

Evaluation of Peripheral Metabolic Status by Determination of Na-K ATPase Pump Activity in Circulating Erythrocytes in Patients with Thyroid Diseases and Nonthyroidal Illnesses

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Abstract. The number of Na-K ATPase units in erythrocytes (RBC) was determined by the maximal ouabain binding assay in 25 normal subjects and patients with hyperthyroidism (n=29), hypothyroidism (8), chronic renal failure (CRF, 19) and with neoplastic disorders (NP, 12). The activity of the pump units was also assessed by measuring ouabain-sensitive ^{86}Rb uptake in some of these subjects. In addition, it was determined in mononuclear cells in normal controls and patients with hyper- and hypothyroidism and CRF. Significant diminution of the number of the RBC pump units was found in hyperthyroidism, while it was increased in hypothyroidism. The binding (O) of old RBC was significantly lower than that (Y) of young RBC and a striking correlation was observed between the % reduction rate ((Y-O)/Y) of the binding and the serum T_4 level in hyperthyroidism ($r=0.85$, $P<0.02$). No difference was observed in pump units of mononuclear cells in normal and hyper- and hypothyroidism. It is suggested that the thyroid hormone-mediated disappearance of the pump units in RBC may play a role in reducing the number of pump units in RBC in hyperthyroidism. The ratio of RBC ^{86}Rb uptake to the number of the pump units in the same cell (U/B) bore a significant relation to serum T_3 ($r=0.48$, $P<0.05$) and T_4 ($r=0.49$, $P<0.05$) indicating that the U/B is a useful index for the peripheral metabolic status. In CRF patients with low T_3 levels, bindings were increased but those in NP with low T_3 was almost normal. The increased bindings were observed in NP patients with normal T_3 . These findings suggest that the metabolic status in CRF and NP patients could not be always assessed by the number of the ouabain sensitive Na-K ATPase pump units. However, the U/B was almost normal in all patients with CRF and NP, suggesting that pump activity as a measure of oxygen consumption and thermogenesis may not be in the hypothyroid state in patients with nonthyroidal illnesses.

Key words: Nonthyroidal illness, Na-K ATPase, Erythrocyte, Metabolic status, Maximal ouabain binding.
(Endocrine Journal 40: 27–33, 1993)

IT IS WELL known that thyroid hormones stimulate the activity of Na-K ATPase in rat liver, kidney, and skeletal muscles [1, 2]. In contrast to these animal data, the reduction in the number of Na-K ATPase in circulating erythrocytes (RBC) of hyperthyroid patients was observed by us [3] and others [4, 5]. Since RBC lacks a nucleus and cannot synthesize new proteins, the number of RBC Na-K

ATPase may be controlled by the rate of degradation.

Moreover, the cation transport activity of RBC was also reduced in hyperthyroid patients [4]. However, further studies are needed to investigate the relationship between the number of Na-K ATPase pump units and the cation transport in human subjects.

In the present study, the maximal ouabain bindings were determined in mononuclear cells as well as circulating RBC from normal subjects and hyperthyroid patients, and, moreover, the bindings were separately determined in young and old

Received: July 1, 1992

Accepted: October 16, 1992

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RBC from these subjects, to compare the degradation rate of RBC pump units. Furthermore, the changes in the amount of cation transported by each pump unit (as expressed by the ratio of the RBC ^{86}Rb uptake to the maximal ouabain binding) were investigated in normal subjects and in patients with hyperthyroidism or hypothyroidism. In addition, to assess the metabolic status in patients with nonthyroidal illnesses (NTI), this ratio was also determined in patients with chronic renal failure (CRF) and with neoplastic disorders.

Subjects and Methods

Blood samples for determining RBC Na-K ATPase pump units and/or ^{86}Rb uptake were obtained from 25 normal subjects and 29 patients with untreated Graves' disease, 8 with untreated primary hypothyroidism, 19 with CRF receiving regular hemodialysis and 12 with neoplastic disorders (originally affected organs: lung, 5; gastrointestinal, 4; liver, 2 and mesothelium, 1). Moreover, blood was drawn from 14 normal subjects and 21 patients with hyperthyroid Graves' disease, 6 with primary hypothyroidism and 14 with CRF for measuring the number of Na-K ATPase pump units in mononuclear cells.

The diagnoses of hyperthyroidism and hypothyroidism were established on the basis of the clinical picture and the determination of T_4 , T_3 and TSH levels in serum. All patients with CRF had been on regular hemodialysis for 1 to 6 months, and blood was drawn at the start of hemodialysis. None of the patients with hyperthyroidism, hypothyroidism or neoplastic disorders had renal diseases. The body weight of normal subjects and the patients were all below 120% of the optimal body weight calculated by the modified Broca's method.

Approximately 20 ml of blood was drawn from each subject into a heparinized syringe. The numbers of Na-K ATPase units in RBC were measured by maximal ouabain binding assay as described by DeLuise, Blackburn and Flier [6]. [^3H] ouabain (specific activity, 2.0MBq/ μg) was purchased from New England Nuclear, Boston, MA, USA, and was used within 6 months after receipt. All assays were done in duplicate. The specific bindings of [^3H] ouabain to RBC from all subjects in the presence of unlabeled ouabain

ranging from 6.25 to 50 nM resulted in a gradual increase and reached a plateau at concentrations from 50 to 250 nM. We therefore regarded the specific bindings with the unlabeled ouabain of 125 nM as the maximal ouabain binding to RBC. This maximal binding represented the number of Na-K ATPase pump units and it was expressed as pmol/ 10^9 RBC. The intraassay coefficient of variation was 13% and the interassay variability was 12% in the present assay. RBC from 5 normal subjects and 7 patients with hyperthyroidism were divided into low and high density portions by discontinuous "percoll" density gradient centrifugation as described by Salvo *et al.* [7]. Thus, the maximal ouabain bindings in relatively light or young RBC and in heavy or old RBC were separately determined in each subject.

Mononuclear cells were separated by the discontinuous "percoll" density gradient centrifugation method of Hjorth [8], and contamination of cells other than mononuclear cells was less than 5%. The numbers of Na-K ATPase units in mononuclear cells were also determined by the method of DeLuise, Blackburn and Flier [6]. The maximal ouabain binding was expressed as pmol/ 10^9 mononuclear cells. The activity of Na-K ATPase in RBC was assessed by measuring ouabain-sensitive ^{86}Rb uptake according to the method of DeLuise, Blackburn and Flier [6]. ^{86}Rb (specific activity, 0.18MBq/ μg) was obtained from New England Nuclear. The pump-mediated uptake of ^{86}Rb was calculated as the difference between the total radioactivity taken up by RBC and that taken up in the presence of excess ouabain (100 μM). All assays were done in duplicate and the activity was expressed as nmol/ 10^9 RBC/h.

Serum concentrations of T_4 , T_3 and TSH were measured with commercial RIA kits (normal ranges were 4.5–13.5 $\mu\text{g/dl}$, 76–160 ng/dl and 0.54–5.9 $\mu\text{U/ml}$, respectively).

Data are expressed as the mean \pm SEM. Student's paired or unpaired *t*-test was used to compare the means for the two groups and the least squares method was employed to analyze the correlation between the two groups.

Results

The principal results obtained are presented in Table 1.

Table 1. Various measurements of red blood cells in normal controls and patients with hyper- and hypothyroidism and with chronic renal failure (CRF) and neoplastic disorders

	T ₄ μg/dl	T ₃ ng/dl	MOB pmol/ 10 ⁹ RBC	Y pmol/ 10 ⁹ RBC	O pmol/ 10 ⁹ RBC	(Y-O)/Y %	⁸⁶ Rb uptake nmol/ 10 ⁹ RBC/h	U/B ×10 ³ /h
Normal control	7.5±0.3	121±4.8	0.48±0.03(25)	0.65±0.11(5)	0.53±0.09(5)	19±3(5)	95±6(11)	208±20(11)
Hyperthyroidism	22.4±1.2*	460±35*	0.26±0.01(29)*	0.28±0.02(7)	0.18±0.02(7)	34±7(7)	92±6(8)	409±53(8)*
Hypothyroidism	2.9±0.7*	93±11*	0.67±0.06(8)*				70±10(3)	132±24(3)
CRF Group I	5.4±0.4*	55±3 *	0.65±0.05(11)*				104±16(4)	162±22(4)
CRF Group II	4.9±0.8*	89±5*	0.44±0.05(8)				86±4(7)	215±21(7)
Neoplasma Group I	6.6±1.0	54±6 *	0.48±0.03(5)				109±17(5)	227±43(5)
Neoplasma Group II	8.5±0.7	83±2 *	0.68±0.07(7)*				99±11(7)	158±29(7)

Mean±SEM. Numbers in parentheses indicate the number of patients studied. MOB, Maximal ouabain binding; Y, Young RBC; O, Old RBC; U/B, ⁸⁶Rb uptake/MOB. **P*<0.01 compared with normal control (unpaired *t*-test). †*P*<0.01 compared with each other values (paired *t*-test).

Maximal ouabain binding to RBC

The maximal ouabain binding to RBC ranged from 0.24 to 0.74 pmol/10⁹ RBC in 25 normal subjects; values averaged 0.48±0.03 pmol/10⁹ RBC (Table 1). In 11 of 29 hyperthyroid patients, the bindings were apparently below the normal range, and the mean value in 29 patients was significantly lower than that in normal subjects (0.26±0.01 *vs.* 0.48±0.03 pmol/10⁹ RBC in the normals, *P*<0.01, Table 1). In 8 patients with hypothyroidism, the maximal ouabain bindings were normal or slightly elevated (Table 1). The mean value (0.67±0.06 pmol/10⁹ RBC) for the bindings in hypothyroidism was high (*P*<0.01). The scatter diagram of the relationship between the maximal ouabain binding and serum T₃ level in normal subjects and in patients with thyroid diseases revealed a significant inverse correlation (*r*=−0.56, *P*<0.01). The relation between the bindings and serum T₄ was also statistically significant (*r*=−0.64, *P*<0.01).

In 5 normal subjects, the bindings of the higher density portion of RBC, obtained by a discontinuous density gradient centrifugation, averaged 0.53±0.09 pmol/10⁹ RBC, values significantly lower than those of the lower density portion in the same subjects (0.65±0.11 pmol/10⁹ RBC, *P*<0.01,

Table 1). The even more marked reduction in the bindings in the higher density RBC was found in 7 hyperthyroid patients (0.18±0.02 in the higher density RBC *vs.* 0.28±0.02 pmol/10⁹ RBCs in the lower density RBC, *P*<0.01, Table 1). The ratios of the differences between the bindings of the lower density RBC and those of the higher density RBC to the bindings of lower density RBC ((Y-O)/Y) tended to increase in the hyperthyroid patients (34±7%), when compared with those in normal subjects (19±3%, Table 1). Moreover, a striking correlation was evident in the plot of serum T₄ levels and the ratios in patients with hyperthyroid Graves' disease (*r*=0.85, *P*<0.02).

In 11 of 19 patients with CRF, serum T₃ levels were markedly diminished and they were classified as CRF group I, and T₃ levels in the remaining 8 patients were almost within the normal range (CRF group II, Table 1). Patients admitted to hospital or those with low physical activity were mainly included in group I. Serum T₄ levels were diminished in 2 in CRF group I and in 4 in CRF group II. In 11 in CRF group I, the maximal ouabain bindings ranged from 0.34 to 0.92 pmol/10⁹ RBC. Despite the wide scatter, the mean value for the bindings in CRF group I was high (0.65±0.05 *vs.* 0.48±0.03 pmol/10⁹ RBC in the normals, *P*<0.01, Table 1). The maximal ouabain

bindings in CRF group II showed some scatter (0.44 ± 0.05 pmol/ 10^9 RBC), but there was no significant difference from the normal value (Table 1).

The marked diminution of serum T_3 levels were observed in 5 of 12 patients with neoplastic disorders and they were classified as NP group I. A patient in this group had low T_4 . Serum T_4 and T_3 levels in the remaining 7 patients were almost within normal limits and were classified as NP group II (Table 1). Patients in group I tended to suffer from severer disease than in group II. In contrast to the results obtained in CRF patients, the maximal ouabain bindings in the NP group I with low T_3 averaged 0.48 ± 0.03 pmol/ 10^9 RBC, and there was no significant difference between the bindings in this group and those in normal subjects (Table 1). The bindings in the NP group II with normal T_3 were higher (0.68 ± 0.07 pmol/ 10^9 RBC), than those in normal subjects (Table 1).

Pump mediated ^{86}Rb uptake by RBC

The RBC ^{86}Rb uptake, which reflects the Na-K ATPase activity, ranged from 64 to 129 nmol/ 10^9 RBC/h in 11 normal subjects; values averaged 95 ± 6 nmol/ 10^9 RBC/h (Table 1). The ^{86}Rb uptake was also determined in 8 hyperthyroid and 3 hypothyroid patients and the mean values were 92 ± 6 and 70 ± 10 nmol/ 10^9 RBC/h, respectively (Table 1). No significant differences in the mean values for RBC ^{86}Rb uptake were found among these 3 groups. The RBC ^{86}Rb uptake bore no significant relation to the maximal ouabain binding in normal subjects and hyperthyroid and hypothyroid patients.

To assess the amount of ^{86}Rb transported by each pump unit, the ^{86}Rb uptake was divided by the number of Na-K ATPase pump units (U/B). In 11 normal subjects, the U/B values ranged from 138 to 335 ($\times 10^3/\text{h}$), the mean being 208 ± 20 ($\times 10^3/\text{h}$, Table 1). In 8 hyperthyroid patients, the ratios ranged from 244 to 718 ($\times 10^3/\text{h}$) and the mean value was significantly higher than that in normal subjects (Table 1). On the other hand, the ratios averaged 132 ± 24 ($\times 10^3/\text{h}$) in 3 patients with hypothyroidism; values did not differ significantly from those in normal subjects (Table 1). As shown in Fig. 1, the ratios bore a significant relationship to the serum T_3 level in 11 normal subjects, 9 patients with hyperthyroid Graves'

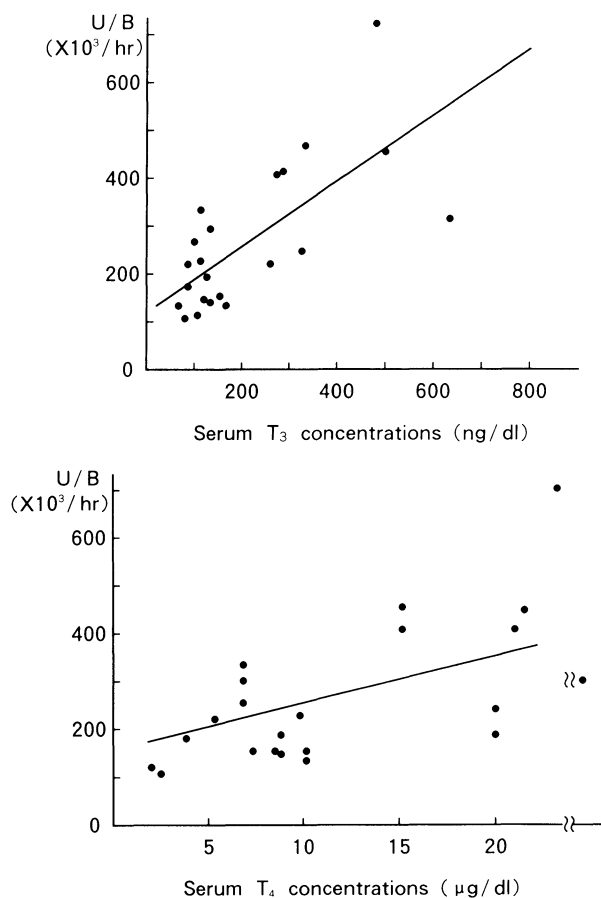


Fig. 1. Correlation of the ratios of ^{86}Rb uptake to the number of Na-K ATPase pump units (U/B) and serum T_3 (upper panel) and T_4 (lower panel) levels in 11 normal subjects, 8 patients with hyperthyroid Graves' disease and 3 with hypothyroidism ($r=+0.48$ (T_3) and 0.49 (T_4), $P < 0.05$).

disease and 3 with hypothyroidism ($r=+0.48$, $P < 0.05$). A significant relationship was also observed between the ratio and T_4 level in serum from these subjects ($r=+0.49$, $P < 0.05$, Fig. 1).

In 11 patients (4 in CRF group I and 7 in CRF group II) with CRF, RBC ^{86}Rb uptake was determined (Table 1). Values for ^{86}Rb uptake in 4 patients in CRF group I with low serum T_3 levels were within the normal range (Table 1). Although the maximal ouabain bindings were slightly increased in 2 of 4 patients, the mean U/B value in 4 patients did not differ significantly from the normal value (Table 1). RBC ^{86}Rb uptake and maximal ouabain binding were within the normal range in 7 patients in CRF group II (Table 1). The ratios were also normal in these patients (Table 1).

In 5 patients in NP group I and 7 patients in NP group II, RBC ^{86}Rb uptake averaged 109 ± 17 and

99 ± 11 nmol/ 10^9 RBC/h, respectively and these mean values were almost the same as that for the normal subjects (Table 1). The maximal ouabain bindings were within the normal range in 5 patients in NP group I with low T_3 levels and the mean ratio did not differ significantly from that of the normal subjects (Table 1). Although the U/B values were diminished in 3 of 7 patients in NP group II with normal T_3 , no significant difference was found between the mean value for the ratio in patients in NP group II and that in normal subjects (Table 1). As anticipated from these results, no significant correlation existed between the U/B and serum T_3 or T_4 level in patients with CRF and with neoplastic disorders.

Maximal ouabain binding to mononuclear cells

As ouabain binding to mononuclear cells with unlabeled ouabain ranging from 50–250 mM showed an increase and a plateau, we regarded the specific bindings with the unlabeled ouabain of 120 mM as the maximal ouabain binding to mononuclear cells. The maximal ouabain binding to mononuclear cells in 14 normal controls ranged from 16 to 58 pmol/ 10^9 cells, averaging 38 ± 3.7 pmol/ 10^9 cells. In 21 patients with hyperthyroidism, it ranged from 10 to 64, and in those with hypothyroidism, 36 to 55 pmol/ 10^9 cells. The mean values in these two groups did not differ significantly from each other. The maximal binding to mononuclear cells did not correlate to that to RBC in normal controls and in patients with hyper- and hypothyroidism.

On the other hand, although the maximal ouabain binding to mononuclear cells in 14 patients with CRF showed a wide scatter from 35 to 105, the mean value (60 ± 17) was statistically significantly higher than that in normal controls. There was, however, no significant correlation between serum T_4 or T_3 and the binding to mononuclear cells.

Discussion

It has been demonstrated that thyroid hormones increase the Na-K ATPase activity in rat liver, kidney and skeletal muscles [1, 2]. Since the ouabain binding is a specific property of Na-K ATPase, [3 H] ouabain binding assay was de-

veloped to measure the number of enzyme units in RBC. In contrast to the results obtained in animal experiments, the maximal ouabain binding was diminished in RBC from patients with hyperthyroidism [3–5]. Although this discrepancy might be ascribed to the tissue specificity or the length of time the tissue was exposed to thyroid hormones, the exact mechanism responsible for the reduction in the number of RBC Na-K ATPase pumps in hyperthyroidism is still unknown.

In the present study, to elucidate a possible effect of thyroid hormones on the rate of disappearance of the enzyme from RBC membrane, the maximal ouabain bindings in light or young and heavy or old RBC, fractionated by a discontinuous density gradient centrifugation, were separately determined in normal subjects and patients with hyperthyroid Graves' disease. The numbers of the pump units in the older RBC were significantly less than those in the younger RBC. Moreover, it was remarkable that the magnitude of this reduction correlated with serum T_4 levels in hyperthyroidism. The present findings suggest that the thyroid hormone-mediated degradation of Na-K ATPase may play a part in reducing the number of pump units in RBC in hyperthyroidism.

On the other hand, there was no difference in the maximal ouabain binding to mononuclear cells in normal controls and hyper- and hypothyroidism. It is suggested that, in mononuclear cells, both synthesis and degradation are affected in thyroid dysfunction, resulting in an unchanged number of Na-K ATPase pump units. Therefore, the maximal ouabain binding to mononuclear cells is not considered to be a useful indicator of peripheral thyroid hormone action.

The increase in myocardial ouabain binding was previously observed in guinea pigs given T_3 and this increase was parallel to the enhanced cation transport without any apparent change in the enzyme kinetics, indicating that T_3 induced an increase in the number of myocardial Na-K ATPase pump units [2, 9]. However, it is still controversial whether or not thyroid hormones stimulate Na-K ATPase activity without any alteration in the number of enzymes. Studies in human subjects have shown a decrease in both the number of pump units and cation transport activity, measured by the ^{86}Rb technique, in RBC from hyperthyroidism [4]. However, it is noteworthy that the

magnitude of the reduction in RBC cation transport was smaller than the alteration observed in the number of pump units [4]. This may mean that the amount of ^{86}Rb transported by each pump unit is increased in patients with hyperthyroidism.

In the present study, the pump mediated ^{86}Rb uptake by RBC was divided by the maximal ouabain binding to calculate the amount of ^{86}Rb transported by each pump unit. This amount was greater in hyperthyroidism than that in normal subjects, and it tended to diminish in hypothyroidism. Of special interest is the relationship between this amount and serum T_3 or T_4 level in normal subjects and in patients with hyperthyroidism and hypothyroidism. It is therefore considered possible from the present results that the amount of ^{86}Rb transported by each pump unit is a useful indicator of the peripheral metabolic status in human subjects. The present findings also suggest that thyroid hormones might have a direct or indirect effect on Na-K ATPase in RBC membrane, although this is a tentative speculation pending more extensive findings.

Serum concentrations of T_3 and T_4 are often decreased in patients with NTI [10], although they are not considered hypothyroid on the basis of clinical findings [11]. Dasmahapatra *et al.* [12] found an increase in the number of Na-K ATPase pump units in RBC from patients with NTI, and they postulated that tissue hypothyroidism might exist in these patients [12]. In the present study, the maximal ouabain binding in RBC was high in CRF patients with low T_3 in serum, while it was within the normal range in the patients with normal T_3 . These findings were consistent with those reported in a previous study. However, in the present study, the number of the pump units was normal in the patients with neoplastic disorders, despite a lowering of serum T_3 levels, and an even greater increase in the number of RBC pump units was found in the patients with normal T_3 levels in serum. Since patients with various disorders were included in NTI, the regulatory effects of biosynthesis and metabolic turnover of Na-K ATPase may be different in several NTI

patients. The present findings suggest that the metabolic status in NTI patients could not always be assessed by the number of ouabain sensitive Na-K ATPase pump units.

A dissociation between the number of RBC Na-K ATPase pump units and its cation transport activity was found in patients with anorexia nervosa [13], and a diminution in the amount of ^{86}Rb transported by each pump unit was observed in the anorectic patients [13]. In the present patients with CRF and with neoplastic disorders, the ratio of RBC ^{86}Rb uptake to maximal ouabain binding did not differ significantly from normal subjects, regardless of whether the T_3 level in serum was normal or diminished. Izumo *et al.* [14] reported that the cation transport turnover rate of the RBC Na-K pump is impaired in CRF by a circulating factor, which could be removed by hemodialysis. In the present study, despite a significant increase in the number of pump units, RBC ^{86}Rb uptake values were almost normal in CRF patients with lowered T_3 levels. This is consistent with the findings by Izumo *et al.* [14]. Moreover, an increase in the number of RBC pump units in patients with neoplastic disorders, who had normal T_3 levels in serum, was not always accompanied by an increase in enzyme activity. However, the deviation from the normal range was less pronounced in the maximal ouabain binding of RBC from these patients, so that the amount of cation transported by each pump unit is almost normal. An increase in pump units in mononuclear cells in CRF suggests that the degradation of the pump is slowed down in these patients, but the clinical significance remains unclear.

The present findings regarding the pump activity have additional interest in view of problems in the metabolic status of NTI patients. It is conceivable from the present results that Na-K ATPase pump activity as a measure of oxygen consumption and thermogenesis may not be in the hypothyroid state in NTI patients. Further studies on the pump activity in tissue other than in peripheral blood cells are needed to evaluate the real metabolic status in NTI.

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