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The plasma membrane Ca^{2+}-ATPase protein from red blood cells is not modified in preeclampsia

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Abstract

Plasma membrane Ca^{2+}-ATPase activity diminishes by about 50% in red blood cells during preeclampsia. We investigated whether the number of Ca^{2+}-ATPase molecules is modified in red cell membranes from preeclamptic pregnant women by measuring the specific phosphorylated intermediate of this enzyme. Also, we isolated the Ca^{2+}-ATPase protein from both normotensive and preeclamptic pregnant women and estimated its molecular weight, and its cross-reactions with specific polyclonal and monoclonal (5F10) antibodies against it. We measured the Ca^{2+}-ATPase activity in a purified state and the effect of known modulators of this ATPase. It was found that the phosphorylated intermediate associated with PMCA is similar for red cell ghosts from normotensive and preeclamptic women, suggesting a similar number of ATPase molecules in these membranes. The molecular weight of the Ca^{2+}-ATPase is around 140 kDa for both normotensive and preeclamptic membranes, and its cross-reactions with specific antibodies is similar, suggesting that the protein structure remains intact in preeclampsia. Calmodulin, ethanol, or both calmodulin plus ethanol, stimulated the Ca^{2+}-ATPase activity to the same extent for both normotensive and preeclamptic preparations. Our results showed that the reduced Ca^{2+}-ATPase activity of the red cell membranes from preeclamptic women is not associated with a defective enzyme, but rather with a high level of lipid peroxidation.

Keywords: Preeclampsia; Ca^{2+}-ATPase; Human red blood cell; Lipid peroxidation

1. Introduction

Preeclampsia is an important complication that can develop during pregnancy after the 20th week of gestation and affects approximately 7% to 10% of pregnant women. It is characterized by generalized arteriolar vasospasm, abnormalities in plasma volume, high blood pressure and proteinuria, and might occasionally be accompanied by renal failure, stroke, liver failure, pulmonary edema, and coagulopathy. The disease can progress to eclampsia (seizures) and it is indeed a very important cause of morbidity and mortality among pregnant women and fetuses [1].

Although the etiology of preeclampsia remains unknown, there is growing evidence that placental ischemia plays a causal role in the elevated blood pressure, which typically resolves rapidly following delivery [2]. The placental ischemia could promote lipid peroxidation reactions [3,4] and their byproducts have been reported to be elevated in the serum of preeclamptic women [5,6]. Circulating blood cells, such as erythrocytes and platelets, could be oxidized during transit through the placenta, propagating in this way the lipid peroxidation at sites distal to

Abbreviations: PMCA, plasma membrane calcium-stimulated adenosine triphosphatase; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances

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Table 1
Clinical data from 60 healthy pregnant women (normotensives) and 60 pregnant women with severe preeclampsia

Patients	Normotensives	Preeclamptics
Age (year)	20.9±3.5	19.7±2.3
Race (*n*)		
Hispanic	60	60
Non-Hispanic	0	0
Parity	0	0
BMI (kg/m^2)	30.1±0.8	31.7±0.9
Gestational age (wk)	38.7±0.5	37.3±0.5
Systolic blood pressure (mm Hg)	112.9±4.2	172.5±5.6 *
Diastolic blood pressure (mm Hg)	67.8±3.8	113.9±2.4 *
Protein excretion (g/24 h)	0.15±0.06	5.77±0.45 *
Oliguria (number of women)	0	60
Preexistent renal disease	0	0
Diabetes mellitus	0	0

Values are means±S.E.
* $P<0.001$ (vs. normotensives).

areas of initial damage. By affecting the integrity of the cell membranes [3], lipid peroxidation could decrease membrane fluidity and thereby could reduce the activity of membrane enzymes, such as the PMCA [7]. In fact, we have previously shown that the PMCA activity of maternal and neonatal red cell ghosts [8,9], myometrium [4] and syncytiotrophoblast basal (fetal-facing) plasma membranes [10,11] of preeclamptic pregnant women, is reduced in about 50% as compared to the ATPase activity of the same tissues from normotensive pregnant women. Since these membranes show higher levels of lipid peroxidation, it has been suggested that this process is responsible for the diminution of PMCA activity in pre-eclampsia [7]. However, modifications on the protein structure of the ATPase could also drive to inhibition of its activity. In the present work, we isolated and purified the PMCA protein of red blood cell membranes from both normotensive and preeclamptic pregnant women, in order to study if this ATPase is defective in this illness. It was found that the red cell membrane PMCA protein is not affected by preeclampsia and the diminution in the activity of this enzyme in this disease is related to an enhanced level of lipid peroxidation of the plasma membranes.

2. Materials and methods

2.1. Blood donors

Sixty normotensive and sixty severe preeclamptic pregnant women of the Maternity Hospital "Concepción Palacios" in Caracas, participated in this study in accordance with the ethical standards established by the Declaration of Helsinki. The study protocol was approved by the Institutional Review Board of the Maternity "Concepción Palacios" and by the Bioethics Committee of IVIC, and all women gave informed signed consent. The study was performed at admission, before any medical treatment, and before delivery. All the women had similar demographic backgrounds, and belonged to urban population of Caracas. Gestational age was estimated from the date of the last menstrual period and confirmed by ultrasonography. Normotensive pregnant women had no history of hypertension and no evidence of hypertension or proteinuria during their pregnancy. The pregnant women with severe preeclampsia were defined by detection of proteinuria (>5 g/day), a blood pressure >160/110 mm Hg, oliguria (≤500 ml/24 h), headache and epigastric pain. The blood pressure was measured twice, 6 h apart at bed rest; the diastolic level was measured at Korotkoff phase V. Any woman that, according to her medical history, was under medical treatment to control blood pressure, or if she was taking >1 g of elemental calcium per day during pregnancy, or if she had a history of chronic hypertension, diabetes, calcium metabolism disorders, or any other chronic medical illness, was not considered for this study. Only women giving birth a single newborn with Apgar scores of 7–10 were included. The clinical data of normotensive and preeclamptic pregnant women are presented in Table 1.

Ten ml of venous blood were collected into heparinized collection tubes from either normotensive or preeclamptic pregnant women before delivery (antepartum). Blood samples were obtained by venipuncture with the patients in lateral decubitus position and immediately transported to our laboratory on ice. Each blood sample was centrifuged at 12,000×*g* for 1 min at 4 °C and the buffy coat and the plasma were discarded. Hemoglobin-free red blood cell ghosts were prepared from the packed red blood cells, following a modification of the method of Heinz and Hoffman [12]. Briefly, the packed red blood cells were hemolyzed (10:1) in a solution containing 10 mM Tris–HCl, 1 mM EDTA (pH 7.5 at 0 °C), and 5 mM phenyl methyl sulfonyl fluoride. Then the ghosts were washed twice with a solution of 10 mM HEPES–KOH, and stored in a solution containing 130 mM KCl, 10 mM HEPES/KOH, pH 7.4, 0.5 mM $MgCl_2$, and 50 μM $CaCl_2$, and kept at −70 °C until use.

2.2. Purification of the erythrocyte PMCA by calmodulin affinity chromatography

The procedure described by Benaim et al. [13] was followed. Red cell ghosts pooled from either 20 normotensive pregnant women or 20 preeclamptic pregnant women were used (150–250 mg of ghost protein). Briefly, red cell ghosts (5 mg/ml) were solubilized on ice for 10 min by adding 1 mg of Triton X-100/mg of protein. Nonsolubilized material was removed by centrifugation at 100,000×*g* at 2 °C for 35 min. Phosphatidylcholine and $CaCl_2$, were then added to the supernatant to final concentrations of 0.5 mg/ml and 100 μM, respectively, and the mixture was applied to a calmodulin-Sepharose 4B column (3–4 ml bed volume), which was equilibrated in a buffer containing 0.4% (w/v) Triton X-100, 130 mM KCl, 20 mM HEPES/KOH, pH 7.4, 1 mM $MgCl_2$, 100 μM $CaCl_2$, 2 mM dithiothreitol, and 0.5 mg/ml of phosphatidylcholine. The column was loaded and washed at a rate of about 40–50 ml/h. The column was then washed with 40 ml of the equilibration buffer and with 40 ml of a similar buffer containing 0.05% instead of 0.4% Triton X-100 and Na^+ instead of K^+. Finally, the purified PMCA was eluted in a buffer containing 0.05% (w/v) Triton X-100, 130 mM NaCl, 20 mM HEPES/NaOH, pH 7.4, 1 mM $MgC1_2$, 2 mM Na-EDTA, 2 mM dithiothreitol, 5% glycerol (v/v), and 0.5 mg/ml of phosphatidylcholine. Na^+ was substituted for K^+ to avoid SDS precipitation in the preparation of samples for SDS-polyacrylamide gel electrophoresis. 0.15–0.25 mg of purified erythrocyte PMCA (concentration about 0.1–0.2 mg/ml) was routinely obtained. The EDTA contained in the active fractions eluted from the column was neutralized by the addition of $MgCl_2$ to a final concentration of 2 mM. $CaCl_2$ was added to a final concentration of 50 μM. The purified ATPase was stored under N_2 atmosphere at −80 °C.

2.3. ATPase assays

Aliquots of purified PMCA (about 1–2 μg of protein/ml) were incubated in a medium containing 130 mM KCl, 20 mM HEPES/KOH, pH 7.2, 1 mM ATP, 1 mM $MgCl_2$, 1 mM EGTA and the appropriate concentrations of $CaCl_2$ to obtain the desired free calcium concentration. The final concentration of calcium ions was calculated by using an iterative computer program as described previously [14]. Since the rates of ATPase activity were linear over 45 min incubation at 37 °C, the reaction was arrested at 45 min by the addition of 8% (final concentration) cold trichloroacetic acid. When ghost preparations were used, the mixture was centrifuged and the supernatant was kept for inorganic phosphate determination. The latter was carried out according the colorimetric method of Fiske and SubbaRow [15], modified by the use of $FeSO_4$ as reducing agent. A coupled-enzyme assay system was used to measure the PMCA activity during purification of the enzyme, as described previously [16]. The medium contained 10 μM free Ca^{2+}, 120 mM KCl, 30 mM HEPES/KOH, pH 7.4, 2.5 mM $MgCl_2$,

1 mM ATP, 0.2 mM NADH, 0.5 mM phosphoenolpyruvate, 1 unit of pyruvate kinase and 1 unit of lactic dehydrogenase, and the reaction was monitored at 37 °C in a final volume of 1 ml. The difference in absorbance between 366 and 550 nm was plotted versus time using a dual-wavelength spectrophotometer (SLM Aminco DW-2000). The PMCA activity was calculated as the difference between the amount of phosphate liberated in the tubes containing Ca^{2+} minus that liberated in the tubes without Ca^{2+}. The results are expressed as nanomoles of inorganic phosphate liberated per milligram of protein per minute, after subtraction of a blank run in parallel without the membrane suspension, which was added after the addition of the trichloroacetic acid.

2.4. SDS-polyacrylamide gel electrophoresis and Western blot analysis

Proteins were separated on SDS-polyacrylamide gel electrophoresis according to Laemmli [17]. They were transferred to 45 μm nitrocellulose membranes (Immobilon-NC-Haft-Nitrocelulose, Sigma) [18] and blocked for 2 h with 3% gelatin from pig skin in TBS (10 mM Tris–HCl, pH 7.0, 500 mM NaCl) followed by a wash for 5 min in TBS and two washes for 5 min each in TBS-T (TBS + 0.05% Tween-20). The membrane was then incubated with the primary antibody (diluted 1/1000) in TBS-T plus 1% gelatin from pig skin) for 90 min. Two different primary antibodies against the PMCA from human red blood cells were used: a polyclonal antibody prepared in our laboratory and a commercially available monoclonal antibody (5F10, Sigma). The membrane was then washed 3 times for 5 min with TBS-T. The incubation with the secondary antibody (alkaline phosphatase coupled to an anti-rabbit or anti-mouse antibody; Promega Corp., Madison, WI) and the staining were performed according to the manufacturer's protocol (ProtoBlot AP, Promega Corp.).

2.5. Formation of the PMCA phosphoenzyme intermediate

The phosphoenzyme intermediate of the PMCA was measured by a modification of the method described by Knauf et al. [19]. In brief, 40 μl of red cells ghosts (2 mg/ml protein) were added to the incubation medium containing (final concentrations): 12 μM $MgCl_2$; 30 mM Tris–HCl (pH 7.4 at 0 °C); 12 μM ATP with traces of [γ-^{32}P] ATP, with a specific activity of 1 mCi/100 ml and less than 0.1% hydrolysis (Amersham, Inc.) and according to the experimental needs, 125 μM $CaCl_2$. The assay was carried out at 0 °C and it was stopped after 1 min by the addition of 400 μl of ice-cold 5% (w/v) trichloroacetic acid containing 0.1 mM ATP and 1 mM Pi (as orthophosphoric acid). The denatured proteins were collected by centrifugation (16,000×*g*) for 2 min and washed twice with the above solution. The final precipitates were dissolved with 500 μl of 0.2% Triton X100. The suspension was transferred to scintillation vials containing 4.5 ml of instagel and the radioactivity was measured in a beta counter (LKB Wallac, Rackbeta, Liquid Scintillation Counter, model 1217). The phosphorylated intermediate is expressed as picomoles of phosphorus per milligram of protein.

2.6. Lipid peroxidation measurements

The amount of lipid peroxidation of the red blood cell ghosts was estimated by measuring the thiobarbituric acid-reactive substances (TBARS), carried out following the method of Feix et al. [20]. The absorbance was measured at 532 nm and the thiobarbituric acid-reactive substances values were calculated by use of a malondialdehyde standard curve prepared by acid hydrolysis of 1,1,3,3-tetramethoxypropane. The TBARS are expressed as nanomoles of malondialdehyde per milligram of protein. Total lipids were extracted from red blood cell ghosts according to the method of Folch et al. [21].

2.7. Statistical analysis

Statistical analysis was performed by the Student's *t* test. All results are expressed as means ± S.E. and (*n*) represents the number of experiments performed with different preparations. Each preparation was assayed in quadruplicate for all determinations. The PMCA activity was calculated from paired data. A *P* value ≤0.05 was accepted as statistically significant.

3. Results

As already described [8,9], and now shown in Table 2, the PMCA activity of red cell ghosts from preeclamptic women without any treatment is lower than that of normotensive pregnant women. However, the stimulating effect of calmodulin and ethanol, two well-known stimulators of the PMCA [22], was similar for both preparations, reaching in both cases a stimulation of about 30%. Also similarly, the stimulating effects of calmodulin and ethanol showed to be additive.

The level of lipid peroxidation of red cell ghosts, as assessed by TBARS and conjugated dienes, is higher for the preeclamptic women without any treatment. TBARS changed from 0.41 ± 0.07 nmol MDA/mg protein for normotensives to 0.91 ± 0.09 nmol MDA/mg protein for preeclamptics ($P < 0.001$). On the other hand, the conjugated dienes changed from 1.44 ± 0.16 nmol hydroperoxides/mg lipids for normotensives to 2.14 ± 0.10 nmol hydroperoxides/mg lipids for preeclamptics ($P < 0.001$).

Regardless of the higher level of lipid peroxidation of the red cell ghosts of the preeclamptic women, direct modifications of the PMCA protein – involving a diminution in the number of functional protein and/or structural changes – could explain the enzyme-decreased activity observed with this disease. To study this possibility, we carried out two different kind of experiments: (1) determination of the PMCA phosphoenzyme intermediate for both red cell ghosts from normotensive and preeclamptic pregnant women and (2) purification of the PMCA protein of red cell ghosts from either normotensive or preeclamptic pregnant women, in order to determine their activities and to test the effects of calmodulin and ethanol on both preparations.

The level of PMCA phosphoenzyme intermediate is quite similar for both normotensive (1.59 ± 0.08 pmol P/mg protein) and preeclamptic women (1.67 ± 0.09 pmol P/mg protein), suggesting a similar number of ATPase molecules in the red cell membranes.

The percentage of recovery of the purified fraction of PMCA of red cell ghosts from normotensive (0.090 ± 0.011%) and preeclamptic pregnant women (0.103 ± 0.003%) is similar. The electrophoretic patterns of the proteins of these preparations of

Table 2
Effect of 0.1 μM calmodulin and 5% ethanol on the PMCA activity of red cell ghosts from normotensive and preeclamptic pregnant women

Addition	Normotensive	% Stimulation	Preeclamptic	% Stimulation
None	19.63 ± 0.69	–	8.39 ± 0.45	–
calmodulin	25.55 ± 0.47 *	30.0	10.96 ± 0.32 *	30.6
ethanol	26.03 ± 0.65 *	32.6	11.32 ± 0.56 *	34.9
calmodulin + ethanol	32.12 ± 0.80 *, [a]	63.6	14.69 ± 0.64 *, [a]	75.0

ATPase activity is expressed as nmol Pi × mg $prot^{-1}$ × min^{-1}. Values are means ± S.E. of determinations carried out with different preparations from different women ($n = 60$).

[a] $P < 0.001$ (vs. either calmodulin or ethanol).

* $P < 0.001$ (vs. no addition).

PMCA are shown in Fig. 1. It can be seen that the two preparations are quite similar, showing a main peak around 140 kDa, which is in agreement with the molecular weight of the PMCA [23]. There is also a second peak, which has been previously seen with similar preparations and it has been suggested to be part of the active PMCA [24]. The structural integrity of the purified preparations of PMCA was assessed by testing the cross reactions with specific polyclonal and monoclonal (5F10) antibodies against the PMCA. For both samples, the PMCA-specific band at around 140 kDa became evident in Western blotting stained with the 5F10 or a polyclonal antibodies specific for the PMCA (data not shown).

The stimulatory effects of calmodulin and ethanol on the pure preparations of PMCA of both samples were tested. The results of these experiments are shown in Table 3. It can be seen that either calmodulin, ethanol or both calmodulin plus ethanol, stimulated the PMCA activity to the same extent for both normotensive and preeclamptic preparations.

4. Discussion

The present study was carried out in order to evaluate the possibility of a defect of the PMCA protein itself or a reduction in the number of ATPase molecules, besides the increased plasma membrane lipid peroxidation during preeclampsia. The facts that the amount of PMCA phosphoenzyme intermediate is similar for the red cell ghosts from normotensive and preeclamptic pregnant women, and that the ATPase activities are similar for both samples after purification and resuspension of the protein (Table 3), clearly indicate that the number of PMCA molecules of the red cell membrane does not change in preeclampsia. On the other hand, the PMCA protein does not seem to be modified in the red cell membranes of preeclamptic pregnant women. This conclusion is based on the following evidences: (1) the activities of the membranes or the purified ATPases from normotensive or preeclamptic women are stimulated by calmodulin, ethanol or ethanol plus calmodulin in a similar proportion (Table 3); (2) preeclampsia does not modify the K_m for Ca^{2+}, optimal pH and temperature of the PMCA [7]; and (3) the molecular weight of the PMCA (Fig. 1) and the cross reactions with specific polyclonal and monoclonal (5F10) antibodies against the PMCA are similar for both purified preparations of PMCA, either from normotensive or from preeclamptic pregnant women. Therefore, the diminution of PMCA activity in preeclampsia may be considered to be the result of the high level of lipid peroxidation of the cell tissues of the preeclamptic women. This condition, by producing modifications in the protein–lipid interactions, might affect directly the activity of the PMCA. Further support for a causal relationship between lipid peroxidation of red cell membranes and PMCA activity in preeclampsia, is provided by the finding that both, lipid peroxidation of the red cell membrane and PMCA activity, return to normal values when all the symptoms of preeclampsia disappear in the postpartum [25].

The PMCA is a crucial enzyme for the cytoplasmic control of cell calcium. The impaired function of the PMCA in preeclampsia can disrupt the maintenance of appropriate Ca^{2+} gradients, leading to cellular dysfunction. Thus, an enhanced concentration of cytoplasmic Ca^{2+} in the vascular smooth muscle cells could increase muscle tension, resulting in vasoconstriction and, consequently, hypertension. Interestingly, alterations in smooth muscle Ca^{2+} regulation in preeclampsia have been previously reported [26–28]. Therapeutic treatments – such as antioxidants – leading to protection of PMCA protein–lipid interactions may help to regulate some of the symptoms of this pathology.

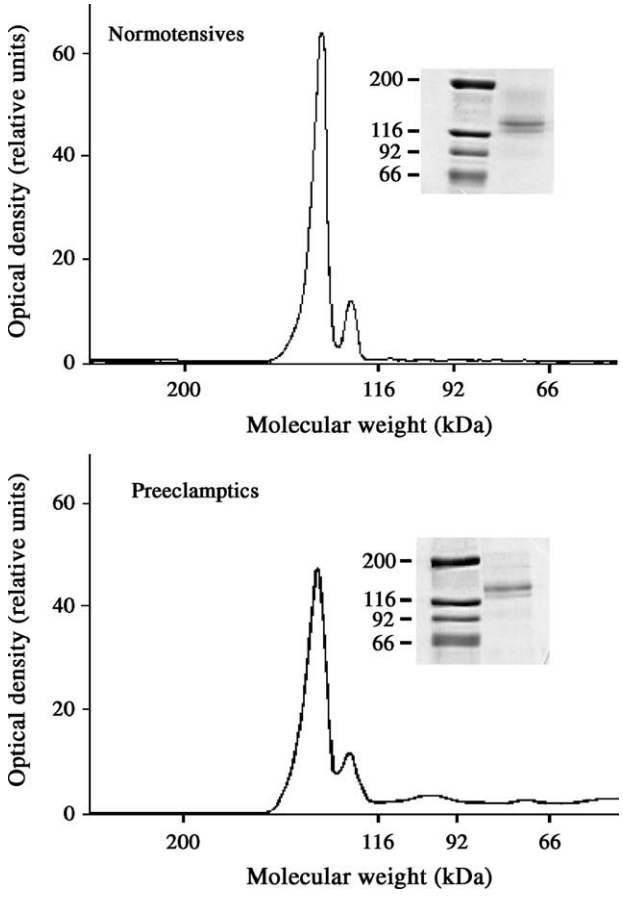

Fig. 1. Electrophoretic pattern and its densitometric scan of purified fractions of red cell ghosts from normotensive and preeclamptic pregnant women. Standards: myosin (200 kDa), β-galactosidase (116.2 kDa), phosphorylase b (92.5 kDa), bovine serum albumin (66.2 kDa).

Table 3
Effect of 0.1 μM calmodulin and 5% ethanol on the activity of purified PMCA of red cell ghosts from normotensive and preeclamptic pregnant women

Addition	Normotensive	Preeclamptic
None	1.24±0.15	1.17±0.08
Calmodulin	1.85±0.15 *	1.83±0.12 *
Ethanol	1.86±0.09 *	1.91±0.09 [a]
Calmodulin+ethanol	2.83±0.15 [a, b]	2.87±0.18 [a, b]

ATPase activity is expressed as μmol Pi×mg $prot^{-1}$ min^{-1}. Values are means±S.E. of determinations carried out with different purified fractions obtained from pools of red cell ghosts from 20 different women (n=3).
[a] $P<0.01$ (vs. no addition).
[b] $P<0.01$ (vs. either calmodulin or ethanol).
* $P<0.05$ (vs. no addition).

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