ONLINE DATA SUPPLEMENT

ARGINASE CONTRIBUTES TO ENDOTHELIAL CELL OXIDATIVE STRESS IN RESPONSE TO PLASMA FROM WOMEN WITH PREECLAMPSIA

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Methods and Results

Subjects

Pregnant women were recruited at the time of delivery and non-pregnant subjects were recruited at the time of abdominal surgeries at the Royal Alexandra Hospital, Edmonton, Canada. The protocols were approved by the University of Alberta Ethics Committee (Study ID # MS1_Pro00000880) and the studies were conducted according to the principles of the Declaration of Helsinki. All subjects provided informed consent prior to inclusion in the study. Preeclampsia (n=12) was characterized by the de novo onset of hypertension and proteinuria after the 20th week of gestation. Hypertension was defined as a blood pressure of > 140/90 mm Hg on two occasions 6h apart and proteinuria of > 500 mg in a 24 hour collection or > +2 on a dip stick.[1] Normal pregnant subjects (n=12) were normotensive throughout pregnancy and non-pregnant subjects (n=12) were normotensive. None of the subjects had a history of chronic hypertension, renal, liver or other metabolic diseases and were not on any medications. Either blood samples at the time of admission or fat biopsies during surgeries were collected. Blood was collected by routine forearm venipuncture at the time of admission (before delivery) in tubes containing EDTA. Blood samples were immediately centrifuged at 2000*g* for 20 minutes and then aliquoted under sterile conditions and stored at -80°C. Omental fat biopsies were obtained, at the time of caesarean section for normotensive pregnant and preeclamptic women, or abdominal surgeries for non-pregnant women who were admitted for indications such as dysfunctional uterine bleeding, ovarian cyst, menorrhagia, and dysmenorrhea. They were snap frozen in liquid nitrogen and stored in – 80^{o}C.[2] The patient characteristics are shown on tables 1 and 2 of this online data supplement.

Immunohistochemistry

Omental fat biopsies were cut into 10-μm sections and mounted on glass slides at –20°C, and stored at –80°C before staining. All vessels in the entire section on the slide were counted. Sections were immunostained using antibodies against arginase I and arginase II (1:100; Santa Cruz Biotechnology Inc). Preliminary studies were conducted using serial dilutions of each antibody to determine optimal concentrations that allowed for a clear resolution. Negative controls were stained with nonspecific IgG (1:100; Vector Laboratories) or stained without a primary antibody. Following the addition of primary antibodies, anti-rabbit Alexa Fluor 488 was used at 1:200 for 40 min. Slides were counterstained with mounting medium containing DAPI and were imaged using IX81 Olympus fluorescence microscope with FITC and DAPI filters.

Western blot

We compared the effects of plasma from non-pregnant, pregnant and women with preeclampsia on arginase and NOS expression. After human umbilical vein endothelial cells (HUVECs) were treated with plasma for 24 hours in 6 well plates, the cells were washed with PBS and scraped with lysis buffer containing Tris·HCl (25 mmol/L, pH 7.5) and 0.5% Triton X-100. Protein content was determined using bicinchoninic acid reagent. 10 μg of the protein was loaded on to a 10% gel. The gel was run at 120 V and the protein was transferred onto a nitrocellulose membrane, blocked with 5% fat free milk and the membranes were probed with primary antibodies for arginase I and II and eNOS and iNOS (rabbit anti-human) (1:200 Santa Cruz Biotechnology), overnight at 4^{o}C. The primary antibody was then detected with a peroxidase-conjugated anti-rabbit secondary antibody (1:5,000). Membranes were scanned with Fluor S Max Multimager (Bio-Rad) and the densitometric analysis was conducted using the software.

To examine the effect of peroxynitrite or SIN-1 on arginase expression, we used a similar approach after treating endothelial cells for 6h. For this set of experiments, equal amounts (10 μg) of protein were loaded after estimating protein concentration with the bicinchoninic acid reagent. To confirm equal loading, ponceau S stain was used on the nitrocellulose membrane after transferring protein from the gel. We have previously used this approach for loading controls[3, 4] as both peroxynitrite and SIN-1 alter the expression of loading control proteins such as α-tubulin, actin and others.

Arginase activity assay

Arginase activity was measured by determining the amount of urea generated by the enzyme according to the method of Corraliza et al.[5] After cells were treated with either plasma from three groups of women, or peroxynitrite or SIN-1, the cells were washed with PBS containing EDTA (2 mmol/L). The cells were scraped in PBS and pelleted in an eppendorf centrifuge tube. The pellet was lysed in PBS containing 0.1% triton X-100, pepstatin (5 μg), aprotinin (5 μg) and EDTA (2 mmol/L). 50 μl of the lysate was added to Tris-HCl (25 mmol/L, pH 7.5) containing $MnCl_2$ (5 mmol/L) and incubated at 56ºC for 10 min to activate the enzyme. 50 μl of this heat-activated lysate was incubated with L-arginine (0.5 mol/L) at 37ºC for 60 min. The reaction was stopped by adding 400 μl of an acid mixture (1 H_2SO_4: 3 H_3PO_4: 7 H_2O). 25 μl of α-isonitrosopropiophenone was added to the above mixture and incubated at 100ºC for 45 min for color development. The mixture was further cooled at room temperature in the dark for 10 min. Urea concentration was measured using a colorimeter at 550/540 nm with 200 μl of the aliquot.

NOS activity assay

NOS activity was measured based on the biochemical conversion of L-arginine to L-citrulline. We have used the highly sensitive radioactive NOS activity assay kit (Cayman Chemical Company, Ann Arbor, MI) to detect L-citrulline formation in the picomolar range. After cells were treated with plasma from non-pregnant, pregnant and women with preeclampsia, in the presence or absence of BEC, the arginase inhibitor, endothelial cell protein was prepared according to the manufacturer's instructions. The reaction was conducted by incubating endothelial cell protein with 100 μmol/L of L-arginine and ^{14}C L-arginine (50 μCi/ml) for 60 min in the presence or absence of calcium to measure calcium dependent (eNOS and nNOS) and calcium-independent (iNOS) NOS activities.[6] The amount of L-citrulline formed per μg of protein per hour is reported.

Total nitrite/nitrate measurement

NOS activity was measured by utilizing the Griess reaction as well. Briefly, after cells were treated with plasma from non-pregnant, pregnant and women with preeclampsia, in the presence or absence of BEC, the arginase inhibitor, the cell culture supernatant was collected and total nitrite/nitrate levels were measured using the Nitrite/Nitrate fluorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI).

Effect of L-arginine on nitrotyrosine formation in endothelial cells treated with plasma

We examined the effect of increasing concentrations of L-arginine (0 – 5 mmol/L) on nitrotyrosine formation in endothelial cells treated with 2% plasma from non-pregnant, pregnant and women with preeclampsia (Figure 1). L-arginine increased nitrotyrosine formation in a concentration dependent manner in endothelial cells treated with plasma from all three groups with the maximal response occurring at 2 mmol/L. However, nitrotyrosine formation in cells

treated with plasma from women with preeclampsia is significantly greater when compared to both non-pregnant and pregnant plasma treated cells at all concentrations used.

References

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Table 1.

Characterisitcs of subjects from whom fat biopsies were obtained

Characterisitics	Non-Pregnant	Pregnant	Preeclamptic
Maternal age (y)	32.1 ± 2.4	29.8 ± 1.7	29.7 ± 2.1
Prepregnant weight (kg)	70.5 ± 3.2	68.6 ± 5.77	74.6 ± 8.0
Term BP			
Systolic BP	111.6 ± 1.0	106.7 ± 3.4	154.9 ± 6.8†,*
Diastolic BP	79.3 ± 3.4	74.1 ± 1.7	99.2 ± 3.6†,*
Parity	1.6 ± 0.7	1.9 ± 0.3	1.4 ± 0.2
Proteinuria	NA	NA	3.1 ± 0.4
Gestational age at delivery (wk)	NA	38.8 ± 0.24	33.4 ± 2.1*
Infant birth weight (g)	NA	3416 ± 152.3	1994 ± 271.4*

The characteristics of subjects, 6 from each group of women are shown in the table. Data are shown as mean ± SEM. † denotes P<0.01 vs. non-pregnant women; * denotes P<0.01 *vs*. pregnant women.

Table 2.

Characteristics of subjects from whom plasma samples were obtained

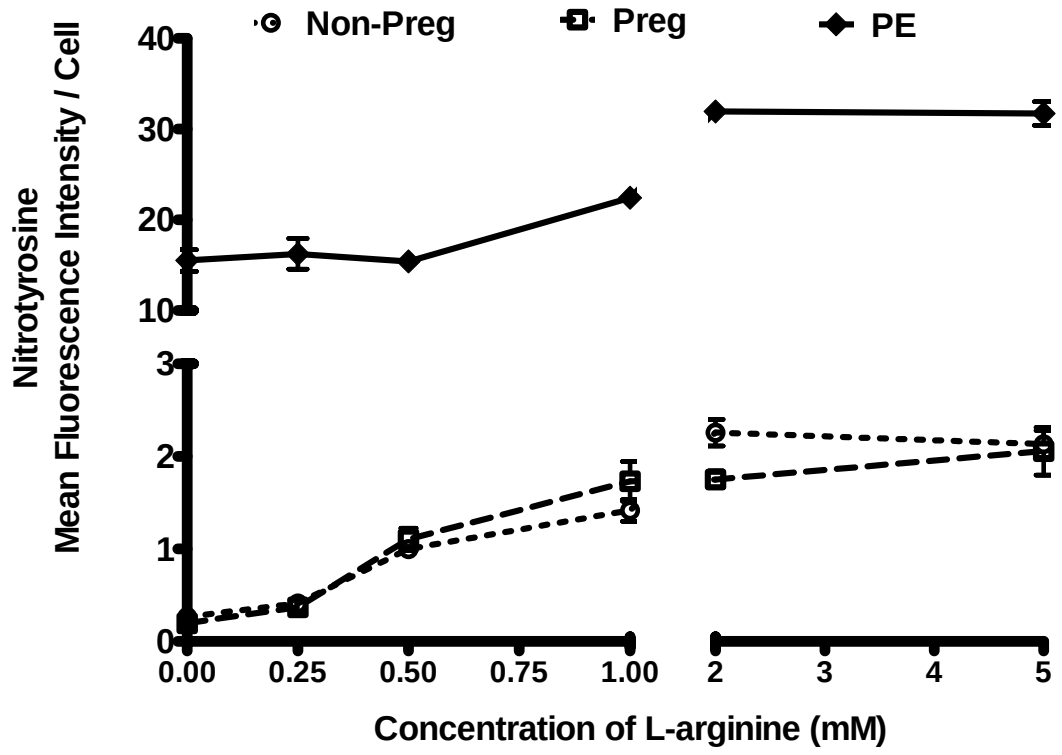
Characterisitics	Non-Pregnant	Pregnant	Preeclamptic
Maternal age (y)	30.4 ± 2.1	28.1 ± 1.6	30.8 ± 4.4
Prepregnant weight (kg)	60.3 ± 2.8	60.9 ± 4.6	63.8 ± 4.4
Term BP			
Systolic BP	115.6 ± 3.6	106.2 ± 5.4	148 ± 3.5†,*
Diastolic BP	78.8 ± 0.8	74.2 ± 2.3	102.1 ± 4.1†,*
Parity	1.0 ± 0.0	1.6 ± 0.2	1.5 ± 0.4
Proteinuria	NA	NA	3+
Gestational age at delivery (wk)	NA	38.5 ± 0.2	33.1 ± 2.2*
Infant birth weight (g)	NA	3296 ± 118	2011 ± 209.2*

The characteristics of subjects, 6 from each group of women are shown in the table. Data are shown as mean ± SEM. † denotes P<0.01 vs. non-pregnant women; * denotes P<0.01 *vs*. pregnant women.

Figure legend

Figure 1. Effect of increasing concentrations of L-arginine on nitrotyrosine formation. Endothelial cells treated with plasma from non-pregnant, pregnant and women with preeclampsia (n=6 / group) for 24h were also incubated with increasing concentrations of L-arginine (0, 0.25. 0.5, 1, 2 and 5 mmol/L) during the same period. Formation of nitrotyrosine was assessed using immunohistochemistry with antibody to nitrotyrosine and the results are depicted in the summary graph.

Figure 1.


Non-Preg
Preg
PE
Nitrotyrosine
Mean Fluorescence Intensity / Cell
40
30
20
10
3
2
1
0
0.00
0.25
0.50
0.75
1.00
2
3
4
5
Concentration of L-arginine (mM)