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Erythrocyte Deficiency in Calpain Inhibitor Activity in Essential Hypertension

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SUMMARY The calpain–calpain inhibitor system was evaluated in erythrocytes of patients with essential hypertension and normotensive controls, either with or without a family history of hypertension. Calpain levels were similar in the controls and hypertensive patients, whereas the inhibitor activity level was significantly reduced in the latter (301.8 ± 26.4 vs 220 ± 14 U/mg hemoglobin, $p < 0.001$). Borderline hypertensive patients and a few controls with a history of hypertension showed low inhibitor activity. Similar results have recently been reported in genetically hypertensive rats of the Milan strain. A significant inverse correlation ($r = -0.43$, $p < 0.001$) was found between mean arterial pressure and calpain inhibitor. Although the pathophysiological significance of these observations is not yet clear, they suggest a new area of investigation into the molecular mechanisms underlying essential hypertension and its complications. (Hypertension 12: 474–478, 1988)

KEY WORDS • erythrocytes • calpain • calpain inhibitor • essential hypertension

THE biochemical mechanisms underlying both the pathogenesis of essential hypertension and the high frequency of associated organ lesions are poorly understood. Previous studies have shown that erythrocytes from spontaneously hypertensive rats of the Milan strain[1] have similar amounts of the neutral Ca^{2+}-dependent proteinase calpain and 10 times less activity of its natural inhibitor,[2, 3] as compared with normotensive rats. Similar results were also obtained by measuring calpain and its inhibitor activity in renal tissues from the same rat strain.[4] These data suggest that, both in erythrocytes and kidney, calpain has lost at least part of the components of its regulatory system. Calpain specifically degrades membrane-bound cytoskeletal proteins (i.e., membrane-bound protein kinase C, epidermal growth factor, adrenergic receptors, actin binding proteins, neurofilament-associated and microtubular-associated proteins, and a few soluble proteins).[5] Based on this specificity, it may be postulated that calpain is involved in signal transduction and processes controlling cellular volume, shape, and deformability. Some of the cellular abnormalities observed in hypertensive rats and in patients with essential hypertension[6–12] may be attributed to the action of an uncontrolled or less regulated calpain activity in the cell.

In the present study, we found that the level of calpain inhibitor in red blood cells from patients with essential hypertension is decreased in comparison with that found in normotensive subjects. Moreover, a significant inverse correlation between calpain inhibitor levels and mean arterial pressure (MAP) was observed.

Subjects and Methods

Four groups of subjects were studied: Group 1 was composed of 23 normotensive subjects without a family history of hypertension; Group 2 was composed of 17 normotensive subjects with at least one hypertensive parent; Group 3 was composed of 19 patients with borderline hypertension; and Group 4 was composed of 49 patients with established hyper-

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tension. Hypertensive patients were recruited in the outpatient clinics of the Institute of Medical Science, University of Milan, or the Division of Nephrology, Scientific Institute of Internal Medicine, University of Genoa. The same criteria were applied for excluding secondary hypertension in both institutions. All patients underwent routine biochemical analyses of blood and urine, including plasma renin activity and urinary aldosterone. Further investigations were performed only when abnormalities were found in these analyses or when other symptoms or signs suggestive of secondary hypertension were present. None of the patients were on drug treatment at the time of the study. They either had never been treated for hypertension or, because hypertension was so mild, had been taken off therapy at least 4 months before the study. Subjects in Groups 1 and 2 were recruited in both clinics from the laboratory or medical staff or from epidemiological studies that were in progress. Normotensive subjects had a systolic blood pressure of 140 mm Hg or less and a diastolic blood pressure of 90 mm Hg or less in the three visits preceding the study. Patients with borderline hypertension were those with at least a normal value of blood pressure in one of the three different measurements. The family history was assessed by one of us or by the family physician, who measured the blood pressure of the parents on at least one occasion. Blood samples used for the present study were collected from an antecubital vein after a 12- to 14-hour overnight fast.

Chemicals

^{125}I-labeled sodium (100 mCi/ml) was obtained from Amersham (Arlington Heights, IL, USA). Bovine serum albumin and reagents for hemoglobin (Hb) determination were purchased from Sigma Chemical (St. Louis, MO, USA). Diethylaminoethyl cellulose (DE 52) was obtained from Whatman (Clifton, NJ, USA), and Ultrogel and AcA34 were obtained from LKB (Bromma, Sweden). Human acid denatured globin was prepared as described elsewhere.[13] Calpain was purified from human erythrocytes and assayed as previously reported.[14] The specific activity of the purified enzyme was 600,000 U/mg. One unit of enzyme activity was defined as the amount causing the release of 1 nmol of amino group per hour in the assay conditions.

Assay of Calpain Inhibitor Activity

Packed red blood cells (2 ml), deprived of leukocytes and platelets,[14] were lysed, and the cytosolic fractions were obtained as previously described.[2] An aliquot containing 200 mg of Hb was submitted to diethylaminoethyl ion-exchange chromatography.[2] Samples (50 μl) of the eluted fractions were heated at 90 °C for 3 minutes and assayed for calpain inhibitor activity, as reported elsewhere.[15] The total amount of calpain inhibitor was calculated from the area under the eluted peak. One unit of inhibitor was defined as the amount inhibiting 1 unit of calpain activity.

Preparation of Anticalpain Monoclonal Antibody

Monoclonal anticalpain antibody was prepared as described elsewhere.[16]

Radioimmunoassay for Human Erythrocyte Calpain

Purified calpain was labeled with ^{125}I as reported by Krantz et al.[17] The specific radioactivity was 12 $\times$ 10^3 dpm/ng of calpain. Iodinated calpain (10 ng) was incubated in 0.3 ml of 20 mM sodium phosphate buffer, pH 7.5, containing 2 mM NaN_3, 1 mM EDTA, and 0.2 mg of bovine serum albumin (Medium A) in the presence of 18 μg of MoAb c 56.3 and 20, 40, or 60 μl of the erythrocyte cytosolic fraction containing 15 mg/ml Hb. Each determination was performed in triplicate. The incubation mixtures were rotated end over end at 4 °C for 20 hours, then 10 μl of antiserum (66 mg/ml) to mouse IgG was added. After 2 hours at 20 °C, the mixtures were centrifuged at 1000 *g* for 10 minutes. The precipitates were washed three times with 1 ml of Medium A and counted in a Packard gamma counter (Downers Grove, IL, USA).

Blood Chemistry Tests

Creatinine, urea, electrolytes and other standard blood chemistry evaluations were performed on serum according to routine methods.

Statistical Analysis

Statistical analysis was performed using the SPSSPC+ package (Chicago, IL, USA) on an AT IBM personal computer (Armonk, NY, USA). The analysis used was one-way analysis of variance with the multiple a posteriori comparison Student-Newman-Keuls and least significant difference tests and simple and multiple linear regression analyses.

Results

Major clinical data of the four groups of subjects are reported in Table 1. Serum levels of sodium and potassium, uric acid, creatinine clearance, and body size indexes were similar in the four groups, whereas the mean age and blood pressure were significantly different. Calpain inhibitor activity in the erythrocytes is shown in Figure 1. The mean values (±SD) of inhibitor activity were 301.8 ± 26.4 U/mg Hb in Group 1, 273 ± 60 U/mg Hb in Group 2, 222 ± 71 U/mg Hb in Group 3, and 220 ± 14 U/mg Hb in Group 4, with wide overlapping of the individual values among the four groups. As calpain inhibitor correlated negatively with age ($r = -0.26$, $p = 0.007$) and mean age values were different in the four groups (see Table 1), the residuals of the regression of calpain inhibitor with age were used for comparison among the groups. The one-way analysis of variance with the a posteriori comparison Student-Newman-Keuls test of the residuals of calpain inhibitor with age was significantly different ($p < 0.05$) in Group 1, as compared with the other three groups.

TABLE 1. *Clinical Characteristics of the Subjects*

Variables	Group 1 (n = 23)	Group 2 (n = 17)	Group 3 (n = 19)	Group 4 (n = 49)
Age (yr)	30.9±11.7*	23.5±9*	30.5±10*	42.1±11
Weight (kg)	66.3±15	66.5±14	75.2±17	72.2±13
Height (cm)	167±9	167±3	173±9	170±9
Quetelet index (weight/height^2)	23±4	23±4	25±5	25±4
MAP (mm Hg)	93.9±4.8*	91.7±7.5*	106.4±5.1	121.1±9.9†, ‡
Uric acid (mg/dl)	5.1±0.7	4.5±1.14	4.9±0.86	5.01±1.27
Creatinine clearance (ml/min)	128.1±44.5	135.9±45.9	136.2±38.0	134.8±45.7
Serum sodium (mEq/L)	138.2±2.7	136.5±1.7	139.9±2.9	141.0±10.0
Serum potassium (mEq/L)	4.13±0.05	4.24±0.47	4.4±0.48	4.25±0.48

Values are means ± SD. Group 1 = normotensive subjects with no family history of hypertension; Group 2 = normotensive subjects with a family history of hypertension; Group 3 = borderline hypertensive patients; Group 4 = essential hypertensive patients.

All statistical comparisons were by one-way analysis of variance.

*$p < 0.05$, compared with Group 4 values.

†$p < 0.05$, compared with Group 2 values.

‡$p < 0.05$, compared with Group 1 values.

The backward multiple regression analysis, performed on calpain inhibitor with MAP and age as independent variables, showed that the only significant variable was MAP ($r = -0.43$, $p < 0.001$). The determination coefficient (r^2) decreased from 19.1% to 18.8% when the contribution of age was removed. The correlation of calpain inhibitor with MAP is shown in Figure 2.

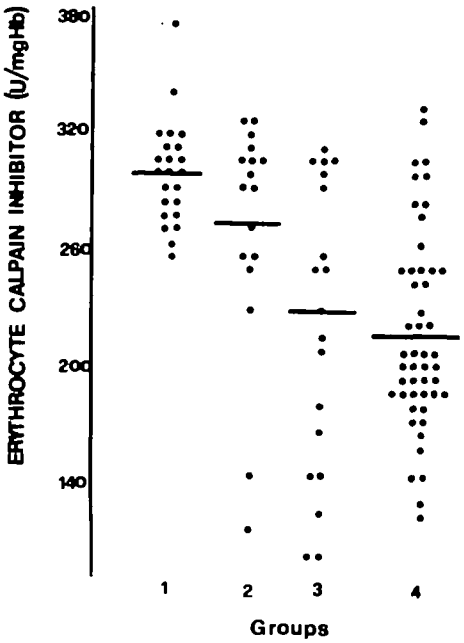

FIGURE 1. *Distribution of calpain inhibitor values in the four groups studied. Group 1 = normotensive subjects with no family history of hypertension; Group 2 = normotensive subjects with a family history of hypertension; Group 3 = borderline hypertensive patients; Group 4 = hypertensive patients; Hb = hemoglobin.* $p < 0.001$, *Group 1 versus Groups 3 and 4 (by one-way analysis of variance with least significant difference test).*

The mean levels of calpain in the erythrocytes of Groups 1, 3, and 4 were similar (81 ± 11.4 ng/mg Hb in Group 1, 82 ± 11.2 ng/mg Hb in Group 3, and 80.5 ± 11.2 ng/mg Hb in Group 4; Figure 3).

Discussion

A number of mechanisms that reduce the Ca^{2+} requirement of calpain to close to the physiological range have been identified.[18] Exposure to Ca^{2+} and substrate, such as human globin, or binding to the inner face of the erythrocyte membrane produces a rapid autoproteolytic conversion of the native calpain to a smaller form with an approximately 200 times lower Ca^{2+} requirement. This process is regulated by the presence in the cell of a natural inhibitor that blocks the catalytic activity as well as the autoproteolytic activation of calpain.[14] In this study we found that, whereas the erythrocyte levels of calpain were similar in normotensive and hypertensive subjects, the calpain inhibitor activity was significantly reduced in the erythrocytes of a large

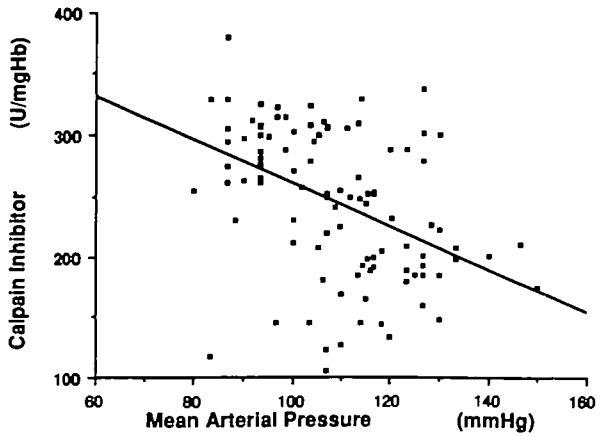

FIGURE 2. *Correlation between MAP and calpain inhibitor for the total population. Hb = hemoglobin.*

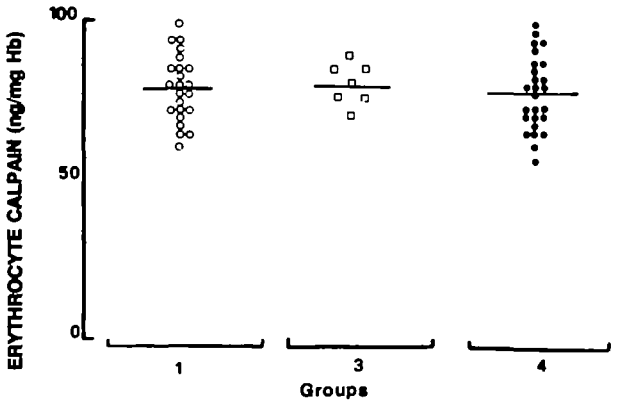

FIGURE 3. *Distribution of calpain levels in 23 normotensive subjects with no family history of hypertension (Group 1), 7 borderline hypertensive patients (Group 3), and 26 hypertensive patients (Group 4). Hb = hemoglobin.*

number of patients with essential hypertension. Low values of inhibitor were also found in subjects with borderline hypertension as well as in a few normotensive subjects with a family history of hypertension. These data demonstrate the existence of an altered calpain–calpain inhibitor system in the erythrocytes of patients with essential hypertension. Interestingly, similar abnormalities have also been found in both erythrocytes and renal tissue of genetically hypertensive rats of the Milan strain, which show other similarities with human essential hypertension.[1-4] It is therefore reasonable to speculate that reduced levels of calpain inhibitor also may occur in human tissues other than red blood cells, including the kidney. Furthermore, low levels of calpain inhibitor were also observed in a few Group 2 subjects, and one-way analysis of variance showed that Group 2 did not differ statistically from Groups 3 or 4. Accordingly, it seems unlikely that decreased inhibitor levels are a consequence of the hypertensive state.

It may be hypothesized that the negative correlation between calpain inhibitor levels and blood pressure is due to a common factor influencing both these variables independently. Alternatively, the reduction of calpain inhibitor activity could be a biochemical abnormality directly related to the genetic mechanisms underlying hypertension that trigger the subsequent sequence of biochemical, cellular, and organ dysfunctions responsible for the blood pressure rise. Data have not yet been reported that support one rather than the other of these hypotheses. However, since it has been shown that calpain is involved in receptor turnover and binding,[5] (including the adrenergic receptor[19]), protein kinase C activation, and modification of the protein composition of the membrane skeleton,[5] and it has been suggested that active ion transport across the renal tubules could be influenced by this enzyme,[20] the relation between calpain inhibitor and blood pressure may be mediated through one of these mechanisms. A synthetic calpain inhibitor of bovine cardiac muscle calpain has the ability to reduce experimental myocardial infarction size in vivo.[21] This finding suggests that calpain or other thiolproteinases have a role in myocardial necrosis. It is now widely recognized that normalization of blood pressure with antihypertensive therapy produces only minor changes in the incidence of cardiac disease, which on the other hand is directly correlated with blood pressure in the unselected population.[22, 23] This discrepancy might be explained by postulating that a rise in blood pressure and decrease in calpain inhibitor are somehow associated in the same patient by pleiotropic or linkage mechanisms. Antihypertensive therapy could leave unmodified the low levels of inhibitor; thus, any increase in cytosolic calcium, which may occur after hypoxia and ischemia,[24] may produce more tissue damage in hypertensive subjects because of a more facilitated activation of calpain.

The results obtained in the present study do not shed light on the possible pathophysiological role played by a modified calpain–calpain inhibitor system in essential hypertension. However, they suggest a new area of research aimed at clarifying the molecular mechanisms underlying the development of essential hypertension or those responsible for organ lesions accompanying this disease.

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