Adrenomedullin mRNA expression in placenta of preeclamptic women

Ekspresja mRNA adrenomeduliny w łożysku u kobiet ze stanem przedrzucawkowym

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Summary

Introduction: Adrenomedullin (ADM) is indicated to be a biologically active polypeptide released by endothelium with strong hypotensive, long-acting vasodilatator properties. It is suggested that development of preeclampsia is partly related to decreased ADM influence on blood vessels.

Aim: The purpose of this study was to evaluate the adrenomedullin mRNA expression in placenta of preeclamptic women and additionally to assess the correlation between ADM mRNA expression and -1984A>G ADM gene polymorphism.

Material and methods: 26 preeclamptic (PE), 20 with gestational hypertension (GH) and 43 normotensive healthy pregnant women have been involved into the study. The placenta samples were collected instantly after delivery from the central part of maternal side. The ADM gene expression was measured with the real-time polymerase chain reaction (rt-PCR). The results were standardized according to the reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The –1984A>G ADM gene polymorphism was determined by PCR/RFLP assay.

Results: Lower expression of ADM mRNA in PE group (0.881 ± 0.254 vs. 1.039 ± 0.391 in controls, ns) has been investigated. In PE group the placental ADM mRNA expression was slightly higher at women carrying AA genotype (0.890 ± 0.263 vs. 0.842 ± 0.231 , ns). In the control group higher placental ADM mRNA expression in women with AG+GG genotype of -1984A>G ADM gene polymorphism (1.249 ± 0.431) in comparison to women carrying AA genotype (1.036 ± 0.356 , ns) was observed. The study also revealed negative correlation between placental ADM mRNA expression and systolic blood pressure in hypertensive pregnant women (p=0.020).

Conclusion: Reduced mRNA expression for ADM in the placenta connected with reverse correlation of systolic blood pressure in preeclamptic women suggests the significant role of disturbances in placental secretion of ADM in etiology of preeclampsia.

Key words: preeclampsia / gestational hypertension / adrenomedullin / / mRNA expression /

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Otrzymano: 15.04.2011 Zaakceptowano do druku: 20.07.2011

Streszczenie

Wstęp: Adrenomedullina (ADM – adrenomedullin) jest biologicznie aktywną substancją wydzielaną przez endotelium, jest również silnym hipotensyjnym, długo działającym wazodilatatorem. Sugeruje się, że rozwój stanu przedrzucawkowego częściowo związany jest z obniżonym wpływem ADM na naczynia krwionośne.

Cel pracy: Celem pracy była ocena ekspresji mRNA dla adrenomedulliny w łożysku kobiet ze stanem przedrzucawkowym oraz dodatkowo ocena korelacji pomiędzy ekspresją ADM mRNA a polimorfizmem -1984A>G genu ADM.

Materiał i metoda: Do badania włączono 26 kobiet ze stanem przedrzucawkowym (PE – preeclampsia), 20 kobiet z nadciśnieniem ciążowym (GH – gestational hypertension) i 43 zdrowe kobiety ciężarne (grupa kontrolna). Próbki łożyska pobierano bezpośrednio po porodzie z centralnej części po stronie matczynej. Ekspresję genu ADM mierzono za pomocą reakcji łańcuchowej polimerazy w czasie rzeczywistym (rt-PCR – real-time polymerase chain reaction). Wyniki standaryzowano dla genu referencyjnego dehydrogenazy gliceroaldehydo-3-fosforanowej (GAPDH – glyceraldehyde-3-phosphate dehydrogenase). Polimorfizm –1984A>G genu ADM wyznaczano metodą reakcji łańcuchowej polimerazy/polimorfizmu długości fragmentów restrykcyjnych (PCR/RFLP – polymerase chain reaction/restriction fragment of lenth polymorphism).

Wyniki: W pracy zaobserwowano niższą łożyskową ekspresję mRNA genu ADM w grupie kobiet ze stanem przedrzucawkowym (0,881±0,254 vs 1,039±0,391 w grupie kontrolnej, ns). W grupie kobiet ze stanem przedrzucawkowym ekspresja ADM mRNA w łożysku była nieco wyższa u kobiet nosicielek genotypu AA (0,890±0,263 vs 0,842±0,231, ns). W grupie kontrolnej odnotowano wyższą łożyskową ekspresję mRNA genu ADM u kobiet z genotypami AG+GG polimorfizmu –1984A>G ADM (1,249±0,431) w porównaniu do kobiet nosicielek genotypu AA (1,036±0,356, ns). Analiza pokazała również negatywną korelację pomiędzy łożyskową ekspresją mRNA genu ADM a ciśnieniem skurczowym krwi u wszystkich badanych ciężarnych z nadciśnieniem (p=0,020). Wnioski: Zmniejszona ekspresja mRNA dla adrenomedulliny w łożysku w połączeniu z istnieniem odwrotnej korelacji ekspresji z ciśnieniem skurczowym u kobiet ze stanem przedrzucawkowym sugeruje istotną rolę zaburzeń w wydzielaniu łożyskowym adrenomedulliny w etologii preeklampsji.

Słowa kluczowe: stan przedrzucawkowy / nadciśnienie ciążowe / adrenomedullina / / ekspresja mRNA /

Introduction

Preeclampsia (PE), appearing in 4-6% of all pregnant women, is a serious obstetrical problem and reason of maternal/ fetal morbidity and mortality. Regardless of the fact that much attention has been paid to PE etiology, there are still issues connected with it that need to be clarified. Currently, PE is believed to be a multifactorial disease and the search for new markers involved in pathophysiology of preeclampsia and their correlation with the severity of the disease is of outmost importance.

It is well known that the imbalance between active substances released from endothelial cells, especially as a result of pathological changes in placental vessel, contributes to PE.

In recent years it has been indicated that adrenomedullin (ADM), vasoactive polypeptide (52 amino acid, isolated in 1993) [1], could play an important role in the regulation of blood pressure and vessel tension in preeclamptic women. ADM is synthesized and released mainly by the cardio-vascular system (highest expression observed in endothelial and smooth muscle cells of vessels) [2, 3]. Thus, ADM is indicated to be a biologically active substance released by endothelium. Moreover, it is a strong hypotensive, long-acting vasodilatator and the blood pressure value depends on ADM serum concentration [4]. Vasodilatation is supported by ADM action through specific adrenomedullin receptors, cAMP rising in smooth muscle cells and regulation of nitric oxide synthesis. Furthermore, in endothelial cells ADM inhibits synthesis of endothelin-1, simultaneously inhibiting proliferation effect on smooth muscle cells. Moreover, ADM inhibits proliferation of heart muscle cells [5].

ADM level is elevated in several disorders connected with pathological changes in vasculature and this observation allows to assume that also in case of PE, where pathophysiology is deeply connected with impairments of utero-placental circulation in early stages of pregnancy, activity of ADM could play an important role. Indeed, ADM has been recently studied as a possible factor contributing to the development of preeclampsia. It is suggested that development of preeclampsia is partly related to decreased ADM influence on blood vessels [6]. Additionally, during pregnancy endothelium of placental vessels seems to be an additional place of ADM synthesis. It was also suggested that ADM takes part in the development of placental circulation in early gestation. ADM activity results in placental arteries relaxation in normotensive pregnant women, as well as preeclamptic women. The range of relaxation depends on ADM plasma concentration [5, 6].

The purpose of this study was to evaluate the adrenomedullin mRNA expression in placenta of preeclamptic women and, additionally, to assess the correlation between ADM mRNA expression and -1984A > G ADM gene polymorphism.

Material and methods

Patients

26 preeclamptic (PE group), 20 with gestational hypertension (GH group) and 43 normotensive healthy (control group) pregnant women have been included into the study. The patients were enrolled in research at the Division of Perinatology and Women's Diseases, Poznan University of Medical Sciences, between January 2008 and January 2010. All women were informed about the study aim and gave their written consent for the participation in the research. The Bioethical Committee of Poznan University of Medical Sciences approved the study (03/08).

The women were enrolled into both, PE and GH, investigated groups according to the American College of Obstetricians and Gynecologists (ACOG) criteria (PE – hypertension observed after 20th week of gestation, systolic blood pressure \geq 140mmHg, diastolic \geq 90 mmHg returned to correct value within 3 months after delivery, proteinuria \geq 30mg/dL in urine sample, GH - blood pressure equal or higher than 140/90 mmHg, without proteinuria). The clinical data of investigated patients are listed in Table I.

In all women physical parameters (systolic/diastolic blood pressure), laboratory tests (urea, uric acid, blood urea nitrogen, total protein blood level, level of electrolytes: Na, K, Cl, proteinuria), the newborn status, course of pregnancy and obstetrical history have been analyzed. Patients with chronic hypertension and other cardio-vascular diseases, diabetes mellitus, renal, endocrinological and collagen vascular diseases, and multiple pregnancy were excluded from the study. Considering the presence of inflammatory changes in the placenta from which the tissue samples were collected after delivery, women with premature rupture of membranes, intra-amniotic infection, as well as smokers, were also excluded.

Tissue sample collection:

The placenta samples were collected instantly after the delivery (vaginal or cesarean section) from the central part of maternal side. The indications for cesarean section in the GH group were: hypertension not responding to pharmacological treatment, fetal distress, breech presentation; in the PE group: preeclampsia/eclampsia, fetal distress in the second period of the delivery, hypotrophia; in the control group: breech presentation, placenta praevia, cervical dystocia, orthopedic and ophthalmic indications. The samples were frozen in liquid nitrogen and directly after that stored at the temperature minus 80°C.

Genetic analysis

The ADM gene expression was measured with the realtime polymerase chain reaction (rt-PCR) with the use of specific primers (Table II).

The amount of transcript was evaluated on the basis of standard curve prepared with dilutions of complementary DNA (cDNA) obtained after reverse transcription of mRNA in cDNA amplification. The results were standardized according to the reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

Table I. Clinical and physical data of investigated patients.

		GH + PE	GH	PE	Control group	р
		n = 46	n=20	n = 26	n = 43	•
	Age (years)					
	mean ± SD	30.4±4.6	30.5±4.7	30.4±4.7	29.5±4.9	<i>p</i> >0.05*
	range	18-40	18-39	21-40	18-39	
	median	31	30,5	31	30	
6	Sestational age at					
	$\frac{ueuvery}{mean \pm SD}$	34.9±3.9	36.9±3.2	33.1±3.7	39.1±1.8	p<0.05*
	range	25-42	28-42	25-40	37-42	
	median	36	37,5	33	39	
Systolic blood						
p	ressure (mmHg)					
	mean ± SD	172.1±16.8	164.8±15.9	178.8 ± 14.9	113.1±11.1	<i>p</i> <0.05*
	range	140-200	140-200	150-200	90-135	
	median	170	160	180	110	
	Diastolic blood					
pressure (mmHg)						
	mean ± SD	107.8 ± 11.6	105.2 ± 11.5	110.3 ± 11.3	71.4±8.2	p<0.05*
	range	90-150	90-150	90-140	60-85	
	median	110	100	110	70	
	Birth weight (g)					
	mean ± SD	2541.1±1157.1	3451.5±705.4	1840.8±926.6	3505.8±522.8	<i>p<0.05</i> *
	range	620-4580	2160-4580	620-3940	2430-4610	
	median	2525	3325	1690	3440	
Placental weight						
	(g)	405.4.216.2	(27.0.101.7	204.2 179.2	(24.4.120.6	<0.05¥
	mean \pm SD	495.4±216.3	627.0±191.7	394.2±1/8.3	034.4±120.6	<i>p</i> <0.03*
	range	160-1200	400-1200	160-840	420-900	
	median	470	580	365	620	

GH-gestational hypertension, PE-preeclampsia

*-p value calculated for difference between whole GH+PE and control groups

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 Table II. Primers used in rt-PCR.

Gene	Primer sequence
ADM	5'-CCC TgA TgT ACC Tgg gTT Cg-3' 5'-gCC CAC TTA TTC CAC TTC TTT Cg-3'
GAPDH	5'-CAA gTg ggg CgA TgC Tgg-3' 5'-gCA GAg ggg gCA GAg ATg A-3'

Reverse transcription

The reverse transcription was carried out with SuperScript First Strand Synthesis System set (Invitrogen, USA). The procedure was performed in the laminar chamber, the preparations were kept in ice. 2μ g RNA, 1μ l deoxyribonucleotide triphosphate (dNTP) (concentration 10mM), 1μ l oligo(dT)₂₀ (concentration 50 μ M) and water (to final volume 10 μ l) were mixed, whirled for 30 sec and finally put in 65°C for 5 min.

Afterwards the test-tube was put in ice for 1 min and whirled. Next the test-tube was put in ice and 2µl buffer, 2µl DTT

(concentration 0,1M), 4µl MgCl₂ (concentration 25mM), 1µl RNase OUT (40 U/µl) and 1µl reverse transcriptase SuperScript III RT (200U/µl) were added. Subsequently, the test-tube was shaken, whirled and put in 65°C. cDNA synthesis was conducted in PTC-200 (MJ Research, USA) thermocycler in 50°C for 50 min. Afterwards the samples were placed in 85°C for 5 min. to stop the reaction. Next 1µl RNase H was added and the test-tube was kept in 4°C for 20 min. Obtained cDNA was used as a matrix for rt-PCR.

Real-time PCR

rt-PCR was used to evaluate changes in ADM gene expression. The reaction was carried out with cDNA obtained by reverse transcription, specific primers and Fast Start DNA Master Sybr Green I (Roche Diagnostic, Germany) set (volume of mixture 10 μ l). LightCycler real time PCR detection system (Roche Diagnostics, Germany) was used in amplification. LightCycler3 Run Version 5.32 and LightCycler Data Analysis Version 3.5.28 were used in the analysis of the results and in comparison of relative differences of matrix initial amount.

The level of analyzed transcripts was standardized according to GAPDH gene (reference gene).

Table III. Conditions of rt-PCR for ADM expression.

Stage	ADM		GAPDH	
Siuge	Temp. (°C)	Time (s)	Temp. (°C)	Time (s)
Initial denaturation	95	600	95	600
Denaturation	95	5	95	5
Starters hybrydization	58	10	65	10
Synthesis of complementary DNA	72	8	72	8
Final synthesis	70	20	70	20

Table IV. Studies concerning placental ADM mRNA expression at preeclamptic women and healthy controls...

Reference Race Population		PE/Controls	Results	р
Kanenishi et al., 2000 [8]	Asian Japan	12/7	lower placental ADM mRNA expression in PE	<i>p</i> <0.005
Knerr et al., 2002 [10]	Caucasian Germany	21/34	lower placental ADM mRNA expression in PE	<i>p</i> <0.05
Gratton et al., 2003 [11]	Caucasian Canada	20/21	higher placental ADM mRNA expression in PE women who delivered after 28 g.w.	<i>p</i> <0.05
Al-Ghafra et al., 2006 [13]	Caucasian Australia	26/38	no differences	ns
Gao et al., 2006 [9]	Gao et al.,Asian10/7lower placental A2006 [9]China10/7expression		lower placental ADM mRNA expression in PE	<i>p</i> <0.05

PE – preeclampsia

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Figure 1. Melting temperature of rt-PCR product for ADM gene (a) (Tm 86,79°C) and melting temperature of rt-PCR product for GAPDH reference gene (b) (Tm 84,90°C).



Figure 2. rt-PCR reaction for ADM gene (a) and for GAPDH reference gene (b) depending on different cDNA concentrations, both with standard curve for ADM and GAPDH genes).

Composition of reactive mixture for amplification of ADM gene transcripts: $7,1\mu$ l water, $0,4\mu$ l MgCl₂ (final concentration 2mM), $0,25\mu$ l primer F (final concentration $0,5\mu$ M), $0,25\mu$ l primer R (final concentration $0,5\mu$ M), 1μ l LightCycler Fast-Start Reaction Mix SYBR Green I (10 x concentrated), 1μ l cDNA. The composition of reactive mixture for reference GAPDH gene differed only with starters (Table II).

Primers were designed with OLIGO v. 5 program. For both genes rt-PCR was conducted in specific conditions (Table III).

The plots of melting temperature (Tm) determination for rt-PCR product and determination of standard curve for ADM gene and GAPDH reference gene are presented in Figures 1 and 2.

Analysis of ADM polymorphism

The -1984A > G ADM gene polymorphism was determined by PCR/RFLP assay previously described [7].

Statistical analysis

For statistical analysis, after data collection, the Statistical Package for Social Science v. 17.0 (SPSS Inc., Chicago, Illinois, USA) were used. Mean values for clinical and biochemical parameters were compared by U-Mann-Whitney test and one-way ANOVA. p value lower than 0.05 was considered as statistically significant.

Results

In the performed investigation slightly lower placental ADM mRNA expression in the whole study group (PE+GH) than in the control group ($0.949\pm0.283 vs 1.039\pm0.391$) was observed. The same level of placental ADM mRNA expression in the GH and the control group ($1.039\pm0.300 vs 1.039\pm0.391$, ns) was noted. The most interesting results the authors noted in the PE group, where lower expression of ADM mRNA ($0.881\pm0.254 vs$. 1.039 ± 0.391 in controls, ns) has been investigated (Figure 3).

The entire study group (GH+PE) and the control group were further divided into subgroups, depending on investigated genotype of -1984A>G ADM gene polymorphism. In women carrying AA genotype (n=36) the placental ADM mRNA expression was only slightly higher (0.958±0.295) than in women carrying at least one mutated allele (AG + GG genotype) (n=10) (0.920±0.245, without statistically significant differences, ns). Likewise, in the PE group (n=26) the placental ADM mRNA expression was slightly higher in women carrying AA genotype (0.890±0.263 vs 0.842±0.231 in AG+GG genotypes carriers, ns). In the control group higher placental ADM mRNA expression in women with AG+GG genotypes (1.249±0.431) in comparison to women carrying AA genotype (1.036±0.356, ns) was observed (Figure 4).

Additionally, we correlated the physical and biochemical parameters with ADM mRNA expression. The study revealed negative correlation between placental ADM mRNA expression and systolic blood pressure in hypertensive pregnant women (GH + PE, n=46, with statistically significant difference p=0.020), what was not observed in the control group. We have not observed any other significant correlations between ADM mRNA expression and analyzed clinical/biochemical parameters in both groups.

Discussion

Only few studies on the role of ADM in the utero-placental circulation indicated the correlation between PE and placental ADM expression and even then they mainly concerned evaluation of placental ADM expression in normal and PE pregnancies. Our study, although without statistically significant differences, revealed lower expression of mRNA for ADM in placentas of PE women. Similar results can be found in other contemporary analysis concerning this problem.

Kanenishi *et al.* evaluated placental ADM mRNA expression in women with PE (n=7) and healthy normotensive pregnants (n=12). The ADM expression was remarkably lower in placentas of women with preeclampsia with significant difference (p<0.005). The ratio of unchanged villi and total amount of villi was significantly lower in women with preeclampsia in comparison to placenta samples of healthy pregnant women (p<0.0001). In amnion and outer villi of trophoblast, ADM mRNA expression was similar in both analyzed groups. Results of this study suggest decrease of ADM mRNA expression in syncytiotrophoblast villi in pregnancy complicated by preeclampsia [8]. Similar conclusions are found in the study made by Gao *et al.*, where average ADM mRNA expression was lower in preeclamptic patients in comparison to normotensive controls [9].

Another interesting study was conducted by Knerr *et al.* The authors evaluated placental expression of ADM, calcitonin gene related peptide (CGRP) and its receptor genes in PE (n=17),

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Figure 3. Placental mRNA ADM expression in analysed groups.



Figure 4. Placental mRNA ADM expression connected with ADM genotypes in investigated groups.

women with HELLP syndrome (*hemolysis, elevated liver enzymes, low platelets*) (n=4) and control group consisting of 34 healthy pregnant women. The study revealed decreased ADM and CGRP mRNA expression in the course of preeclampsia and HELLP syndrome, while ADM receptor and CGRP receptor mRNA expression remained unchanged [10].

Slightly different conclusions were found in the study of Gratton *et al.*, who analyzed placental ADM mRNA expression in PE and normal pregnancy. The study involved 41 patients: PE pregnant women who delivered before 28 gestational week (gw) (7 patients) and after 28 gw (13 patients), such as normotensive

pregnant women who delivered before 28 gw (6 patients) and after 28 gw (15 patients). Directly after labor, 8 samples from central villi and 4 samples from peripheral villi/fetal membranes were taken. The highest ADM mRNA expression was found in chorionic decidua, and the lowest – in chorion and amnion. There were no differences as to the mRNA expression between peripheral and central part of the placenta, as well as gestational week of delivery in the groups of normotensive women. In case of preeclamptic patients who delivered after 28 gw, ADM mRNA expression was remarkably higher in chorionic decidua in comparison to normotensive women. Moreover, the strongest ADM mRNA expression was found in smooth chorion cells and the lowest – in amnion of normotensive pregnant women [11].

In the study made by Li et al., the authors analyzed the influence of cytokines on placental ADM synthesis. Cytotrophoblast samples were taken from 8 healthy normotensive pregnant and 10 preeclamptic women. The samples were kept for 3 days alone or with the following substances: 10 ng/ml endothelial growth factor (EGF), 1 ng/ml transforming growth factor β (TGF- β 1), 10 ng/ml tumor necrosis factor- α (TNF- α) or 100 U/ml of interferon- γ (IFN- γ). The study revealed that ADM synthesis in culture medium was essentially lower in samples from PE women. There was also lower placental ADM mRNA expression in samples from preeclamptic patients. Results showed that EGF stimulated ADM synthesis in trophoblast of normotensive pregnants but not in preeclamptic women. TNF- α , TGF- β or IFN- γ did not affect the ADM synthesis in both groups. ADM synthesis was decreased in the course of preeclampsia, probably because of insensibility to EGF. Authors noted also no compensatory increase of ADM plasma concentration in preeclamptic women [12].

Al-Ghafra *et al.* analyzed the ADM level and mRNA expression in fetal membranes (but not in placenta) in PE patients and in healthy normotensive pregnant women. The study revealed significant ADM concentration increase in chorionic decidua and amnion in preeclamptic patients. What is more interesting, the authors proved that ADM mRNA expression in chorionic decidua was increased in case of preterm deliveries complicated by PE [13].

We have also noted negative correlation between ADM mRNA expression and systolic blood pressure (p=0.020). Makino *et al.* analyzed mRNA expression of both ADM receptor components – CRLR and RAMP2, which probably is a part of ADM receptors system in placenta, fetal membranes, uterus and umbilical vessels. In women with preeclampsia RAMP2 mRNA expression was lower in umbilical artery and uterus and higher in fetal membranes. The CRLR mRNA expression was remarkably lower in umbilical artery and uterus. There was also negative correlation between RAMP2 mRNA expression in umbilical artery and uterus vessels [14].

Previously, we had demonstrated interesting results connected with ADM concentration and -1984A>G ADM gene polymorphism [7]. In the presented study we have additionally assessed the connection of mRNA ADM expression and this polymorphism. The whole group of hypertensive pregnant women was divided into subgroups according to the carried genotype (AA, AG, GG) of this polymorphism and ADM mRNA expression was compared among those subgroups.

In PE group the placental ADM mRNA expression was slightly higher in women carrying AA genotype (0.890±0.263) than in women with AG or GG genotype (0.842±0.231, not statistically significant, ns). On the contrary, the placental ADM mRNA expression was higher in women carrying AG genotype (1.249±0.431 vs 1.036±0.356 in AA carriers, ns). These results probably suggest that carrier of wild A allele connected with decreased level of ADM mRNA expression could be involved in PE pathogenesis.

To the best of our knowledge, this is one of very few studies concerning ADM mRNA expression in placenta from preeclamptic women and the first study to focus on the correlation between -1984A>G ADM gene polymorphism and placental ADM mRNA expression. Although this research is most intriguing and may be very helpful in clarifying the etiology of preeclampsia, the obtained results require future evaluation.

Conclusions

Reduced mRNA expression for ADM in the placenta connected with reverse correlation of systolic blood pressure in preeclamptic women suggests a significant role of disturbances in placental secretion of ADM in the etiology of preeclampsia.

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