = 24)			GC (n = 24)			GN (n = 24)		
Time (d)	0	7 BS	180 AS	0	7 BS	180 AS	0	180	360
Fasting glucose (70-110 mg/dL)	96 ± 8	81 ± 3	82 ± 6	87 ± 8	88 ± 5	89 ± 5	80 ± 3	83 ± 4	88 ± 6
Total cholesterol (<190 mg/dL)	142 ± 14	166 ± 10	159 ± 17	147 ± 13	148 ± 20	157 ± 14	139 ± 21	142 ± 13	144 ± 15
Triglycerides (50-170 mg/dL)	96 ± 10	121 ± 18	132 ± 15	130 ± 13	140 ± 9	129 ± 14	133 ± 11	120 ± 19	112 ± 23
Serum CPK (10-70 UI/L)	34 ± 7	42 ± 8	44 ± 10	32 ± 9	40 ± 7	30 ± 7	37 ± 9	42 ± 7	43 ± 10
Serum AlPh (50-190 UI/L)	196 ± 18	157 ± 10	142 ± 12	103 ± 21	97 ± 17	105 ± 13	89 ± 17	92 ± 14	100 ± 18
Serum K (3.5-5.0 mmol/L)	4.0 ± 0.2	4.1 ± 0.2	3.9 ± 0.2	4.1 ± 0.2	3.9 ± 0.1	4.1 ± 0.2	4.0 ± 0.1	4.1 ± 0.2	3.9 ± 0.2
Serum Na (135-145 mmol/L)	140 ± 1	138 ± 1	140 ± 1	141 ± 1	137 ± 2	140 ± 1	138 ± 2	139 ± 1	140 ± 1
Heart rate (beats/min)	103 ± 6	76 ± 4	78 ± 3	74 ± 6	77 ± 3	77 ± 5	72 ± 4	74 ± 3	76 ± 4
Blood pressure (mm Hg)									
Systolic	135 ± 7	123 ± 3	120 ± 3	120 ± 4	121 ± 3	120 ± 4	117 ± 2	119 ± 3	117 ± 3
Diastolic	81 ± 2	76 ± 2	77 ± 2	73 ± 3	74 ± 3	75 ± 2	72 ± 3	75 ± 1	75 ± 1

NOTE. Results are means ± SD; Normal values are indicated in parentheses for biochemical parameters.
Abbreviations: 7 BS, 7 days before thyroidectomy; 180 AS, 180 days after thyroidectomy.

Table 2. Summary of Results of Thyroid Function Tests

Variable	GG (n = 24)			GC (n = 24)			GN (n = 24)		
Time (d)	0	7 BS	180 AS	0	7 BS	180 AS	0	180	360
Free T_3 (2.6-5.6 pg/mL)	21.7 ± 2.7*†	3.7 ± 0.5	3.6 ± 0.6	3.7 ± 0.3	3.8 ± 0.5	4.0 ± 0.6	4.0 ± 0.3	3.8 ± 0.4	4.1 ± 0.7
Free T_4 (6.0-14.0 pg/mL)	45.1 ± 8.1*†	9.4 ± 1.2	9.2 ± 1.3	10.0 ± 1.3	9.4 ± 1.5	9.5 ± 1.4	8.9 ± 1.9	9.6 ± 0.9	9.2 ± 1.5
TSH (0.3-4.0 μUI/mL)	<0.01†	2.0 ± 0.6	2.2 ± 0.7	2.1 ± 0.4	2.1 ± 0.7	2.3 ± 0.4	2.3 ± 0.7	2.0 ± 0.4	2.1 ± 0.5

NOTE. Results are means ± SD; Normal values are indicated in parentheses for thyroid function tests.
*$P < .05$ *v* GC.
†$P < .05$ *v* GN.

Beckmann Ready-Solv scintillation fluid (Beckmann Instruments, Geneve, Switzerland; LS 9800).

Statistical Analysis

The number of binding sites and the dissociation constant were calculated by Scatchard analysis.[6] Precision of the assay (expressed as coefficient of variation) determinated by analysis in duplicate was 8.7% for Na^+,K^+ATPase activity and 5.5% for the number of ouabain-binding sites.

Statistical analysis was performed using ANOVA followed by *t* test for paired or unpaired data if necessary ($P < .05$ was considered statistically significant). Correlations were obtained by simple regression analysis.

RESULTS

Clinical data of the subjects studied are listed in Table 1. Hyperthyroid subjects had elevated FT_4, FT_3, and reduced TSH at the time of diagnosis of Graves' disease (Table 2). Thionamide therapy restored a normal level of FT_4 and FT_3 in all patients after 60 days (data not shown). TSH values also increased, but did not reach the normal level. Following replacement therapy, GG and GC were all euthyroid after 60 days from surgery (data not shown) and remained euthyroid 180 days after surgery (Table 2).

As expected, Na^+,K^+ATPase activity in RBCs was impaired in all GG patients at the time of diagnosis of Graves' disease; it was normal in all subjects 7 days before surgery, but 180 days after surgery, the activity of Na^+,K^+ATPase was newly impaired in GG patients.

The number of ouabain-binding sites showed a parallel pattern with the activity of Na^+,K^+ATPase (Table 3). Glycemia, serum lipids (triglicerydes, total and high-density lipoprotein-cholesterol), ions, AlPh, CPK, blood pressure, and heart rate were normal in GG and GC 180 days after surgery (Table 1).

We can observe a linear regression between the time lapsing from the onset of the symptoms caused by thyrotoxicosis and the moment of the total thyroidectomy and the decline in Na^+,K^+ATPase activity in GG ($r^2 = .904$; $P < .05$) (Fig 1).

DISCUSSION

In this work, we have demonstrated that in women treated by total thyroidectomy for Graves' disease and placed on replacement therapy with levothyroxine, the activity of Na^+,K^+ATPase in RBCs is still reduced 180 days after surgery. The decline in Na^+,K^+ATPase activity observed during untreated hyperthyroidism and, in GG patients, 6 months after surgery, may be reasonably justified by the reduction in the number of sodium pumps. In turn, the decrease in the number of sodium pumps cannot be interpreted as a consequence of a condition of hypothyroidism or hyperthyroidism, as all of the GG patients reassessed during replacement therapy with levothyroxine appeared to be clinically and biochemically euthyroid and no symptoms or other parameters tested (ECG, lipids, electrolytes, and enzymes) showed evidence of subclinical hypothyroidism or hyperthyroidism. Consequently, this circumstance requires some other explanations. First and foremost, we have to emphasize that in patients subjected to thyroidectomy for NDNG, there are no defects in the activity of Na^+,K^+ATPase even during replacement therapy, and in GG patients, the extent of the ATPase defect is correlated with the time lapsing from the onset of the symptoms linked to thyrotoxicosis and the moment of the total thyroidectomy.

We know that the breadth of the cellular response to the thyroid hormones normally depends on the number of T_3 receptors.[15,16] This means that a pathological increase in thyroid hormones level (such as occurs during the thyrotoxic phase of Graves' disease) could cause a compensating reduction in the

Table 3. Summary of Results of Na^+,K^+ATPase Activity, Number of Oubain-Binding Sites, and Dissociation Constant

Variable	GG (n = 24)			GC (n = 24)			GN (n = 24)		
Time (d)	0	7 BS	180 AS	0	7 BS	180 AS	0	180	360
Na^+,K^+ATPase activity (mmol Pi $h^{-1} \cdot L^{-1}$ RBCs)	1.35 ± 0.23*†	2.07 ± 0.22	1.77 ± 0.16*†	2.08 ± 0.20	2.1 ± 0.25	2.09 ± 0.26	2.07 ± 0.27	2.09 ± 0.30	2.09 ± 0.24
No. of ouabain-binding sites	201 ± 18*†	305 ± 27	265 ± 20*†	308 ± 26	309 ± 16	308 ± 20	313 ± 17	311 ± 22	310 ± 18
Dissociation constant (nmol/L)	3.21 ± 0.43	3.11 ± 0.61	3.14 ± 0.50	3.10 ± 0.47	3.21 ± 0.39	3.16 ± 0.62	3.11 ± 0.43	3.20 ± 0.53	3.13 ± 0.62

NOTE. Results are means ± SD.
*$P < .05$ *v* GC.
†$P < .05$ *v* GN.

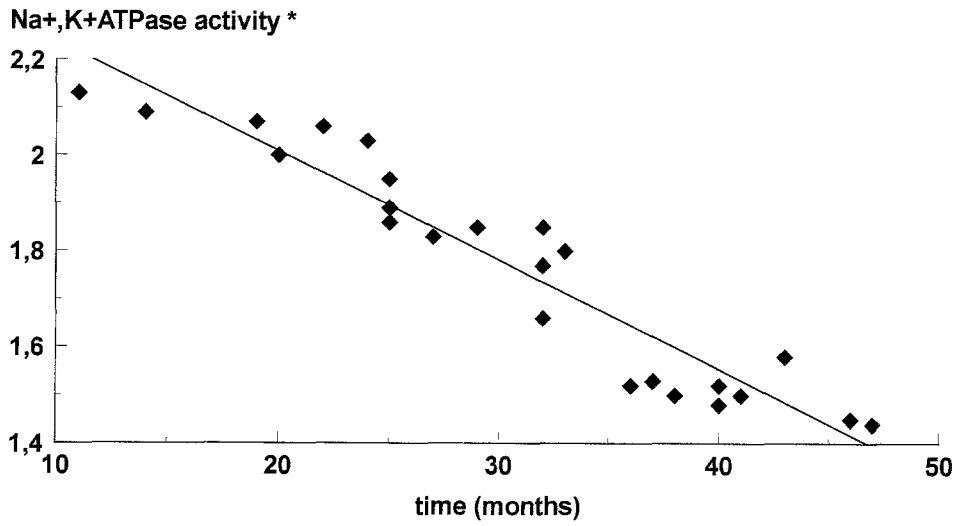

Fig 1. Linear regression between time lapsing from onset of symptoms caused by thyrotoxicosis and moment of surgical thyroidectomy and activity of Na^+,K^+ATPase in RBCs. *Micromoles phosphate generated per hour per liter RBCs. Regression data: $Y = -0.022 \times + 2.465$; $r^2 = .904$; $P < .05$.

number and/or affinity of the receptors, as a countermeasure to the effect of the excess thyroid hormones. Whereas the circulating erythrocytes are unnucleated cells, it was obviously not possible to evaluate the state of the receptors in this study. However, we can suggest—though without proof—that a hypothetical reduction in density and/or affinity of the T_3 receptors, occurring during marrow maturation and related to prolonged hyperthyroidism, may explain the decline in the effect of the thyroid hormones, with a mechanism similar to a downregulation phenomenon.

The present findings would suggest that, in the regulation of the expression of Na^+,K^+ATPase by thyroid hormones, a theoretical control device of the energy balance may be involved, and that in the final analysis, this could act on the ATP expenditure. The net result of the activation of this could bring about a saving in energy expenditure during the thyrotoxic phase of Graves' disease and the recovery period after thyroidectomy, suggesting the possibility that this mechanism operates at the peripheral level (ie, in RBCs cells and/or in bone marrow) modifying the sensibility to thyroid hormones even in the presence of normal levels of FT_3 and FT_4,[6,8,9] thus determining a resetting of various enzymatic activities, such as Na^+,K^+ATPase. Finally, if we consider the Na^+,K^+ATPase activity as a indicator of the peripheral action of the thyroid hormones,[6,17-19] it appears evident that in GG patients, at least one of the peripheral FT_3 and FT_4 actions is not completely normalized during levothyroxine replacement therapy. Alternatively, since thyroid hormones promote the synthesis of the pump units,[20] it may be possible that the reduction in ouabain-binding sites is due to the inability of the replacement therapy to ensure euthyroidism in all tissues, at least in GG patients. This was recently demonstrated in thyroidectomized rats.[21,22] In particular, the thyroid hormone may act on the RBCs during the period of maturation in the bone marrow in a contest of clinical and biochemical situations of euthyroidism only in a specific condition of genetic or immunologic predisposition. Moreover, as suggested later, the peripheral level of thyroid hormones may be different from the level of thyroid hormones in single organs (eg, bone marrow). Another possibility is that the hormonal thyroid variations, when they are minimal, do not precede an alteration of Na^+,K^+ATPase, which may be damaged, when considered as an energetic cellular system,[23] by some other kind of noxia (eg, stress, viruses)[24,25] that induce and perpetuate the energetic, hormonal, and clinical imbalance in thyroidectomized patients.

In summary, our data demonstrate impaired Na^+,K^+ATPase activity in RBCs of thyroidectomized Graves' patients during euthyroidism due to levothyroxine replacement therapy, which is linked to a reduction of the number of ouabain-binding sites. Considering those findings, it is difficult to determine whether the condition may be reversible, but it is not improved by replacement therapy with levothyroxine. Consequently in GG patients 180 days after thyroidectomy, replacement therapy with levothyroxine was unable to normalize all of the peripheral indicators of thyroid hormone activity.

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