Effect of pravastatin on NO synthase activity in preeclamptic placentas

Ph. D thesis

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Introduction

Preeclampsia is a common and severe pregnancy condition that develops in 4.5 to 11.2% of pregnancies. Its significance is well illustrated by the fact that it is responsible for more than half a million fetal and neonatal deaths and more than 70,000 maternal deaths worldwide each year.

Despite intensive research in recent decades, the exact etiology of preeclampsia remains unclear, as has its causal treatment and effective prevention unsatisfactorily addressed. Insufficient NO production is known to contribute to the development of preeclampsia.

The placenta mainly expresses the endothelial nitric oxide synthase (eNOS) isoenzyme. Nitric oxide diffuses freely between cells, passing from the endothelium to the medium layer of the vascular wall, which is rich in smooth muscle cells. NO is an effective antihypertensive agent that causes vasodilation of the blood vessels in the placenta, passes paracrinely into the myometrium, and helps maintain uterine muscle relaxation during pregnancy. It plays a role in the endovascular invasion of trophoblasts, which is an essential part of physiological placentation.

There may be several reasons for the decreased effective NO level. Oxidative stress in women with preeclampsia could be one reason. There are several ways to quickly change eNOS activity. On the one hand, the substrate supply of eNOS bound to caveolae, can be ensured by arginine transporters. The activity of the enzyme is enhanced by an increase in intracellular Ca2 + and BH4 concentrations and decreased by

an endogenous competitive inhibitor of the enzyme, asymmetric dimethyl arginine (ADMA). Ser1177 activating phosphorylation and Thr495 dephosphorylation, which activate eNOS, are the two most significant regulatory modes associated with phosphorylation. The activity of eNOS is also modified by protein-protein relationships. Binding of Hsp90 activates, caveolin inhibits eNOS. Elevated intracellular Ca2 + levels cause dissociation of caveolin, thereby activating eNOS. NOS requires BH4 to operate. The concentration of placental BH4 is in the half-saturation range (Km 0.11 μM), so BH4 levels can significantly modify enzyme activity. Measuring the eNOS activity of preeclamptic placenta, it was confirmed in some cases that the eNOS activity of preeclamptic placenta does not increase at physiological BH4 levels, and very high BH4 concentrations are required to reach maximal activity. (termed BH4 resistant)

As NOS activity increases, NO levels increase, so one possible way to treat preeclampsia is to increase NOS activity. Pravastatin may also be effective in normalizing NO levels.

Statins (3-hydroxy-3-methylglutaryl-CoA reductase inhibitors) are the most commonly used cholesterol-lowering drugs. Their economic importance is shown by the fact that they are among the most widely traded medicines in the world. In addition to their primary effect, they also have pleiotropic effects. Their pleiotropic effects include proangiogenic, anti-inflammatory, antioxidant, neuroprotective, and antithrombotic properties, including endothelial protection. Statins increase eNOS expression by prolonging the half-life of eNOSmRNA, reducing caveolin-1 protein expression and thus its eNOS

inhibitory effect. They can activate the phosphatidylinositol-3-phosphate (PI3K) / Akt pathway and promote eNOS function by Ser1177 phosphorylation and Thr495 dephosphorylation.

Of the statins, pravastatin is most commonly used to treat preeclampsia because, in addition to its beneficial effects, the number of adverse events observed was most favorable with this statin.

Objectives

1. To Investigate the rapid mechanism of action of pravastatin on NOS activity in healthy and preeclamptic placentas.

2. To develop a new method for the determination of tetrahydrobiopterin sensitivity of NOS in preeclamptic placentas.

3. To investigate whether pravastatin may be suitable for increasing the NOS activity of tetrahydrobiopterin-resistant preeclamptic placenta to a physiological level.

Methods

To investigate the relationship between pravastatin and eNOS, we followed the strategy of examining whether pravastatin can modify the relationship between eNOS-caveolin, eNOS-Hsp90, changes in microsomal arginine uptake, and changes in eNOS Ser1177 phosphorylation. The Ser1177 and Thr495 phosphorylation status of eNOS changes reciprocally, so it is sufficient to study the phosphorylation status of one of them,

in this case Ser1177. The putative association of caveolin and Hsp90 with pravastatin was investigated in the presence of their inhibitors (caveolin-Ca and Hsp90-geldanamycin).

The potential BH4 insensitivity of NOS activity in preeclamptic placenta has been known since 2000. In the study performed by Kukor et al. using placenta homogenate, the BH4 insensitivity of the preeclamptic samples was 70%. Increased oxidative stress in preeclamptic placenta, despite the protection provided by the reducing agent, may inactivate the physiologically concentrated BH4 used in the measurement, thereby causing a decrease in enzyme activity. Therefore, the methodological way used had to be modified to determine the true BH4 insensitivity.

Selection of participants and recording of clinical data

Healthy and preeclamptic pregnant women were involved in the study. All pregnancies were taken care of at the 2nd site of the Clinic of Obstetrics and Gynecology, Semmelweis University (formerly: 2nd Department of Obstetrics and Gynecology, Semmelweis University, Budapest, Üllői út 78 / A). The ethical authorization for the study was granted by ETT TUKEB (authorization number: 48995–2016 / EKU). Clinical data were collected from the medical records of the participating patients from the MedSol system.

Placental microsome preparation

Immediately after delivery, at least 30 grams of placental tissue sample was taken from different parts of the placenta by scissors, then the sample was put in ice and immediately

transferred to the biochemistry laboratory. As we know that the microsome fraction has the highest specific NOS activity in human placenta, microsome was prepared without delay. This microsome fraction was used for the experiments. Samples were separated from the microsome fraction for protein determination and NOS activity was determined immediately, and the remaining samples were stored in several aliquots at -80 oC until NOS BH4 sensitivity was determined and used for Western blot analysis. Samples were stored in liquid nitrogen before the examination of arginine uptake.

Determination of protein

The protein content of the microsome was determined by the Lowry method. The standard curve was prepared using bovine serum albumin (range 0.005-0.2 mg / ml).

Measurement of NO synthase activity

NO synthase activity was determined under conditions providing maximal enzyme activity at various BH4 concentrations, incubated at 37 oC for 10 min. NOS activity was measured in placental microsomes using C14 arginine substrate in healthy (n = 6-9) and preeclamptic (n = 6-9) samples. The activity was stopped by ice-cold NAME solution. C14 arginine was separated from the product of the enzyme reaction C14 citrulline on a Dowex 50-W cation exchange resin. The radioactivity of 3 * 1 ml eluate was measured after mixing with 5-5 ml of scintillation fluid. A sample containing 1 mM NAME was used as a background.

During microsome preparation, most of the water-soluble, low molecular weight materials remain in the cytosolic fraction, including BH4. BH4 is sensitive to oxidation when is quickly converted to dihydrobiopterin (BH2), which no longer binds to the enzyme and does not increase its activity. Activity was always determined using a standard microsome sample from BH4-sensitive, healthy placenta to avoid oxidation-induced artifacts. Enzyme activity was measured at 20 nM (basic acitivity), 200 nM (physiological), and 50 nM (pharmacological) BH4 concentrations. To minimize BH4 oxidation, it was added to the reaction mixture just immediately before starting the reaction. The effect of pravastatin was tested at a concentration of 10 μM.

Investigation of e11OS Ser1177 phosphorylation

Phosphorylation of Ser 1177 in eNOS was examined by Western blot. Western blot analysis of total eNOS and eNOS phosphorylated on Ser1177 was performed on the same membrane.

Determination of arginine uptake of the placental microsome

Rapid filtration technique was used to determine placental microsomal arginine uptake. The method was optimized for arginine uptake. Arginine uptake of microsome samples was measured for 1 min using C14 arginine. The measurement was also performed in the presence of alamethicin (50 μg/mg protein) to determine the intravesically membrane-bound radioactivity. Microsomal arginine uptake was calculated from

the difference in radioactivity between alamethicin-free and alamethicin samples.

Statistical analysis

Statistical analysis of the results was performed with Excel Data analysis software. Significance test was performed by t-test. The difference was considered statistically significant if $p<0.05$. Data were presented as mean ± SD, usually 3-6 different samples were used per experiment.

Results

Effect of pravastatin on eNOS activity

For the study, 6 preeclamptic and 6 healthy placenta were collected at the Department of Obstetrics and Gynecology, Semmelweis University (Budapest, Üllői út, department 78 / A). In the experiments, 6 microsomes prepared from preeclamptic placentas and 6 healthy samples (as controls) were used. There was a significant difference between the two groups in terms of blood pressure, protein excretion, gestational age at birth and birth weight. The age of the mothers did not differ significantly between the control and the preeclamptic groups.

Placental microsome NOS activity

Most of the placental NOS activity (approximately 85%) is Ca-dependent eNOS. Due to the inflammatory condition characteristic of preeclampsia, it has been suggested that iNOS is induced in preeclampsia. It has been shown in the previous years that in addition to the induction of iNOS, the level of

eNOS mRNA also increases significantly, therefore eNOS isoform determines placental NO synthesis even in preeclampsia.

The activity of eNOS increased by 2.0–2.5-fold in 50 μM BH4 in both control and preeclamptic samples compared to baseline activity at 20 nM BH4.

Maximum eNOS activity was further increased by 10 μM pravastatin in healthy subjects by 28% and in preeclamptic specimens by 32%. The effect of pravastatin was similar in healthy and preeclamptic samples. It was observed that the effect of pravastatin was more intensive in the presence of 50 μM BH4 than in the presence of 20 nM BH4. This effect was detected in both the control and preeclamptic groups, but was not significant in either. This minimal effect may be explained by the oxidation of BH4. The effect of pravastatin is concentration dependent, the maximum effect of pravastatin can be measured at a concentration of 10 μM with 1 mM Ca2 + and 50 μM BH4. Pravastatin 100 and 500 μM already has an inhibitory effect. The caveolin, which inhibits eNOS, dissociates from eNOS at 1 mM Ca 2+ concentration, so the effect of pravastatin can be assumed to be independent of caveolin.

Pravastatin effect and Hsp90

The activity of eNOS is increased by the heat shock protein Hsp90. The Hsp90-independent effect of pravastatin was studied in the presence of its inhibitor, geldanamycin. eNOS activity is also increased by 10 μM pravastatin in the presence of 100 nM geldanamycin. When eNOS activity measured in

the presence of geldanamycin was the reference (100%), the mean eNOS activity of a healthy placental microsome was increased by 10 μM pravastatin by 30% using 1 mM Ca 2+, 20 nM BH4 (basal activity); 25% using 1 mM Ca 2+, 50 μM BH4 ($p<0.05$). A similar effect of pravastatin was observed in preeclamptic samples. NOS basic activity increased by 37%; BH4-stimulated activity by 28%.

This result suggests that the effect of pravastatin may be independent of the effect of Hsp90.

Effect of pravastatin on eNOS Ser1177 phosphorylation

Samples were incubated under the same conditions that were used to measure eNOS activity without the use of C14 arginine. The eNOS Ser1177 phosphorylation of the samples was examined by Western blot analysis. We measured that 10 μM pravastatin did not change the phosphorylation state of eNOS Ser1177 during 10 min incubation under the measurement conditions.

Effect of pravastatin on placental microsomal arginine uptake

ENOS is also associated with amino acid transporters (y +, y + L, B0 +) in the cell membrane. Thus, the arginine supply of eNOS is also provided from the extracellular space, the blood. Decreased serum arginine levels have been observed in preeclamptic cases, which may contribute to an increase in blood pressure. Insufficient arginine supply to eNOS may also be exacerbated by elevated ADMA levels observed in preeclampsia. This is because ADMA is not only a

competitive inhibitor of NOS, but also competes with arginine for its amino acid transporter binding site, inhibiting arginine uptake. The uptake of arginine by the placental microsome in the first minute changes linearly with time. Arginine uptake was also increased by 10 μM pravastatin in healthy (38 ± 9%, $p<0.05$) and preeclamptic (34 ± 11%, $p<0.05$) placental microsomes. The effect was found to be similar in both healthy and preeclamptic samples.

Investigation of BH4 resistance in preeclamptic placenta

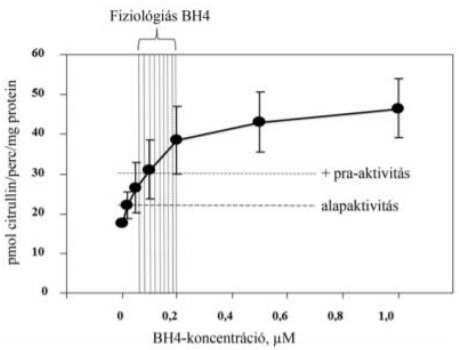
To determine placental BH4 resistance, we examined placental eNOS activity in samples from 9 healthy and 9 preeclamptic pregnancies. There were significant differences between the control and preeclamptic groups in terms of blood pressure, proteinuria, gestational age at birth, and birth weight. The age of the mothers did not differ significantly in the two groups, nor did the average weight gain during pregnancy.

BH4 resistance was assayed using the previously described method of measuring eNOS activity. Of the NOS activity of the 9 preeclamptic samples, one was BH4 resistant. The clinical picture of a BH4-resistant patient did not differ from the preeclamptic cases studied. Physiological BH4 concentration of 0.20 μM in this sample did not significantly increase NOS activity, while the activity of healthy placental microsomes was increased by an average of 60% ($p<0.01$), and the NOS activity of BH4-sensitive preeclamptic samples was increased by 67% ($p <0.01$) compared to basic activity at 0.02 μM BH4.

50 μM BH4 more than doubled the NOS activity of all three groups (healthy, BH4-sensitive-, and BH4-insensitive preeclamptic).

10μM pravastatin increased NOS activity by 32–38% in both healthy and BH4-sensitive and BH4-insensitive samples. The effect was similar at all three BH4 concentrations.

In the case under study, the BH4 insensitive placenta has basal NOS activity, so the enzyme is able to bind BH4. Basal activity cannot be increased by physiological BH4 concentration (60–180 nM), but it could be increased by 50 μM BH4. This suggests that the affinity of the enzyme to BH4 was decreased in this case. The relatively mild (34%) NOS activity-increasing effect of pravastatin is sufficient to increase the activity of the enzyme in the physiological range.



BH4 concentration dependence of NOS activity in healthy placental microsomes and the effect of pravastatin on NOS activity. The hatched part indicates the mean BH4 concentration in healthy primordial and mature placenta. n = 9 ± SD

Conclusions

Research suggests that pravastatin treatment may be effective in high-risk pregnancies for preeclampsia.

1. The aim of this study was to determine the mechanism by which pravastatin can rapidly increase NOS activity in human placenta. As a result, a previously unknown property of pravastatin has been described, that pravastatin may increase placental arginine uptake.

2. A new methodological method has been developed for the determination of tetrahydrobiopterin sensitivity of NOS in preeclamptic placenta.

3. In the investigated tetrahydrobiopterin resistant case, it was established that pravastatin may be suitable for increasing the NOS activity of the placenta to a physiological level.

In order for pravastatin to be used therapeutically in preeclampsia, a number of studies remain to be performed, including the mechanism of action, the evaluation of the advantages and disadvantages of therapy in a large number of patients, and the indication for treatment. The above results are at the level of basic research, but may provide a basis for the selection of individuals to be included in pravastatin therapy (BH4-resistant preeclampstic cases) and the refinement of therapy (pravastatin treatment + arginine supplementation).

List of own publications

Publications on the topic of the dissertation

1. Alasztics B, Kukor Z, **Pánczél Z**, Valent S. A praeeclampsia kórélettana a kétlépcsős modell tükrében [The pathophysiology of preeclampsia in view of the two- stage model]. Orv Hetil. 2012; 153(30): 1167-1176
2. **Pánczél Z**, Kukor Z, Supák D, Kovács B, Kecskeméti A, Czizel R, Djurecz M, Alasztics B, Csomó KB, Hrabák A, and Valent S. Pravastatin induces NO synthesis by enhancing microsomal arginine uptake in healthy and preeclamptic placentas. BMC Pregnancy Childbirth. 2019; 19(1): 426 IF: 2,239
3. **Pánczél Z**, Supák D, Kovács B, Kukor Z, Valent S. Pravasztatin hatása tetrahidrobiopterin érzékeny és -rezisztens praeeclampsiás placenták NO szintáz aktivitására [Effect of pravastatin on tetrahydrobiopterin sensitive and resistant NO synthase activity of preeclamptic placentas]. Orv Hetil. 2020; 161(10): 395–401. IF: 0,564

Other publications

1. **Pánczél Z**, Sára L, Tóth P, Hubay M, Keller E, Langmár Z, Pajor A. Spontán aortaruptura várandósság alatt [Spontaneous aortic rupture during pregnancy]. Orv Hetil. 2011; 152(23): 929-933