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Effects of detyrosinated tubulin on Na^+,K^+-ATPase activity and erythrocyte function in hypertensive subjects

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This paper is dedicated in memory of Marina Rafaela Amaiden ("Rafa") who will remain forever in our hearts.

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ABSTRACT

Formation of tubulin/Na^+,K^+-ATPase (NKA) complex in erythrocytes of hypertensive subjects results in a 50% reduction in NKA activity. We demonstrate here that detyrosinated tubulin, which is increased in hypertensive erythrocytes membranes, enhances the inhibitory effect of acetylated tubulin on NKA activity. Moreover, we report a reduced content and activity of the enzyme tubulin tyrosine ligase in erythrocytes of hypertensive subjects. Such alterations are related to changes in erythrocyte deformability. Our findings indicate that the detyrosination/tyrosination cycle of tubulin is important in regulation of NKA activity, and that abnormalities in this cycle are involved in hypertension development.

1. Introduction

Tubulin is biosynthesized with a tyrosine residue at the carboxy terminus of its α-chain, and then is called *tyrosinated tubulin* (Tyr-tubulin) [1]. After tubulin is polymerized into microtubules, a tubulin tyrosine carboxypeptidase (TTCP) removes the carboxy-terminal tyrosine to produce *detyrosinated tubulin*, which is also known as Glu-tubulin because glutamic acid becomes exposed as the carboxy-terminal amino acid [2,3]. Following depolymerization from microtubules, detyrosinated tubulin can be tyrosinated again through the action of *tubulin tyrosine ligase* (TTL), and so the cycle continues [4,5]. Balance between the two isotypes involved in this cycle is essential for normal cell development. Abnormalities in detyrosinated tubulin levels have been implicated in various pathologies, including hypertension and tumor progression [6–8].

We reported recently that tubulin is found in three fractions (membrane, cytoplasmic and sedimentable fraction) of human erythrocytes [6] and formation of an Ac-tubulin complex with Na^+,K^+-ATPase (NKA) in the membrane [6] could be responsible of lower NKA activity in erythrocytes from hypertensive subjects observed by other groups [9,10].

We demonstrated previously that tubulin must be in acetylated form to interact with NKA [11]. However, no studies to date have examined the possible roles of other post-translational modifications of tubulin, particularly detyrosination, in the regulatory mechanism mentioned above. Based on our finding of increased detyrosinated tubulin levels in hypertensive erythrocyte membranes [6], we hypothesized that the detyrosinated tubulin isotype, in addition to Ac-tubulin, is involved in the regulation of NKA activity. The results of the present study demonstrate that: (i) although acetylation of tubulin is essential for complex formation with NKA, detyrosinated tubulin appears to play an important role

Abbreviations: GluTub, detyrosinated tubulin; AcTub, acetylated tubulin; TyrTub, tyrosinated tubulin; NKA, Na^+,K^+-ATPase; TTCP, tubulin tyrosine carboxypeptidase; TTL, tubulin tyrosine ligase; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride

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in regulation of enzyme activity; (ii) the increased level of detyrosinated tubulin in hypertensive erythrocytes is due to decreased content and activity of TTL but not TTCP; (iii) membrane levels of detyrosinated tubulin are related to NKA activity and erythrocyte deformability, which are reduced in hypertensive subjects [12,13].

In view of these results, we propose that the reduced TTL activity in hypertensive erythrocyte membranes is responsible for increased levels of detyrosinated tubulin, which causes inhibition of NKA activity and erythrocyte deformability, and that both effects are closely related to the development of arterial hypertension.

2. Materials and methods

2.1. Materials

Nitrocellulose membrane, nocodazole, parthenolide, DMSO, carboxypeptidase pancreatic A bovine, PMSF, Triton X-100, SDS, pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, ATP, NADH, and Tween were obtained from Sigma–Aldrich (St. Louis, MO, USA). Lumigen™ PS-3 detection kit and high-performance chemiluminescence film were from GE Healthcare Life Sciences (Piscataway, NJ, USA).

2.2. Antibodies

Mouse anti-Ac-tubulin mAb 6-11B-1, mouse anti-α-tubulin mAb DM1A, anti-Tyr-tubulin mAb Tub1-A2, peroxidase-conjugated mouse or rabbit IgG, and fluorescein-conjugated mouse IgG were from Sigma. Rabbit polyclonal Ab H-300 specific to α-subunit of NKA was from Santa Cruz Biotechnology. Rabbit anti-TTL polyclonal Ab ID3 was kindly provided by Dr. J. Wehland (Max Planck Institute for Biophysical Chemistry, Goettingen, Federal Republic of Germany). Rabbit anti-detyrosinated tubulin polyclonal Ab was prepared as described by Gundersen et al. [14].

2.3. Human subjects and erythrocyte preparation

Patients at the Hospital Regional de Río Cuarto (Argentina) were recruited for this study. All procedures were performed in compliance with relevant laws and institutional guidelines and using an informed and written consent protocol approved by the Committee on Research Ethics (CoEdi) of the Universidad Nacional de Río Cuarto, which are in accord with the standards set by the Declaration of Helsinki. Demographic and hemodynamic data of the patients are shown in Table 1. Fresh blood samples were collected using 3.4 mM EDTA as an anticoagulant and stored for 96 h at 4 °C.

Table 1
Demographic and hemodynamic data of the studied population.

Parameters	H (n = 35)	N (n = 20)	P
Age (years)	43 ± 11	39 ± 14	–
Sex, male/female	22/13	11/9	
Weight (kg)	86 ± 17	79 ± 12	NS
Systolic BP (mmHg)	167 ± 18	121 ± 11	<0.05
Diastolic BP (mmHg)	109 ± 10	73 ± 7	<0.05
Triglycerides (mg/dl)	268 ± 13	103 ± 8	<0.01
Diabetes (yes/no)	0/35	0/20	–
Hypercholesterolemia (yes/no)	3/32	0/20	–
Antihypertensive treatment (yes/no)	19/16	–	–
Urea (mg/dl)	33 ± 7	34 ± 8	NS
Creatinine (mg/dl)	0.75 ± 0.13	0.71 ± 0.14	NS
Na^+ (mM)	141 ± 13	144 ± 10	NS
K^+ (mM)	5 ± 1	4 ± 2	NS

H hypertensive patients, N normotensive patients, NS not significant.

Blood sample pooling was always omitted. Individual blood samples were incubated (when required) with 50 μM nocodazole for 1 h at 37 °C or with 20 μM parthenolide for 2 h at 37 °C. In other experiments, erythrocytes were treated with 30 mM glucose in the presence or absence of 0.5 mM tyrosine for 2 h at 37 °C. In experiments where hypertensive blood samples were used, blood samples of patients with and without anti-hypertensive treatment were used. Following treatment, erythrocytes were isolated by conventional centrifugal separation and used immediately. Hypertensive subjects were selected based on a history of blood pressure consistently > 160/110 mm Hg.

2.4. Blood pressure measurement

Blood pressure was measured using a mercury sphygmomanometer after 10 min of rest in the seated position, 20–30 min after obtaining blood samples in the opposite arm to that subjected to venesection. Five consecutive BP readings were obtained using an appropriately sized cuff, 30–60 s apart. The average of the 5 readings was taken as the BP.

2.5. Isolation of cytosolic and membrane fractions from human erythrocytes

Different fractions of human erythrocytes were isolated following the method described by Amaiden et al. [6]. Briefly, erythrocytes isolated from 2 ml human blood were resuspended in 3 ml lysis buffer (7.5 mM sodium phosphate buffer, pH 7.5, containing 1 mM EDTA and 20 μg/ml PMSF) and incubated for 15 min in ice-water. The lysate was centrifuged (20000×g) for 20 min at 4 °C. The pellet was washed three times with 6 ml lysis buffer without PMSF, resuspended in 0.3 ml lysis buffer (membrane fraction), and stored at −20 °C until use, except when membranes were used as NKA source, in this case, they were used immediately. The supernatant fraction from the 20000×g centrifugation was immediately centrifuged at 100000×g for 30 min at 30 °C. The supernatant, containing cytosolic fraction, was passed through a Sephadex G-25 column pre-equilibrated with sodium phosphate buffer for TTL activity determination.

2.6. Animals

Male Wistar rats were housed (five per cage) under controlled temperature and humidity, with a cycle of 12 h light/12 h dark, and food and water ad libitum. All experimental protocols followed the guidelines of the Committee on Research Ethics (CoEdi) of the Universidad Nacional de Río Cuarto regarding the care and use of laboratory animals, minimization of suffering, and the number of animals used. Rats were maintained without treatment. Rats of 30- to 60-days-old were sacrificed and brains were removed to obtain membranes and tubulin. Rats were sacrificed by decapitation without anesthesia avoiding animal suffering.

2.7. Rat brain membranes

Rat brain membranes were obtained according to [15]. Brains from 30- to 60-day-old Wistar rats were homogenized at 4 °C in 5 volumes of Tris-sucrose buffer (10 mM Tris, pH 7.4, containing 0.24 M sucrose) and centrifuged at 1000×g for 5 min. The pellet was discarded, and the supernatant was centrifuged at 16000×g for 20 min at 4 °C. The pellet was washed twice with Tris-sucrose buffer and resuspended in water (3 ml per g of original tissue). The suspension was stirred for 30 min in ice-cold water and centrifuged at 16000×g for 20 min. The pellet was resuspended in TBS (1 ml per g of original tissue), and the suspension was used immediately.

2.8. Rat brain tubulin preparation

Brains from 30- to 60-day-old rats were homogenized at 4 °C in 1 volume of MEM buffer containing 1 mM $MgCl_2$. The homogenate was centrifuged at 100000×*g* for 45 min, and the pellet was discarded. Tubulin was purified by two cycles of assembly/disassembly followed by phosphocellulose chromatography. Purity and quality of tubulin was evaluated by SDS–PAGE and Coomassie Blue R250 staining (only one 55 KDa protein band in the gel was seen) and its ability to polymerize in microtubules (with GTP and taxol), respectively [15,16]. To obtain tubulin with a high content of the detyrosinated isotype, MAP-free tubulin was incubated for 40 min at 37 °C with 10 μg/ml bovine carboxypeptidase pancreatic A (CPA) as described by Barra et al. [17], and the preparation was kept on ice until use. Tubulin without CPA treatment was used as tubulin with low content of the detyrosinated isotype.

2.9. Incorporation of [^{14}C]tyrosine into tubulin

[^{14}C]Tyrosine was incorporated into tubulin as described by Alonso et al. [18]. In brief, the incubation medium contained, per ml, 0.9 ml rat brain extract (passed previously through a Sephadex G-25 column equilibrated with 10 mM sodium phosphate buffer, pH 7), 2.5 μmol ATP, 100 μmol KCl, 10 μmol $MgC1_2$, and 0.32 μmol (3 μCi) [^{14}C]tyrosine. The mixture was incubated at 37 °C for 30 min, passed again through the Sephadex G-25 column, measured for radioactivity bound to protein, and stored at 4 °C until use.

2.10. Determination of TTL activity in human erythrocytes

A cytosolic fraction of erythrocytes (300 μl) and 500 μg unlabeled three-cycle microtubule protein from rat brain were added to the reaction mixture (2.5 mM ATP, 100 mM KCl, 12.5 mM $MgCl_2$, and 0.32 mM (0.75 μCi) [^{14}C]tyrosine) and incubated at 37 °C. After 5 min of incubation, a 100-μl aliquot of the mixture was inactivated by addition of 20% trichloroacetic acid (100 μl). The mixtures were heated at 90 °C for 20 min, tubes were centrifuged at 10000×*g* for 30 min, and radioactivity incorporated into tubulin was measured by filtration through DE81 disc filter paper as described for [^{3}H]colchicine binding [19]. In control tubes, buffer solution was used instead of erythrocyte cytosolic fraction.

2.11. Determination of tubulin tyrosine carboxypeptidase activity

Determination of TTCP was done according [20]. The incubation system for this assay contained 0.3 ml erythrocyte cytosolic fraction and 100 μl (50 μg, 15000 cpm) [^{14}C]tyrosinated tubulin purified from rat brain and pre-incubated at 37 °C in the presence of 1 mM GTP and 10% glycerol for microtubule formation. The mixture was incubated at 37 °C for 30 min, inactivated by addition of 20% trichloroacetic acid (0.1 ml), and centrifuged at 10000×*g* for 30 min at 4 °C. Radioactivity was measured in an 80-μl aliquot of the supernatant fraction. Radioactivity released during incubation without tubulin tyrosine carboxypeptidase was subtracted from experimental values. Released [^{14}C]tyrosine was expressed as pmol of tyrosine released per mg protein in the erythrocyte cytosolic fraction.

2.12. Immunofluorescence microscopy

Erythrocytes were stained by indirect immunofluorescence as described by DeWitt et al. [21]. The primary antibodies used were mAb DM1A (dilution 1:1000), mAb Tub-1A2 (dilution 1:1000), or polyclonal anti-Glu-tubulin (dilution 1:200) to visualize tubulin, or polyclonal ID3 (dilution 1:100) to visualize TTL. The secondary antibody was fluorescein-conjugated anti-rabbit IgG (dilution 1:50) or anti-mouse IgG (dilution 1:50). Coverslips were mounted in Fluor-Save and observed with a fluorescence microscope (Nikon, Eclipse 50i). As we demonstrated previously [6], a uniform distribution of fluorescence in erythrocytes indicates a high content of Mem-tub, whereas a fluorescent ring pattern indicates a tubulin localization at the sedimentable fraction.

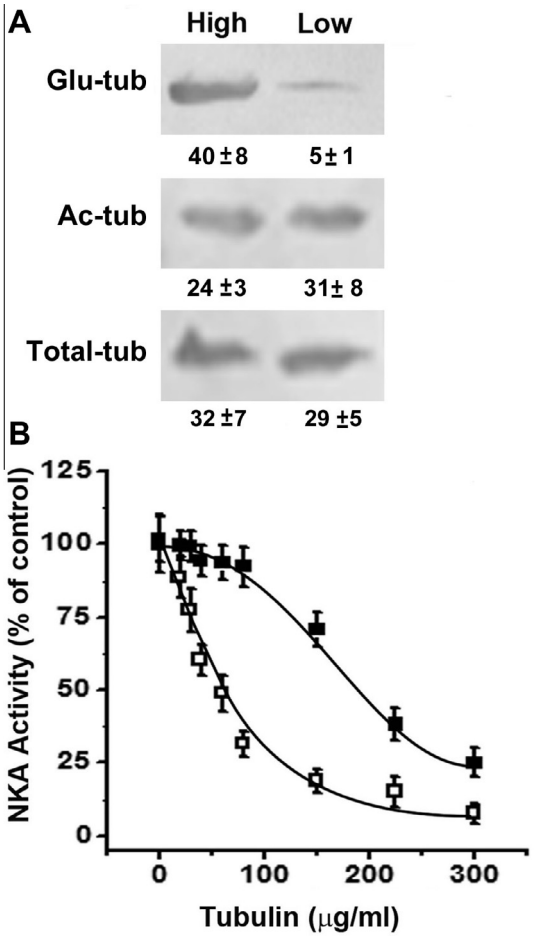


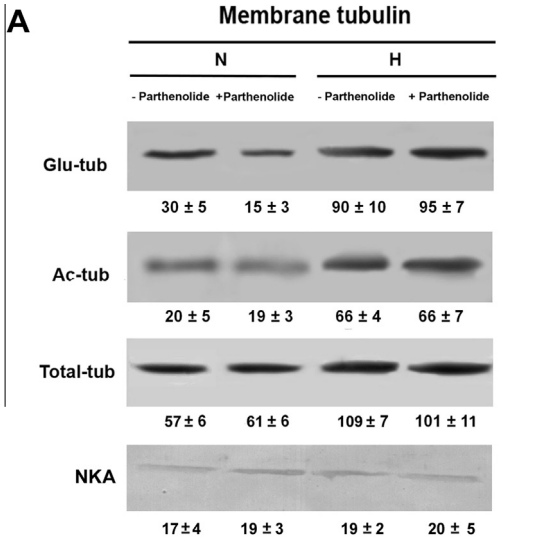
Fig. 1. Effect of detyrosinated tubulin on NKA activity. (A) Equal quantities of total tubulin were used for quantification of acetylated and detyrosinated tubulin by immunoblotting. Three experiments were performed, and regions containing relevant bands from a representative experiment are shown. (B) NKA activity was measured by the NADH oxidation method. Prior to ATP addition, the mixture was preincubated for 25 min at 37 °C with two tubulin preparations: (i) low detyrosinated tubulin content ("Low"; ■); (ii) high detyrosinated tubulin content ("High"; □). The "High" preparation was obtained by incubation of tubulin with 10 μg/ml bovine carboxypeptidase for 40 min at 37 °C. The data are expressed as a percentage of control value; mean ± S.D. from three independent experiments.

2.13. Immunoblotting

Proteins were separated by SDS–PAGE on 12% polyacrylamide slab gel by the method of Laemmli [22], and the gel was transferred to a nitrocellulose sheet. Blots were reacted with mAb DM1A (dilution 1:1000), 6-11B-1 (dilution 1:1000), ID3 (dilution 1:1000), or anti-Glu-tubulin (dilution 1:200). The sheet was reacted with the corresponding peroxidase-conjugated anti-IgG antibody and stained by the 4-chloro-1-naphthol method or the ECL system. Band intensities were quantified by the Scion Image software program.

2.14. NKA activity assay

NKA enzyme activity was determined by the method of Salvador and Mata [23]. Erythrocyte membranes (5–10 μg protein)



B

NKA activity

	- Parthenolide		+ Parthenolide	
	pmol/min mg of prot.	% of control	pmol/min mg of prot.	% of control
N	3.70 ± 0.5	100	7.03 ± 0.7	190 ± 10
H	1.78 ± 0.4	48 ± 11	1.67 ± 0.3	44 ± 20

Fig. 2. Parthenolide effect on NKA activity. Hypertensive (H) and normotensive (N) human erythrocytes were treated with 50 μM nocodazole for 1 h at 37 °C. The nocodazole was then removed, and the samples were resuspended in plasma with (+) or without (−) 20 μM parthenolide. The erythrocytes were lysed, and a membrane fraction was obtained. (A) Membranes were used to determine total, acetylated and detyrosinated tubulin by immunoblotting. A loading control was performed for NKA using rabbit polyclonal H-300 antibody. Three experiments were performed, and regions containing relevant bands from a representative experiment are shown. The values (mean ± S.D. from three independent experiments) shown below the blot represents quantification of tubulin bands by densitometry using Scion Image software. (B) NKA activity was measured by the NADH oxidation method. The data are expressed as a percentage of control value; mean ± S.D. from three independent experiments.

were added to the reaction mixture (50 mM Tris–HCl, pH 7.4, 20 mM KCl, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5 mM EGTA, 0.16 mM NADH, 1 mM phosphoenolpyruvate, 2.5 IU pyruvate kinase, 2.5 IU lactate dehydrogenase) in a final volume of 340 μl. The mixture was kept for 10 min at room temperature, and the reaction was initiated by addition of 1 mM ATP. NADH oxidation was measured for 15 min at room temperature using a recording spectrophotometer at wavelength 340 nm. Control cuvettes were prepared without enzyme or with heat-denatured enzyme. Enzyme activity was estimated as the difference between samples incubated in the absence vs. presence of 1 mM ouabain.

2.15. Determination of erythrocyte deformability

Erythrocyte deformability was determined using the filtration method described by Cabrales [24]. In brief, polycarbonate filters (Nuclepore/Whatman; Pleasanton, CA, USA) with pore size 5 μm, diameter 13 mm, and pore density $4 \times 10^3/mm^2$ were perfused with diluted cell suspensions using an infusion pump. Cell suspensions or medium (buffer) were delivered through the filter at a rate of 1.1, 1.2, 1.3, 1.4, or 1.5 ml/min. The pressure drop across the filter was measured with a differential pressure transducer. Erythrocyte deformability was assessed by determining the parameters β and ψ. β is the ratio of the resistance to flow through a filter pore calculated according to the equation:

$$\beta = (\mathrm{Pi}/\mathrm{Po} - 1)\upsilon/h + 1$$

where Pi is pressure drop across the filter, Po is the pressure drop in the presence of cells, υ is the ratio of mean cell volume to pore volume, and h is the volume of packed erythrocyte fraction of the perfusate. Filtration measurements were performed in triplicate at a hematocrit of 0.12, using different filters, and averaged. β is dependent on the flow used to deform the cells, while ψ is the slope of the curve β vs. flow. An increase in ψ reflects a decrease in erythrocyte deformability and is independent of the flow used to deform the cells.

2.16. Protein determination

Protein concentration was determined by the method of Bradford [25].

2.17. Statistical analysis

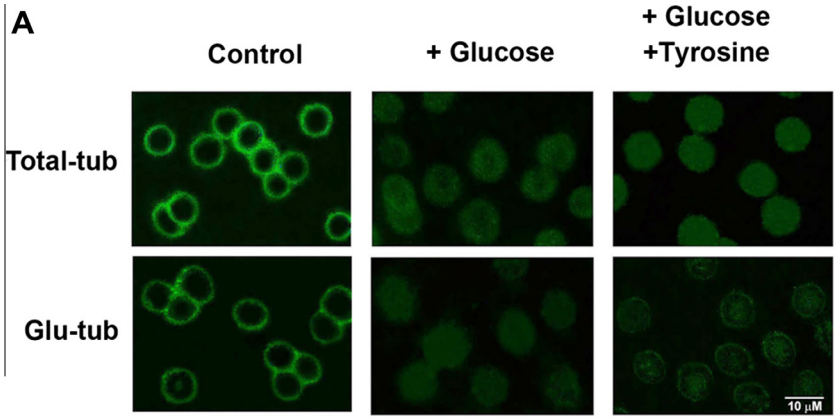
Results were expressed as mean ± SD. Student's *t*-test was used for the comparison of means between two groups. Differences in means were considered statistically significant for $P < 0.05$.

3. Results

3.1. Detyrosinated tubulin enhanced the inhibitory effect of Ac-tubulin on NKA activity

In hypertensive erythrocyte membranes, detyrosinated tubulin is the isotype showing the greatest increase [6], we therefore tested the possibility that detyrosination is involved in NKA regulation by measuring enzyme activity in rat brain membranes treated with two tubulin preparations that contained similar levels of total tubulin and Ac-tubulin but different levels of detyrosinated tubulin. The preparation pre-treated with bovine carboxypeptidase produced a 5-fold increase in detyrosinated tubulin content (Fig. 1A). Both tubulin preparations reduced NKA activity in a dose-dependent manner (Fig. 1B). However, for the preparation containing a higher proportion of detyrosinated tubulin, a concentration of 50 μg tubulin/ml was sufficient to produce a 50% inhibition of NKA activity and maximal inhibition (90%) was achieved with a concentration of 150 μg tubulin/ml. In contrast, for the preparation that had low detyrosinated tubulin content a concentration of 200 μg tubulin/ml was necessary to reach 50% NKA activity inhibition, and maximal inhibition was achieved with a concentration of 300 μg tubulin/ml (2-fold higher than for the first preparation). An incubation system of carboxypeptidase without tubulin was used as a control; this preparation had no effect on NKA activity (data not shown). These findings suggest that while acetylation is essential for tubulin/NKA interaction [11,15], detyrosination of tubulin enhances the inhibitory effect of Ac-tubulin on NKA activity.

To test this hypothesis, we conducted two experiments in which NKA activity of human erythrocytes was assayed in the presence of compounds that modify detyrosinated tubulin content. Parthenolide, which efficiently inhibits TTCP [26], was used in the first experiment (Fig. 2). Erythrocytes were treated with 20 μM parthenolide and detyrosinated, and Ac-tubulin content and NKA activity in the membrane were determined. The erythrocytes were incubated with nocodazole prior to parthenolide treatment to deplete membrane tubulin [6] and remove all tubulin associated with NKA. After the nocodazole was washed out, the cells were incubated with parthenolide (+parthenolide) for 2 h at 37 °C; during



B

	Control	+ Glucose	+ Glucose +Tyrosine
Total-tub (AU)	27 ± 3	29 ± 5	31 ± 4
Glu-tub (AU)	20 ± 3	19 ± 3	10 ± 2
NKA (pmol/min mg of prot.)	3.80 ± 0.6	2.65 ± 0.3	3.60 ± 0.2

Fig. 3. Effect of glucose/tyrosine on membrane detyrosinated tubulin and NKA activity. Human erythrocytes were treated with physiological solution (control), 30 mM glucose (+glucose) or 30 mM glucose and 0.5 mM tyrosine (+glucose + tyrosine) for 2 h at 37 °C. Following treatment, erythrocytes were washed twice and suspended in physiological solution. (A) Erythrocytes from each treatment were subjected to visualization of total tubulin and detyrosinated tubulin (Glu-tub) by immunofluorescence using specific antibodies as described in Section 2. (B) Fluorescence intensity for each cell were quantified using Scion Image software, values shown (mean ± S.D.) are an average of at least 30 cells by experiment from three independent experiments and were expressed as arbitrary units (AU). NKA activity was determined by the NADH oxidation method in membrane fractions. The data are expressed as pmol/min/mg protein; mean ± S.D. from three independent experiments.

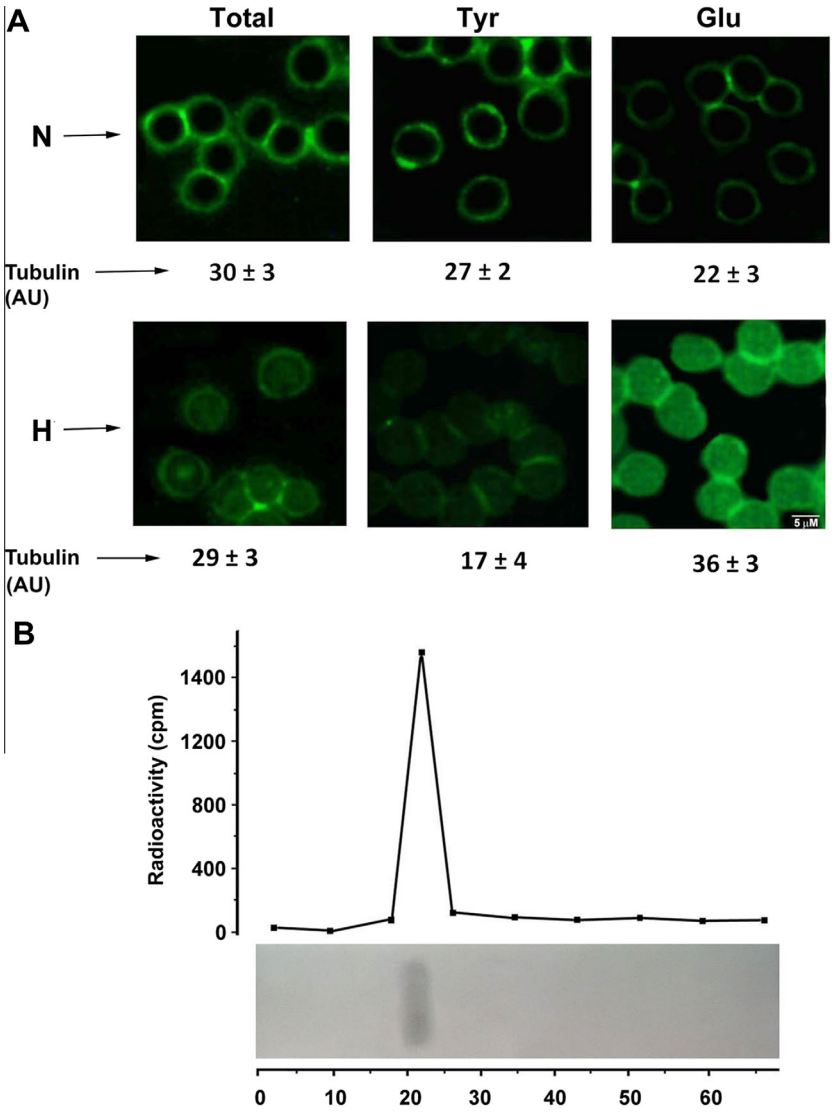
this time, new tubulin became associated with the erythrocyte membranes. As a control, erythrocytes were incubated under the same conditions but without parthenolide (−parthenolide). In the +parthenolide normotensive erythrocytes cells, detyrosinated tubulin levels in membranes were greatly reduced (∼50%), whereas levels of total tubulin and Ac-tubulin were unchanged (Fig. 2A). NKA activity was increased 90% in the +parthenolide cells (Fig. 2B). With hypertensive erythrocytes, parthenolide treatment did not cause significant changes of membrane levels of detyrosinated tubulin, Ac-tubulin, or NKA activity (Fig. 2).

In the second experiment, erythrocytes were treated with high glucose concentration (Fig. 3), which induces increased membrane tubulin content and consequent inhibition of NKA activity in human erythrocytes [27]. As we demonstrated previously, a uniform distribution of fluorescence in erythrocytes indicates a high content of Mem-tub, whereas a ring pattern of fluorescence indicates a low Mem-tub content [28]. The total content of the detyrosinated tubulin isotype was not significantly altered by glucose treatment (Fig. 3B), however, distribution was affected; in non-treated erythrocytes (control) fluorescence was seen as a thin peripheral fluorescence ring, indicating that detyrosinated tubulin was located in sedimentable fraction, whereas after glucose treatment, fluorescence is distributed through the entire cell, indicating a high tubulin content in the plasma membrane tubulin. Typical fluorescence signal for each fraction was previously determined by our group [6,28]. Then results suggest that detyrosinated tubulin was translocated to the membrane fraction following glucose treatment (Fig. 3A). A similar effect was observed for α-tubulin.

Consistently with the above findings, glucose treatment induced a significant reduction of NKA activity (Fig. 3B), suggesting that increased detyrosinated tubulin in the membrane is involved in NKA inhibition. If so, reducing detyrosinated tubulin content following glucose treatment should restore NKA activity. We tested the possibility that tyrosine reverses the effect of glucose on NKA activity. The presence of tyrosine caused a ∼50% reduction of detyrosinated tubulin content, and NKA activity was not significantly affected by glucose treatment (Fig. 3B). The distribution of detyrosinated tubulin was also modified under this condition and the translocation induced by glucose was partially inhibited; some detyrosinated tubulin was still observed in the membrane fraction, but it was also observed (intense fluorescence) in the sedimentable fraction. Taken together, the results from experiments with parthenolide, glucose, and tyrosine confirm that detyrosinated tubulin levels are involved in the regulation of NKA activity in human erythrocytes.

3.2. High detyrosinated tubulin content in hypertensive erythrocytes is correlated with low TTL expression

Our previous study showed that the proportion of detyrosinated tubulin is elevated in hypertensive erythrocyte membranes [6]. Similarly, in the present study, total tubulin content was similar in normotensive vs. hypertensive erythrocytes, but the distribution was altered in the latter group; tubulin was translocated from the sedimentable fraction to the membrane fraction (Fig. 4A). The distributions of the detyrosinated and tyrosinated tubulin isotypes were modified similarly to that of total tubulin: the levels of both isotypes were reduced in the sedimentable fraction and increased in the membrane fraction. In contrast to total tubulin, the levels of the detyrosinated and tyrosinated isotypes were modified in hypertensive erythrocytes: detyrosinated tubulin was increased 50% and tyrosinated tubulin was reduced by 40%.



C

	TTL activity		TTCP activity	
	Tyr incorporation (pmol/min mg of protein)	% of control	Tyr released (pmol/min mg of protein)	% of control
N	0.060 ± 0.01	100	0.0031 ± 0.001	100
H	0.034 ± 0.01	43 ± 5	0.0029 ± 0.001	94 ± 5

Fig. 4. Detyrosinated tubulin content and TTL and TTCP activities in hypertensive erythrocytes. (A) Normotensive (N) and hypertensive (H) human erythrocytes were subjected to visualization of total tubulin, tyrosinated (Tyr) and detyrosinated (Glu) tubulin by immunofluorescence using specific antibodies as described in Section 2. Fluorescence intensity for each cell were quantified using Scion Image software, values shown (mean ± S.D.) are an average of at least 30 cells by experiment from three independent experiments and were expressed as arbitrary units (AU). (B) Products of incorporation of [^{14}C]tyrosine into exogenous tubulin were subjected to immunofluorescence with mAb DM1A on nitrocellulose sheets. Each fraction was used to determine radioactivity. (C) TTL and TTPC activities were determined in cytosolic fractions of hypertensive or normotensive erythrocytes as described in Section 2. The values (mean ± S.D.) from five independent experiments were expressed as pmol/min/mg protein.

To clarify the origin of these changes, we measured the activities of TTCP and TTL in hypertensive and normotensive erythrocytes. TTL activity was assayed by measuring incorporation of [^{14}C]tyrosine into exogenous tubulin. An aliquot of the incubation products was subjected to SDS–PAGE, and the gel was stained with Coomassie Brilliant Blue. Over 95% of the protein was found in a

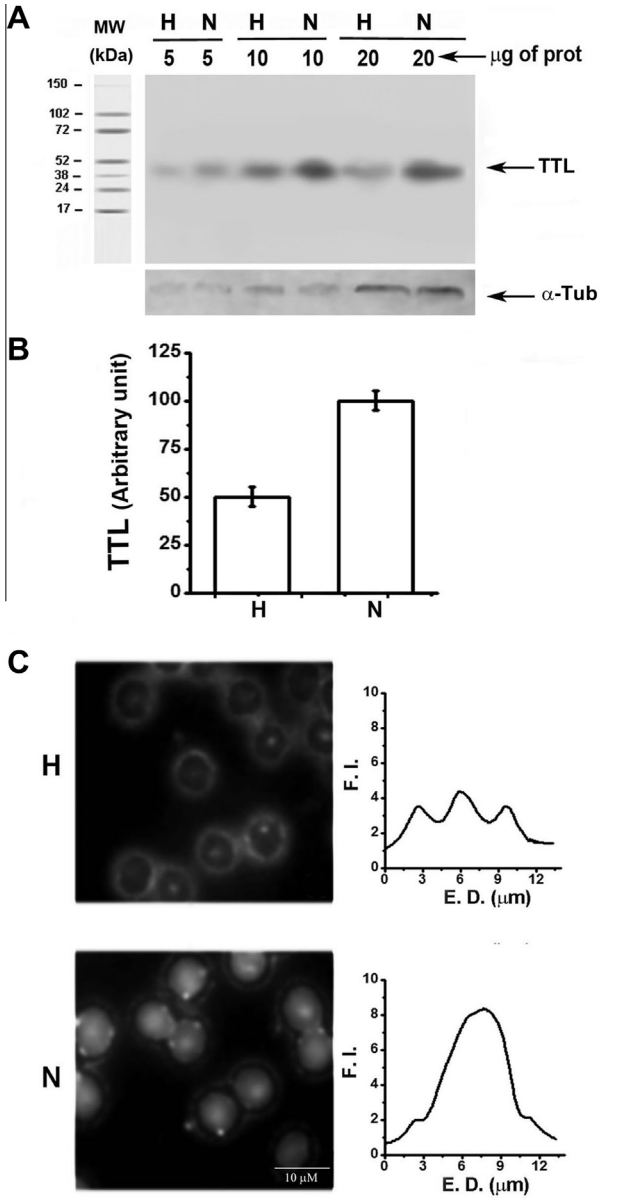


Fig. 5. TTL content in hypertensive human erythrocytes. (A) Cytosolic fractions of hypertensive (H) or normotensive (N) human erythrocytes were obtained and used to determine TTL content by immunoblotting using polyclonal anti-TTL antibody ID3 and ECL system to stain. The same western was probed with DM1A to quantified tubulin as loading control, using 4-chloro-1-naphthol staining method. Three experiments were performed, and regions containing relevant bands from a representative experiment are shown. (B) Bands were quantified using Scion Image software, and values are shown. The data are expressed as arbitrary units; mean ± S.D. from three independent experiments. (C) Hypertensive and normotensive erythrocytes were fixed on coverslips and analyzed by immunofluorescence microscopy (left panel) using ID3, as described in Section 2. Inner fluorescence through the erythrocyte was measured using Scion Image software, and values of a representative cell of at least 30 cells are shown in the right panel. F.I., fluorescence intensity. E.D., erythrocyte diameter.

single band corresponding to tubulin. This band also contained almost all of the radioactivity (Fig. 4B). The incorporation of radioactivity into tubulin indicates the presence of TTL in cytosolic fractions of human erythrocytes. TTL from both groups was able to incorporate [^{14}C]tyrosine; however, the activity was 0.06 pmol/min/mg protein in normotensive erythrocytes and 0.034 pmol/min/mg protein (43% lower) in hypertensive erythrocytes. These findings suggest that the increased detyrosinated tubulin level in hypertensive erythrocytes results from reduced TTL activity.

The observed decrease in TTL activity did not rule out the possibility that the increased detyrosinated tubulin level in hypertensive erythrocytes also involved a modification of TTCP activity. We therefore measured TTCP activity in extracts of normotensive and hypertensive erythrocytes. Both extracts contained measurable TTCP activity. However, in contrast to the results for TTL activity, no significant difference was observed between normotensive and hypertensive erythrocytes (Fig. 4C). This finding suggests that the increased detyrosinated tubulin level in hypertensive erythrocytes is due to reduced TTL activity but not to increased TTCP activity.

To determine whether the reduced TTL activity in hypertensive erythrocytes was correlated with changes in TTL content, we isolated cytosolic fractions of normotensive and hypertensive erythrocytes and analyzed TTL content by immunoblotting. Visual examination of bands revealed a markedly lower TTL content in hypertensive erythrocytes for all protein concentrations analyzed (Fig. 5A). Quantification of bands showed that TTL content was ~50% lower in hypertensive than in normotensive erythrocytes (Fig. 5B). TTL content in erythrocytes was further evaluated by immunofluorescence microscopy. In normotensive erythrocytes treated with anti-TTL Ab ID3, fluorescence corresponding to TTL was dispersed in the cytoplasm, whereas ID3-treated hypertensive erythrocytes displayed a relatively bright zone at the periphery, beneath the membrane (Fig. 5C). This distribution was confirmed by quantification of fluorescence along the erythrocyte diameter. These findings indicate that hypertensive erythrocytes have a different localization of TTL as well as lower TTL content in comparison with normotensive erythrocytes.

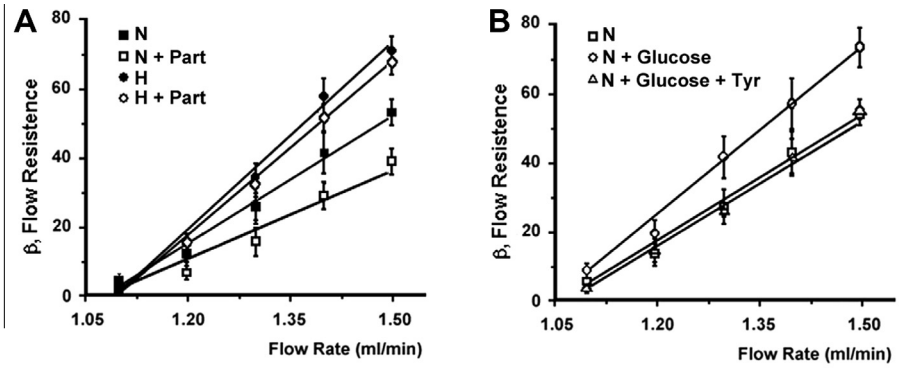
3.3. The increased level of detyrosinated tubulin in human erythrocytes results in modification of deformability

Since previous studies showed that modification of NKA activity is related to changes in erythrocyte deformability [29–31], a rheological property which is closely associated with the development of hypertension [12,13,28]. We therefore analyzed the effect of treatment with parthenolide or glucose/tyrosine on deformability.

In parthenolide-treated normotensive erythrocytes, β and ψ values were reduced by ~40% (Fig. 6A and C), indicating decreased flow resistance and increased deformability. These results are consistent with the decreased membrane detyrosinated tubulin content and increased inhibition of NKA activity by TTCP in parthenolide-treated cells (Fig. 2). No significant change of ψ value by parthenolide treatment was observed in hypertensive erythrocytes, consistent with the lack of effect of parthenolide on membrane detyrosinated tubulin level or NKA activity in these cells (Fig. 2).

In normotensive erythrocytes treated with glucose/tyrosine, correlations were observed among membrane detyrosinated tubulin level, NKA activity, and cell deformability. Glucose, which we showed to increase membrane detyrosinated level (Fig. 2), induced a ~60% increase of ψ and a consequent decrease of deformability (Fig. 6B and C). However, the co-presence of tyrosine reversed the effect of glucose; i.e., ψ values were not significantly different from control values under this condition (Fig. 6B and C). These findings, taken together, reveal an inverse correlation between membrane detyrosinated tubulin content and erythrocyte deformability.

Due to there is a significantly increase of triglycerides content in the blood of hypertensive subjects, we cannot discard the involvement of these lipids on the observed phenomena; however, treatment of erythrocyte from normotensive subjects with glucose or parthenolide showed changes in deformability and NKA activity in response to modifications of levels of detyrosinated tubulin in samples with similar content of triglycerides,



C

DEFORMABILITY

	Control		+Parthenolide		+Glucose		+Glucose +Tyrosine	
	ψ	% of Cn	ψ	% of Cn	ψ	% of Cn	ψ	% of Cn
N	121±7	100	75 ± 10	62 ± 13	160 ± 9	132 ± 9	118 ± 8	97 ± 18
H	170±8	100	150±15	88 ± 8	ND	ND	ND	ND

Fig. 6. Effect of membrane detyrosinated tubulin on deformability of human erythrocytes. (A) A sample of human blood (2 ml) from normotensive (N) or hypertensive (H) subjects was incubated for 2 h at 37 °C in the absence or presence of 50 μM parthenolide (+Part). Aliquots (1 ml) of blood from each treatment were washed, resuspended in physiological solution (the original volume was maintained), and used for determination of flow resistance (β) and deformability (ψ) as described in Section 2. (B) An aliquot (1 ml) of blood from normotensive subjects was treated with 30 mM glucose in the absence (Glu) or presence of 0.5 mM tyrosine (Glu + Tyr), and β and ψ values were measured as in (A). (C) Values for deformability (ψ) from each treatment were calculated from the slope of the respective curve. The % of control (Cn) values were calculated relative to ψ values from N and H without treatment defined as 100%. The values shown are mean ± S.D. from three independent experiments.

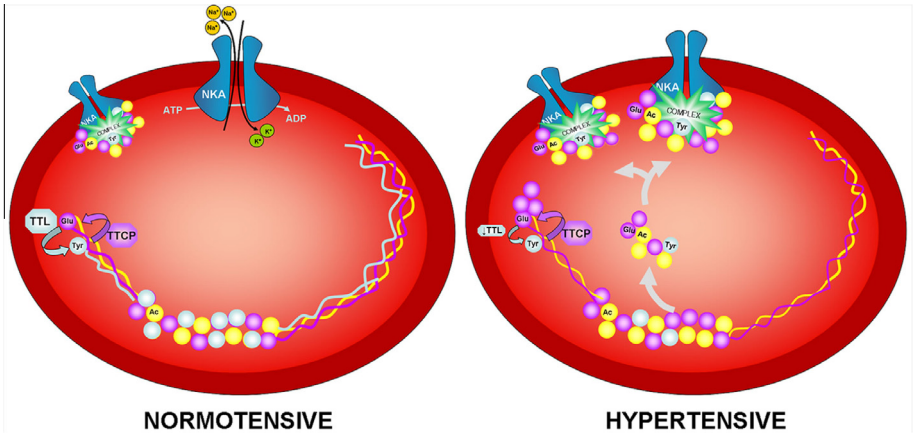


Fig. 7. Proposed mechanism (schematic) of NKA regulation and cell deformability by tubulin in erythrocytes. In hypertensive erythrocytes, decreases in TTL content and activity induce increased detyrosinated tubulin levels in the sedimentable fraction and consequent translocation to the plasma membrane. In the membrane, Ac-tubulin forms a complex with NKA and inhibits its enzymatic activity, and erythrocyte deformability is consequently reduced. In normotensive subjects, an appropriate equilibrium in the detyrosination/tyrosination cycle prevents excessive accumulation of membrane tubulin and promotes correct NKA function and cell deformability.

suggesting that described effect would be independent of the triglycerides content.

4. Discussion

Post-translational modifications of tubulin have an important role as temporal or spatial markers of microtubules indicating binding sites of proteins that regulate a variety of cellular events [32]. In the present study, we demonstrate in vitro that although acetylation is essential to tubulin form a complex with NKA [11,25], an increased level of detyrosinated tubulin is able to inhibit NKA activity.

The effect of detyrosinated tubulin was studied further in whole erythrocytes treated with parthenolide or glucose/tyrosine. Parthenolide treatment of human erythrocytes reduced membrane detyrosinated tubulin level and significantly increased NKA activity. Glucose treatment had the opposite effect; *i.e.*, increased detyrosinated tubulin level and reduced NKA activity. Co-treatment with tyrosine reversed the effect of glucose. Although acetylation of tubulin is essential for complex formation with NKA, results shown that the tyrosination/detyrosination status of tubulin appears to play an important role in regulation of NKA activity. We do not know if detyrosination and acetylation occurs in the same chain, it would be an interesting point to future study. Use of CAD cells could be an helpful tool to elucidate this question, since in these cells acetylated tubulin are absent but levels of this isotype of tubulin appears after treatment of cells with trichostatin A [11].

NKA function generates transmembrane chemical and electrical gradients that are essential for excitation activity of muscle and nerve tissue, as well as the regulation of cell volume and a number

of Na^+-coupled transporters [33,34]. Because of the multiple functions of NKA, alterations of its enzymatic activity are associated with a variety of pathological conditions, including arterial hypertension [35]. We demonstrated previously that detyrosinated tubulin level is increased in hypertensive erythrocyte membranes [6]. We now propose that this alteration is an important factor in modification of NKA activity. The mechanism of the increased detyrosinated tubulin level in hypertensive erythrocytes is unclear. The tubulin tyrosination capacity of cells depends on the equilibrium between two enzymes, TTCP and TTL activities [2,4]. Abnormalities in the cycle have been implicated in various pathological conditions [6,8]. Our present findings indicate that the increased detyrosinated tubulin level in hypertensive erythrocytes is a consequence of reduced tubulin tyrosination capacity. Both the content and activity of TTL are reduced in hypertensive erythrocytes, whereas the activity of TTCP is not significantly different from those in normotensive erythrocytes.

On other hand, we observed correlations among membrane detyrosinated tubulin, NKA activity, and erythrocyte deformability. Glucose, which increases detyrosinated tubulin level, caused reductions of NKA activity and deformability. Co-treatment with tyrosine reversed these effects. In contrast, treatment with parthenolide (which inhibits TTCP and reduces detyrosinated tubulin level) caused an increase of NKA activity and deformability in normotensive erythrocytes. Parthenolide had no effect on deformability, detyrosinated tubulin level, or NKA activity in hypertensive erythrocytes. In view of the high reactivity and numerous targets of parthenolide, we cannot rule out the possibility that the absence of effect of parthenolide on hypertensive erythrocytes is an indirect consequence of its action on some other target, *e.g.*, regulatory enzymes such as kinases or phosphatases [26].

The pathogenesis of essential hypertension is poorly understood. However, because arterial pressure is defined as the product of cardiac output and peripheral resistance, it is clear that hemorheological factors in microcirculation play a primary role. Changes in deformability as observed in the present study may be a consequence of NKA inhibition induced by alteration of detyrosinated tubulin level. Our previous study revealed correlations among erythrocyte membrane tubulin content, deformability, and blood pressure [28]. This phenomena would be independent of the high content of triglycerides observed in blood of hypertensive subjects (Table 1), since we observed changes in deformability and NKA activity in response to modifications of levels of detyrosinated tubulin in samples with similar content of triglycerides (Figs. 1–3). The present results support this concept and suggest that the mechanism involves changes in detyrosinated tubulin level. Moreover, considering that the changes reported in this study are expected to occur in other cell types and due to the ubiquity and functions of NKA in mammalian cells, such as in the vascular wall, renal proximal tubular cells, brain, etc., these changes could be pointed out as contributory factors in the pathogenesis of hypertension.

Based on our previous [28] and present results, we propose the model illustrated in Fig. 7. The reduced TTL content and activity and increased stability of the sedimentable tubulin fraction in hypertensive subjects induce increased levels of detyrosinated tubulin and Ac-tubulin. These tubulin isotypes are translocated through an unknown mechanism to the membrane, where Ac-tubulin forms a complex with NKA. Detyrosinated tubulin plays an important role in enhancing the inhibitory effect of Ac-tubulin on NKA. NKA is involved in decreased erythrocyte deformability, an important factor in the progression of hypertension. No differences in the results were observed between hypertensive subjects treated or not with anti-hypertensive drugs, suggesting that the results were independent of these treatments. In normotensive subjects, an appropriate equilibrium in the detyrosination/tyrosination cycle prevents excessive accumulation of membrane tubulin and promotes correct NKA function and cell deformability. The proposed mechanism clearly indicate an essential role of tubulin in the regulation of the rheological properties of erythrocytes and provides important knowledge because the regulation of this system could be promising therapeutic target for the treatment of hypertension.

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