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Short communication

Plasma L-arginine is markedly reduced in pregnant women affected by preeclampsia

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Abstract

The objective of this study was to determine the concentration of free L-amino acids and in particular of L-arginine in the plasma of pregnant women affected by preeclampsia compared to healthy pregnant women in order to know if an alteration in the concentrations of these amino acids occurs in preeclamspia. Twelve pregnant women affected by preeclampsia and twelve pregnant control women, ages 28–35 years old and at the 35–36 weeks of pregnancy were studied. The blood analysis of free amino acids was carried out by using a high performance liquid chromatographic (HPLC) fluorometric method and OPA-NAC as derivatizing agent for the amino acid determination. In the blood of women affected by preeclampsia L-arginine is markedly reduced compared to controls (about five-fold lower, $P<0.01$). The other amino acids also are significantly reduced, but to lesser extents (about 1.5 times lower, $P<0.05$). Thus, the determination of L-arginine in the blood of pregnant women could potentially constitute an additional marker for the early diagnosis of preeclampsia.

Keywords: L-Arginine; Amino acids; Preeclampsia

1. Introduction

Preeclampsia is an important cause of maternal mortality [1]. Although there is increasing evidence that many of the symptoms of preeclampsia may be attributed to diffuse endothelial dysfunction [2], the cause of this complex multisystem maternal disorder remains unknown. Clinically, preeclampsia is characterized by maternal high blood pressure, proteinuria and edema. Women affected by this disorder also present other disturbances including hyperlipidemia, particularly hypertriglyceridemia [3,4], excessive lipid peroxidation or oxidative stress [5], insulin resistance [4], and an imbalance in the placental production of the eicosanoids, thromboxane and prostacyclin [6].

Whether or not there occurs a reduction or an increase in amino acid concentrations in the blood of pregnant women affected by preeclampsia is controversial. Some authors have found high levels of aromatic amino acids in the blood of the women with preeclampsia, accompanied by elevated concentrations of threonine, arginine, glycine, cysteine and glutamic acid [7]. However, other authors reported

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that aspartic acid, glutamic acid, threonine, glycine and asparagine were at elevated concentrations in this illness, but the remaining amino acids were not changed [8]. In normal human physiological functions, the concentrations of free amino acids must fall into certain ranges, therefore, a variation of this range could be taken into consideration as a signal for a variety of disorders.

Based on preliminary experiments which indicated that L-arginine was found at significantly lower levels in the patients affected by preeclampsia than in controls, we carried out a specific comparative study (this study) for the determination of free amino acids in women with normal elapsed pregnancies and in pregnant woman affected by preeclampsia in order to know whether an alteration of free amino acids exists between the two groups. For this purpose, we used a sensitive and reliable HPLC fluorometric method based on the separation of the amino acids on a C-18 reversed phase column after derivatization with *o*-phthaldialdehyde (OPA) – *N*-acetyl-L-cysteine (NAC) instead of the usual OPA-mercaptoethanol derivatization. The OPA-NAC derivatization gives better separation of arginine from alanine than the OPA-mercaptoethanol derivatization procedure.

2. Experimental

2.1. Collection of the blood and purification of the amino acids

The procedures for this study were approved by the Federico II School of Medicine committee responsible for human experimentation, and all of the women who participated in the study did so voluntarily. Blood samples were collected from 12 healthy women and 12 women affected by preeclampsia (ages 28–35 years at 35–36 weeks of pregnancy) from the district of Naples (Italy). Both the normal women and those with preeclampsia were fasting for 12–18 h before analysis and were not in labour. All subjects delivered within 1–2 weeks of the specimen collection. Women with chronic hypertension, diabetes, renal, or metabolic disease were excluded from this study. The following clinical criteria were used to define preeclampsia: (1) nulliparous, (2) diastolic blood pressure at least 90 mmHg on two occasions at least 4–6 h apart, and (3) 24-h urine excretion at least 300 mg of protein, two random urine specimens obtained 4–6 h apart and containing at least +1 protein by dipstick, a single random urine specimen with a protein–creatine ratio at least 350 mg/g, or a single random urine specimen containing +2 protein by dipstick. These criteria were the same as those used in the trial of calcium for preeclampsia prevention [9]. The 12 healthy controls had uncomplicated pregnancies and no known medical problems or gestation diabetes. All controls had normal pressures during prenatal course and labour as well as no proteinuria.

Whole blood was collected in vacuutainer tubes with ethylenediamine tetraacetate (EDTA) and was placed on ice and centrifuged within 30 min of collection at 2000 *g* for 30 min. The plasma was decanted with a pipette being careful to not disturb the buffy coat or underlying red blood cells and was treated with 1 M perchloric acid (PCA) in a ratio of 1 ml plasma with 4 ml of PCA. After 10 min, the mixture was centrifuged at 30 000 *g* for 30 min, and the supernatant was brought to pH 8.5–9.5 with 1 M KOH. Then it was left in an ice water bath for 30 min to permit the maximum insolubilization of the potassium perchlorate, which was generated from the reaction between PCA and KOH. After that the sample was centrifuged at 30 000 *g* for 10 min and the supernatant was used for the amino acid analyses.

2.2. Amino acid analyses

The determination of the free amino acids was carried out using an HPLC chromatographic fluorometric method based on the separation and quantification of each amino acid on a C-18 column after derivatization of the amino acids with OPA-NAC (*o*-phthaldialdehyde-*N*-acetyl-L-cysteine) reagent instead of the usual OPA-mercaptoethanol reagent in order to obtain better resolution between Arg and Ala. The method was a variation of the procedures described by Aswad [10]. The procedure was as follows: 100 μl of each sample as obtained above was mixed with 300 μl of 0.05 M borate buffer, pH 9.0, and 40 μl of OPA-NAC, prepared by dissolving 10 mg of OPA (*o*-phthaldialdehyde) and 10 mg of NAC (*N*-acetyl-L-cysteine) in 1 ml methanol. After 2

min, 20 μl of this last mixture were injected onto a C-18 Supelcosil HPLC column (0.45×25 cm, Supelco Inc., Belafonte, PA, USA). The column was eluted with a gradient consisting of solvent A (7.5% acetonitrile in 30 mM sodium acetate buffer, pH 5.5) and solvent B (70% acetonitrile in 30 mM sodium acetate buffer, pH 5.5). The gradient was performed as follows: 0% B to 30% B over 30 min; 30% B to 100% B over 5 min; 100% B for 3 min, and 0% B in 1 min. The flow rate during analysis was 1.2 ml/min. The amino acid derivatives were detected fluorometrically using an excitation wavelength of 330 nm and an emission wavelength of 450 nm. In order to identify each amino acid and to determine its concentration, a standard curve consisting of a mixture of 19 amino acids (Sigma) each at the concentration of 0.02 mM (in distilled water), was chromatographed in the same way as the sample (100 μl of the mix of the amino acids + 300 μl of 0.05 M borate buffer, pH 9.0, and 40 μl of OPA-NAC, and after 2 min 20 μl were injected to HPLC).

2.3. Data analysis – accuracy and precision

The accuracy of the method was tested by adding to a serum sample a standard solution of L-Arg in order to obtain: 400 mg, 800 mg, and 1200 mg/100 ml serum, and the analysis of the free amino acids were carried out as described above. The results obtained from this experiment indicated that the % recovery of L-Arg was between 95 and 105%. In order to determine the intra-assay variability of the methods, one serum sample from a woman effected by preeclampsia was analyzed for the content of amino acids five times and the percent coefficient of variation was calculated. In this case, the results indicated that the method was reproducible, and the percent of the coefficient of variation (%C.V.) was between 2 and 5%. Although fluorescence is able to detect nanomolar concentrations of amino acids, the concentration of amino acids found in the derivatized plasma samples was higher, e.g. in the micromolar range (~10 picomoles per 20 μl of derivatized samples injected into the HPLC). The data were statistically analyzed using a StatView II program. The statistical significance was determined with nonparameter statistics by the Mann–Whitney *U* test between two groups. *P*-values <0.05 were considered significant.

3. Results and discussion

In this study we have used a sensitive and reproducible fluorometric method which permitted us to detect with very high precision the concentration of free amino acids in the blood of normal pregnant women and in those affected by preeclampsia. Fig. 1 shows a typical example of the separation of the amino acids that came from a plasma pool of 12 normal pregnant women (top profile) and a plasma pool of 12 women affected by preeclampsia (bottom

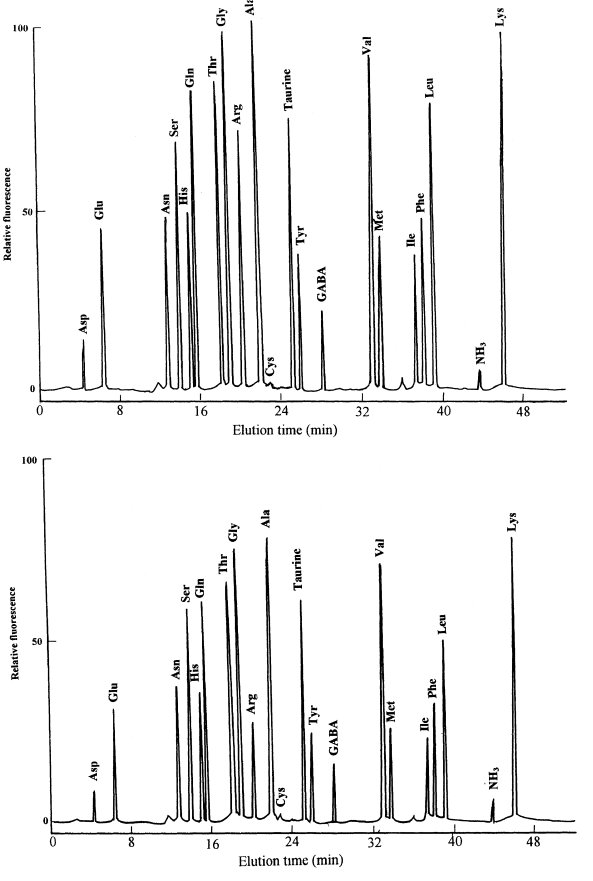

Fig. 1. Analysis of amino acids from a pool of plasma of 12 normal pregnant women (top panel) and of 12 pregnant women affected by preeclampsia (bottom panel) determined according to the procedure described in the Experimental section.

Table 1
Free L-amino acid composition in the plasma of pregnant women affected by preeclampsia and in healthy pregnant women[a]

	Preeclampsia pregnant women (mg/100 ml plasma)	Healthy pregnant women (mg/100 ml plasma)	Ratio healthy/ preeclampsia	*P* value
Aspartic acid	28±15	44±19	1.57	<0.01
Glutamic acid	147±39	215±44	1.46	<0.01
Asparagine	205±54	337±76	1.64	<0.01
Serine	447±86	589±106	1.31	<0.01
Histidine	105±28	180±39	1.71	<0.01
Glutamine	347±66	526±98	1.51	<0.01
Threonine	360±49	468±60	1.30	<0.05
Glycine	519±87	776±130	1.49	<0.01
Arginine	**116±33**	**610±120**	**5.25**	**<0.01**
Alanine	980±188	1,430±367	1.45	<0.01
Taurine	510±88	720±135	1.41	<0.01
Tyrosine	120±36	180±41	1.50	<0.01
GABA	60±19	80±41	1.33	<0.05
Valine	677±110	970±167	1.43	<0.01
Methionine	158±39	280±59	1.77	<0.01
Isoleucine	186±34	287±68	1.54	<0.01
Phenylalanine	216±66	329±78	1.52	<0.01
Leucine	317±77	563±98	1.69	<0.01
Lysine	766±127	1208±202	1.57	<0.01
Total	6723	10,768	1.60	<0.01

[a] The values refer to the mean concentrations and the SD of free amino acids expressed in mg/100 ml plasma as obtained from 12 women in pregnancies affected by preeclampsia ages 28–35 years and 12 normal pregnancies of the same age at 35–36 weeks of pregnancy.

profile) in which each pool consisted of equal volumes of each plasma. In Table 1 is reported the concentrations (mean±SD) of 19 free amino acids determined in the blood of 12 patients affected by preeclampsia and 12 healthy pregnant women aged 28–35 years at 35–36 weeks of pregnancy. As is shown in this table, as a general phenomena, it was observed that in the patients affected by preeclampsia the concentration of most amino acids was significantly reduced by about 1.5 times ($P<0.01$ and 0.05 depending of the amino acid) compared to the control group. Moreover, as a very interesting result obtained in this study, it was observed that L-arginine was highly significantly reduced in the preeclampsia group by about five-fold compared to the control group, and 3.3 times more reduced than the other amino acids. In fact, we found that this amino acid in healthy pregnant women is at a mean concentration of 610±120 mg/100 ml plasma, whereas in the women affected by the preeclampsia, the content of this amino acid was 116±33 mg/100 ml (5.25 times reduced; $P<0.01$).

In order to know if these discrepancies in the concentration of the L-amino acids in general and of L-arginine in particular are real, the determination of some clinical parameters were taken into account. The following analyses were carried out on both normal women and on women affected by preeclampsia. For this purpose on the plasma of the patients chosen in this study were also determined the concentrations of the enzymes: aspartate aminotransferase (AST) and alanine aminotransferase (ALT); the electrolytes: sodium, potassium, chloride, phosphorus and calcium; the nitrogen compounds: urea nitrogen, uric acid, creatine, and plasma electrophoresis of the protein. The results indicate that except for calcium and urea nitrogen which are moderately elevated in the patients affected by

preeclampsia compared to normal pregnant women, no statistical differences occur for all other metabolites examined between the two groups of patients.

4. Conclusions

In conclusion, the results found in this work indicate that in preeclampsia the concentrations of all amino acids are significantly reduced compared to normal controls. However, this discrepancy was observed to be more accentuated for the L-arginine concentration. Therefore, these data could be used as a biochemical marker to further aid in the early diagnosis of preeclampsia.

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