OXIDATIVE STRESS INCREASES PLACENTAL ACTIVIN A SECRETION

THESIS

Submitted for the partial fulfillment of Master Degree in Medical Biochemistry

By
Alaa Abd El Hamid Mohamed Ali
M.B.,B.Ch.
Faculty of Medicine
Bani-Suef University

Supervised by

Prof. Dr. *Dawlat Alsayed Salem*
Professor of Biochemistry
Faculty of Medicine
Cairo University

Prof. Dr. Eman Mohammed Obaia
Ass.Professor of Biochemistry
Faculty of Medicine
Cairo University

Dr. Eman Zain El Abedeen
Lecturer of Gynaecology and Obstetric
Faculty of Medicine
Bani-Suef University

Faculty of Medicine
Cairo University
2009

شدة الأكسدة تزيد افراز المشيمة لأكتيفين أ

رسالة مقدمة من

الطبيب / علاء عبد الحميد محمد

بكالوريوس الطب والجراحة

كلية الطب – جامعة بنى سويف

توطئة للحصول على درجة الماجستير في الكيمياء الحيويه ا لطبية

تحت إشراف

أ.د / د ولت السيد سالم

أستاذ الكيمياء الحيوية الطبية

كلية الطب – جامعة القاهرة

د/ إيمان محمد عبية

أستاذ مساعد الكيمياء الحيوية

كلية الطب – جامعة القاهرة

د/ إيمان زين العابدين

مدرس النساء و التوليد

كلية الطب – جامعة بنى سويف

كلية الطب

جامعة القاهرة

2009

Abstract

Oxidative stress increases placental activin A SECRETION

OBJECTIVE: To evaluate the role of activin A in pathogenesis and severity of pre-eclampsia & investigate whether oxidative stress might be the mechanism of increased secretion of activin A. METHOD: This was a case control study comparing cases of pregnancies complicated with hypertension ,with controls matched for gestational age . We studied serum levels of activiv A and malondialdehyde in 25patients with preeclampsia,25 patients with pregnancy induced hypertension and 25 pregnant women with normal blood pressure. RESULTS: Serum levels of activin A and malondialdehyde was higher in patients with pre-eclampsia than in patients with pregnancy induced hypertension and that of control group. There was highly significant correlation between serum levels of activin A and malondialdehyde in pre-eclampsia group and PIH group. CONCLUSION: levels of activin A increases in pre-eclampsia and oxidative stress might be the mechanism of increased secretion of activin A.

Key words

Preeclampsia, Oxidative stress, Activin A

ACKNOWLEDGEMENT

I would like to express my deep appreciation and utmost thanks to Prof. Dr, ***Dawlat Alsayed Salem*** *Professor of biochemistry , Faculty of Medicine, Cairo University, for her valuable guideness, scientific support, encouragement and kind supervision in every step in this work.*

I would like to express my deepest gratitude to Prof. Dr. **Eman Mohammed Obaia**, *Assistant professor of biochemistry, Faculty of Medicine, Cairo University, for her kind supervision, great support and encouragement.*

I am also, indebted to Prof. Dr. **Eman Zein El Abedeen**, *lecturer of Obstetric and gynecology, Faculty of Medicine, Bani Suef University, for her kind supervision and contribution in presenting this work.*

I would like to extend my deepest gratitude to all my professors, my colleges and my family for their helpful assistance and moral support.

Subjects and methods

The present study was carried out at Bani Suef university ,Maternity hospital .In this study 75 pregnant women were included, their age ranged from 18 to 35 years .They were divided into three groups :

GroupI:

Control group consisting of 25 pregnant women with normal blood pressure, no proteinuria nor other complications.

GroupII:

Pregnancy induced hypertension (PIH) group consisting of 25 pregnant with pregnancy-onset high blood pressure defined as diastolic level >90 mmHg after 20 weeks of gestation in a woman .

Group III:

Pre-eclampsia group consisting of 25 pregnant with pregnancy-onset hypertension along with antenatal proteinuria $\geq$ 300 mg/24h after 20 weeks of gestation not resulting from chronic renal disease .

Pre-eclampsia and PIH were diagnosed according to the criteria proposed by ***Davey and MacGillivray (1986).***

-All patients were enrolled in the study after obtaining a consent.

Inclusion criteria:

1- Maternal age from 18 to 35 years.

2- Gestational age of (26- 40) weeks.

Exclusion criteria:

1- Patients with history of any medical disorders such as hypertension, diabetes mellitus, renal diseases, cardiac diseases, endocrine diseases and autiommune diseases.

2- Gestational age of less than 26 weeks.

Method

All pregnant females who met the inclusion criteria, were subjected to:

1- Detailed history taking, concerning:

-Previous and present medical diseases (cardiac, hepatic, renal, endocrinal, pulmonary … etc).

-Previous pregnancies and their outcome.

-Last menstrual period.

-Any gynecological diseases.

2-Full physical examination, of:

-Vital signs: blood pressure, heart rate, temperature, and respiratory rate.

-Systemic examination of heart, lungs, abdomen, lower limbs, and neurological examination.

3-Laboratory investigations, for:

Platelet count.

Liver enzymes (AST, ALT).

Serum creatinine.

Dipstick test for proteinuria.

Urinary creatinine.

MDA by colorimetric method.

Activin A by ELISA.

4- Determination of gestational age by ulra-songraphy, and the date of last menstrual period, and detailed ultra-sonography to exclude intrauterine growth retardation, anomalies.

The total activin-A in serum of all groups was determined using solid phase sandwich enzyme linked immuno-sorbent assay (ELISA) method, according to manufacturer s instructions (Oxford Bio- Innoviation, United Kingdom).

Sample collection

Blood samples were collected from the ante-cubital vein after at least 2 h bed rest, and allowed to clot in plastic tubes at room temperature. After centrifugation at 1000 g for 15 min, serum from the samples was separated and stored at -80 C until assayed for malondialdehyde and activin A.

Principle of the assay of activin A

This assay employs the quantitative sandwich ELISA (Enzyme-Linked Immuno-adsorbent Assay). Wells of a micro-plate are dry coated with a monoclonal antibody ***(Groome et al.,1991)*** specific for the beta-A subunit of Activin. Samples (including standards etc) are pretreated with SDS solution and heated to 100 for 3 minutes. Hydrogen peroxide solution is then added and the sample stood at room temperature for 30 minutes. Then the sample is added along with assay diluent to the coated microplate, followed by biotinylated monoclonal antibody 1 hour later. The plate is covered and incubated overnight at room temperature. The following day the plate is washed and streptavidin-alkaline phosphatase is aded. After a 2-hour incubation the plate is washed and bound alkaline phosphatase is detected with a sensitive substrate system included in the kit. The red colour intensity is proportional to the concentration of activin-A in the samples

Reagents used:

Two activin-A assay plates (ACT A PLATE) .

Sodium Dodecyl Sulphate (SDS):one vial 15ml.

Bovine Serum Albumin Solution(BSA SOL 5%) .

Activin-A standard (56ng/ml) (ACT A STD) : one vial 15ml

Assay Diluent 010 (ASS DIL): one vial l40ml.

Activin Detection Reagent 1 (ACT A BIOTIN Ab): two vials 0.1 ml .

Activin Detection Reagent 2 (S.A. ALK PHOS) :two vials 0.1 ml .

Wash Buffer (WASH CONC 25X):one vial 0.1 ml.

Substrate (SUB) : one vial .

Substrate Diluent (SUB DIL): one vial 13ml.

Amplifier (AMP): one vial.

Amplifier Diluen (AMP DIL): one vial 13ml.

Stop Solution (STOP SOL): one vial 13ml.

Preparation of the reagents :

Reconstitution of the standard:

1. Into separate labelled 1.5ml microfuge tubes a volume of 125ul of each standard dilution or diluted unknown was placed. Into a further tube a volume of 125 ul of plain diluent was placed as a zero analyte sample. This will be culture medium for standards reconstituted in culture medium, or 5% BSA for standards reconstituted in bovine serum albumin solution.

2. A volume of 125 ul 56% SDS was added to each tube. Make sure the SDS is warmed before use and fully dissolved if it was kept in the refrigerator

3. The tubes were capped and place in water at 100°C for 3 minutes.

4. Then, the tubes were allowed to cool and then a volume of 20 ul of hydrogen peroxide (30% solution) was added to each tube then, mixed.

5. The tubes were allowed to stand at room temperature for 30 minutes.

Standard preparation:

1.A volume of 0.09ml of the reconstituted standard was placed in a tube and a volume of 0.91 ml of the same solution was added as used to reconstitute the standard (ie with BSA solution). Mix. This gives an Activin-A concentration of 5 ng/ml.

2.A volume of 0.2ml of the diluting solution was added in each of 6 small tubes.

3.A volume of 0.2ml of the 56 ng/ml standard was placed into the first tube and mixed.

4. A volume of 0.2ml was serially transferred and mixed each time.

5. The standards for the assay are thus 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 ng/ml

Dilution of the wash buffer:

The wash buffer is provided as 25x concentrate .The concentrate should be warmed to room temperature and mixed until any precipitated salts have dissolved. For use dilute 1 volume of wash buffer concentrate with 24 volumes of distilled water. The resulting solution can be stored at 4 °C for up to 5 days.

Dilution of Activin Detection Reagent 1&2:

-A volume of 1 ml of assay diluent 010 was added to one vial of Activin 1 Detection Reagent and the contents was removed and a further 3ml of assay diluent 010 was added.

- The Activin Detection Reagent 2 was Diluted for use by adding 50 ul of detection reagent 2 to 6 ml of Assay Diluent 010.

Preparation of substrate and amplifier

- The substrate was prepared by adding the substrate diluent to the lyophilized

substrateand mixing for 5 minutes.

- The ampilifier was Prepared by adding the ampilifier diluent to the lyophilized ampilifier and mixing for 5 minutes.

ASSAY PROCEDURE

All reagents are allowed to attain room temperature (range 18 to 26°C) before use.

1. A volume of 25µl of assay diluent 010 was added to each well on the plate.
2. A volume 100 µl of each treated sample was added in separate to the plate.
3. The plate was covered and incubated for 1 hour at room temperature.
4. A volume of 25 µl of diluted prepared Activin Detection Reagent 1 was Addeded to each well of the microplate .
5. The plate was covered and icubated overnight at room temperature.
6. The wells of the microtitre plate were washed by filling each well to the top with Wash Buffer allowing to stand for about 15 seconds, and then decanting or aspirating each well thoroughly. this step was repeateda further 3 times. the plate was inverted to drain on absorbent paper.
7. A volume of 50 µl of prepared detection reagent 2 was added to each well of the plate. And the plate was covered and incubated at room temperature for 1 hour.
8. Washing thoroughly as in step 11was done but with 8 cycles ending with wells filled with wash solution . the plate was left to soak for 15 minutes at room temperature while preparing the substrate, then the wash buffer was removed from the plate wells and the plate was further washed for 2-3 cycles thereafter the plate was Inverted to drain on absorbent paper.
9. To each well of the plate a volume of 50 µl of the substrate solution was added,then the plate was covered and sealed and incubated at room temperature for 2 hours.
10. To each well of the plate a volume of 50 µl of ampilifier solution was added ,then the plate was covered and incubated at room temperature. Colour will appear quite rapidly.

11.Finally we Stopped the reaction by the addition 50 µl of STOP solution to each well.

Calculation

The absorbance values of each well was read at 492 nm using a micro-plate reader (MRX) preferably referencing at 620 nm.

when the 5 ng ⁄ ml standard has reached an absorbance of 2.0(5 to 20 minutes depending ambient temperature)

Estimation of malondialdehyde

This was done by colorimetric method using colorimeter.

Reagents:

Analytically pure chemical substances (manufacturers given in brackets) were used for analysis:

1. T hiobarbituric acid (Merk,Dormastadt,FRG).
2. Thiocholoroacetic acid (Merk,Dormastadt,FRG).
3. 1,1,3,3 Tetrsethoxy propane (Fa Sigma,Munches FRG).

Method:

0.5 ml of serum was shaken with 205 ml of 20% tricholoroacetic acid (TCA) in a 10 ml centrifuge tube.

1 ml of 0.67 thiobarbituric acid (TBA) was added, shaken and warmed for 3 minutes in water bath followed by rapid cooling.

Then 4 ml of N-butyl alcohol was added and shaken and the mixture was centrifuged at 3000 rpm for 10 minutes.

The resultant N-butryl alcohol was taken into a separate centrifugation tube and MDA in the plasma was determined from the absorbance on colourimeter at 535 mm.The standard used in this assay was 1,1,3,3 tetraethoxy propane.

Standard curve preparation:

The method mentioned above was done using a blank solution (water) and solutions containing different concentrations of 1,1,3,3 tetraethoxy propane.The extension values was plotted agaist the cocentrations and regressioc line was drawn.

Statistical Methodology

Analysis of data was done by IBM computer using Statistical Program for Social Science (SPSS) version 13**.**

Description of quantitative parameters as follows**:**

❖ **Mean(X'):**

$$\textbf{(X')} = \frac{sum\ (X)}{n}$$

- Where as; **sum (X)** = Sum of individual values**.**

  n = numbers of measurements**.**

❖ **Standard deviation (SD):**

$$\textbf{SD} = \sqrt{\frac{d^{2}}{n-1}}$$

- Where as; $\mathbf{d^2}$ **=** Sum of squared deviations of the individual values from the arithmetic mean of series**.**

  n – 1 = Degree of freedom (d.f.) for the sample**.**

(Altman, 1992).

❖ **Unpaired student t- test:** Used to compare a quantitative variables between two independent groups in parametric data**.**

$$\textbf{t} = \frac{X_1 - X_2}{\sqrt{\frac{SD_1^2}{N_1} + \frac{SD_2^2}{N_2}}}$$

- where as; $\bar{X}_1$ = mean 1; $\bar{X}_2$ = mean 2; SD_1 and SD_2 = standard deviations for groups 1 and 2, respectively; N_1 and N_2 = the respective number of subjects for each group**.** ***(Altman, 1992).***

❖ **ANOVA:**

Analysis of Variance (ANOVA) test is a single test used to collectively indicate the presence of any significant difference between several groups**.**

The test is based on comparing the variance between the groups to the variance within the groups**.** It is measured as the ratio between these 2 variances ***(Knapp and Miller, 1992).***

❖ **Probability "P":** Indicates level of significance.

From the degree of freedom ($n_1 + n_2 - 2$) and from "t" table, the probability can be estimated**.**

❖ **Limits of significance:**

- P > 0.05 = non significant**.**
- P <0.05 = significant**.**
- P <0.001 = highly significant**.**

❖ **Correlation coefficient " r ":** was used to determine the relation between two variables, either positively or negatively**.**

$$\textit{Correl (X,Y)} = \frac{\sum(x-x')(y-y')}{\sqrt{\sum(x-x')^2 \sum(y-y')^2}}$$

Where as; X & Y are the two variables**.** ***(Knapp and Miller, 1992).***

Receiver operating characteristic (ROC) curve:

It is a graphical plot of sensitivity versus (1-specifity),was used to illustrate the diagnostic properties of a test on a numerical scale.

Specifity: The ability of the test to detect those who are truly diseased (true positive

rate)

Sensitivity: Is the ability of the test to detect those who are free of disease (true negative rate)

PPV: Positive predictive value is the proportion of patients with an outcome or disease if the test is positive,is the percentage of true positive to all positive by the examined test.

NPV: Negative predictive value is the proportion of free cases in negative results.