

ABRIDGEMENT OF THE DOCTORAL DISSERTATION

**MECHANISMS OF ESTROGEN- AND RALOXIFENE-MEDIATED  
CARDIOVASCULAR PROTECTION**

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## INTRODUCTION

The influence on the incidence of cardiovascular diseases, as one of the most important effect of hormone replacement therapy on mortality of the population has been studied extensively for nearly 20 years. But following natural, or artificial menopause due to bilateral ovariectomy, in the lack of estrogen treatment the risk of coronary diseases is twice higher than in women having received estrogen treatment. In menopause the function of endothelium is weakened gradually: the endothelium-dependent vasodilatation in women above the age of 60 can reach the level observed in men. The tight connection between the reduced function of endothelium, hypertonia, hypercholesterolaemia and atherosclerosis is well-known, and it might lead to increased cardiovascular risk. Therefore the dysfunction of endothelium, as a result of the lack of estrogen in menopause, might play an important role in the elevated mortality due to cardiovascular diseases. Based on these we can postulate that hormone replacement therapy might decrease the incidence of cardiovascular diseases in menopause. However, in contrast to the expectations, well-known, extensive, randomized clinical studies, such as Heart and Estrogen/Progestin Replacement Study (HERS) and Women's Health Initiative (WHI) have not confirmed the beneficial effect of hormone replacement in menopause. The major problems were the short duration of the studies and the involvement of only women already suffering from coronary disease and aged between 65 and 70. Long-term estrogen treatment enhances the risk of breast and endometrial cancer. Therefore a large scale research work was initiated in order to find novel compounds that possess the beneficial characteristics of estrogen without being risk factors of cancers.

The modern idea of selective estrogen receptor modulation (SERM) is promising, since we would have a tool for designing drugs that retain their estrogen-agonistic effect in the target tissues, such as bones, circulatory or

central nervous system without stimulating other tissues, like breasts, where estrogens can act as oncogens. One of these SERM compounds is raloxifene, which was studied by us as well.

Carbon monoxide (CO) produced by heme-oxygenase (HO) enzyme system takes part in estrogen-mediated cardiovascular protection. A range of pharmacons can play role in certain inflammatory (5-amino-salicylic acid) and anti-proliferative (paclitaxel) protective mechanisms through the induction of HO system. Several beneficial features of paclitaxel makes it a promising candidate for local drug therapy of extensive arterial smooth muscle cell proliferation in restenosis following balloon angioplasty or stent implantation.

## **PURPOSES**

The clinical use of new generation SERM compounds might be a potential alternative of hormone replacement therapy in certain stages of menopause. The requirement for the long-term application of SERM compounds is that they have to be safe in diseases appear more and more frequently following menopause, such as ischaemic heart disease and certain alterations of the central nervous system.

We studied the effects of the lack of sex steroids, as well as estrogen and raloxifene treatment.

### 1. Examination of the cardiovascular effect of the lack of estrogen

1.1. How does the lack of E<sub>2</sub> influence plasma AVP-level, basal blood pressure and tendency to heart ischaemia? How is blood pressure as response to AVP, vessel contraction and heart perfusion altered in the lack of estrogen?

1.2. How does estradiol monotherapy and raloxifene substitution influence plasma AVP-level, basal blood pressure and tendency to heart ischaemia, as

well as blood pressure as response to AVP, aorta contraction and heart perfusion as compared to the state of the lack of estrogen?

1.3. We studied the estrogen-mediated cardiovascular role of HO enzyme.

2. Local drug therapy of cardiovascular diseases

2.1. By determining the tissue paclitaxel concentration of the dilated artery wall and adjacent segments we intended to confirm the uptake of paclitaxel into the wall of coronary artery following the treatment of single segments of the coronary artery, as well as bifurcations by drug eluting balloon.

**Further plans:** To prove that paclitaxel develops its anti-proliferative effect through the induction of HO enzyme system, and to study the role of the estrogen-mediated mechanism in domestic pig model.

## **METHODS**

### **Experimental processes, treatments**

In our first series of experiments we studied 230-250g-weighted, 10-12-week-old male and female Wistar rats. Females were examined in the morning of estrus and in diestrus phase. A certain group was subjected to bilateral ovariectomy. Distinct groups of the ovariectomized females underwent a 2-week-long estrofem- ( $E_2$ : 0.10 mg/kg/day, per os, once daily) or raloxifene- (R0.11: 0.11 mg/kg/day; R0.33: 0.33 mg/kg/day; R1: 1.0 mg/kg/day, per os, once daily) treatment. The activity of enzymes was blocked by tin-protoporphyrin IX (SnPP: 30.0  $\mu$ g/kg, s.c., pH 7.40, 24- and 1-hour-long pretreatment).

In the second phase of our experiments we determined the paclitaxel concentration in the coronary arteries - left descending (LAD), left circumflex (LCx) and right (RCA) - of 18-30-kg-weighted domestic pigs 1.5, 12, 24 and 48 following Dior-balloon inflation (2×30 s, 6 atm). Furthermore, bifurcation

intervention was performed on 6 arteries as described: first the main, then the side branches were dilated by a Dior-balloon (6 atm, 2x30s), which was followed kissing balloon dilatation.

During the experiments the care and laboratory use of the animals were performed in accordance with the concerning regulations of the EU and with the approval of the Ethical Committee of the Institute.

### **Determination of plasma AVP-level**

Plasma AVP-level was determined by radioimmunoassay (RIA) technique, and expressed in pg/ml.

### **Measurement of surviving aorta ring contraction in rat**

The tension of aorta was measured *in vitro* with the aid of the software ISOSYS. We examined the contraction as response to 2.0 µg/ml AVP and expressed results as the pressure of aorta ring (g/mg aorta ring).

### **Determination of HO enzyme activity**

We measured the amount of bilirubin formed during the conversion of heme using spectrophotometric method, and expressed results as nM bilirubin/h/mg protein.

### **Examination of HO-1 enzyme expression**

The expression of HO-1 enzyme was determined by using Western blot. The results were expressed as percentage following densitometrical analysis.

### **Measurement of basal blood pressure and the response to AVP**

The animals were subjected to phentolamine treatment (10.0 mg/kg, i.p.) following anesthesia performed with 30 % urethane (0.50 ml/100 g, i.p.), and after the stabilization of blood pressure AVP (0.02; 0.06; 0.18 µg/kg, i.v.) was

injected into the lateral tail vein. The increase in blood pressure was determined (maximal increase in % as compared to the basal value) in the right carotid artery. Results were evaluated by the software HAEMOSYS program (Experimetria, UK, London).

### **Determination of heart perfusion according to Langendorff**

10-20 minutes prior to cervical dislocation the animals received heparin injection (500 IU, i.p.), then their hearts were placed to a Langendorff perfusion column. Heart perfusion as response to AVP (1.0; 3.0; 10.0 µg) was measured following a stabilization period of 15 minutes, and results were expressed as % as compared to the basal value.

### **Experimental angina model evoked by adrenalin and phentolamine**

Mean arterial blood pressure and surface II. ECG was measured and analyzed with the aid of HEMOSYS computerized system. The changes in ST depression was used for determining heart ischaemia. In epinephrine-phentolamine model a single dose of epinephrine (10.0 µg/kg), and 30 s later  $\alpha$ -adrenoceptor antagonist phentolamine (15.0 mg/kg) was administered into the tail vein of the animals. Changes in ECG and blood pressure were monitored simultaneously.

### **Determination of paclitaxel concentration**

The concentration of paclitaxel was determined in plasma, LAD, LCx, and RCA by using high-performance liquid chromatography (HPLC), and tissue concentrations were expressed as µM/L.

### **Statistical analysis**

Data are expressed as mean  $\pm$  S.E.M. The results of Western-blot experiments are demonstrated in representative photos. Statistical significance was

determined by Mann W. test, and differences were considered as significant if  $p < 0.05$ .

In experiments with pigs the continuous parameters of the groups were expressed as mean  $\pm$  S.D. Tissue paclitaxel concentrations measured in the distinct arterial segments (proximal, middle and distal) were grouped, and grouped mean values were compared by unpaired t-test. Differences were considered as significant if  $p < 0.05$ . Statistical analysis was performed with the aid of the software SPSS for Windows version 11.5.

## **DISCUSSION**

The lack of endogenous estrogen following experimental menopause resulted in elevated plasma AVP level, increased basal blood pressure and ST depression, which alterations could be normalized by hormone replacement or raloxifene therapy. Treatment with estradiol or raloxifene restored the ability of responding to AVP both in heart and aorta, through significantly reducing the enhanced aorta contraction observed in the lack of the sex steroid, and increased heart perfusion. This alteration means that in the lack of endogenous estrogen the risk of hypertonia is enhanced, and that following hormone replacement blood pressure is normalized. Results gained from measuring surviving aorta contraction in rat show that the reason for the vascular response to AVP is the direct effect of estrogen and raloxifene on the smooth muscle of the vessels. According to our results in ovariectomized rats plasma AVP level and arterial blood pressure are elevated, the increase in surviving aorta ring contraction and the decrease in Langendorff heart perfusion are much more expressed than in intact females. Estrogen replacement or raloxifene treatment abolish the increased blood pressure and the extended pressor response to AVP. By intensifying the expression and activity of NOS and HO enzymes, as well as enhancing the release of other vasodilators, while reducing the synthesis and

secretion of vasoconstrictors, estrogen and raloxifene favor vasodilatation. Supporting these findings we have observed that the lack of endogenous estrogen results in the inhibition of NOS and HO enzymes located in the vessels, which, according to our conclusions, explains the enhanced sensitivity of the vessel system to AVP. The expression and activity of HO enzymes are also significantly lower in the cardiac left ventricle of ovariectomized rats than those in control females possessing intact estrogen formation. While the lack of estrogen resulted in decreased level of HO, the 2-week-long estrogen or raloxifene treatment restored the activity and expression of HO enzymes to the level observed in control females. According to our results concerning cardiac muscle the expression of HO shows sexual dimorphism and and cycle-dependence. We observed a lower level of activity and expression also in males as compared to females in estrus phase. However, having examined the distinct estrogen fullness states we did not detect significant differences in activity between estrus and diestrus phase. Concerning HO-activity in the aorta the results were entirely contrary, which might be explained in differences between the distinct tissues.

Both enzymes up-regulated in an estrogen-mediated manner - NOS and HO – play role in the reduction of the tendency of cardiac muscle to ischaemia. Following treatment with L-NAME – a non-selective NOS inhibitor – or SnPP – inhibitor of HO – cardiac ischaemia, blood pressure and heart perfusion became significantly expressed, which support the estrogen-mediated regulator role of NOS and HO in cardiovascular protection. Based on our results we assume that estrogen and raloxifene substitution lead to an increased level of NO, as well as the reaction products of HO, and these mediators enhance the beneficial effect of each other. Therefore these two mechanisms are considered to be the most important participants in the protection of heart from ischaemia.

In our further experiments we have proved that paclitaxel – a compound used in clinical practice due to its ability to induce HO enzyme system, therefore acting



as an anti-proliferative agent – can be uptaken within a short time into coronary vessels in a concentration that is sufficient for its anti-proliferative effect. According to the literature paclitaxel induces the expression of HO-1 gene efficiently. In the present study, measuring tissue paclitaxel concentration in single or bifurcation segments of porcine coronary artery 1.5, 12, 24 and 48 post-dilatation we confirmed the efficacy of the delivery of the compound by a Dior-balloon. Paclitaxel is supposed to deliver its anti-proliferative effect through the HO system, therefore one of our further plans is to confirm its relationship with this system, and to examine the estrogen-mediated effect in domestic pig model.

## **SUMMARY**

According to our observation SERM raloxifene provides cardiovascular protection in the lack of estrogen through acting as an estrogen agonist in the regulation of HO enzyme system. The effect of estradiol on miogenic tone appears to be an endothelium-dependent response mediated by NO synthesis, and our results demonstrate that estrogen and raloxifene takes part in the protective mechanisms of the cardiovascular system through the up-regulation of HO enzymes. Furthermore, we can state that endogenous AVP is likely to play role in the enhanced vessel contractility in menopause.

Short-term local delivery of paclitaxel into the coronary artery by a drug-coated balloon is sufficient for providing the appropriate tissue concentration of the compound, which can effectively inhibit the neointimal growth of coronary arteries.

## **Publications based on the dissertation**

**Posa A, Hemetsberger R, Petnehazy Ö, Petrasi Zs, Testor M, Glogar D, Gyöngyösi M**

Attainment of local drug delivery with paclitaxel-eluting balloon in porcine coronary arteries  
Coronary Artery Disease (In press)

*Impact factor: 1.507*

**Whittle BJ, Varga C, Posa A, Molnar A, Collin M, Thiemermann C.**

Reduction of experimental colitis in the rat by inhibitors of glycogen synthase Kinase-3  $\beta$   
Br. J. Pharmacology 2006 Mar;147:575-82.

*Impact factor: 3.825*

**Varga C, Laszlo F, Fritz P, Cavicchi M, Lamarque D, Horvath K, Posa A, Berko A, Whittle BJ.**

Modulation by heme and zinc protoporphyrin of colonic heme-oxygenase-1 and experimental inflammatory bowel disease in the rat.  
Eur J Pharmacol. 2007 Apr 30;561(1-3):164-71. *Impact factor: 2.432*

## **Publications related to the dissertation**

**Barta A, Tarjan I, Kittel A, Horvath K, Posa A, Laszlo F, Kovacs A, Varga G, Zelles T, Whittle BJ.**

Endotoxin induces a decrease in isolated rat parotid acinar cell amylase secretion in a nitric oxide independent manner.  
Eur J Pharmacol. 2005;524:169-73.

*Impact factor: 2.432*

**Czako L, Szabolcs A, Vajda A, Csati S, Venglovecz V, Rakonczay Z Jr, Hegyi P, Tiszlavicz L, Csont T, Posa A, Berko A, Varga C, Varga Ilona S, Boros I, Lonovics J.**

Hyperlipidemia induced by a cholesterol-rich diet aggravates necrotizing pancreatitis in rats.  
Eur J Pharmacol. 2007;572:74-81.

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**Szabolcs A, Tiszlavicz L, Kaszaki J, Posa A, Berko A, Varga IS, Boros I, Szüts V, Lonovics J, Takacs T.**

Zerumbone exerts a beneficial effect on inflammatory parameters of cholecystokinin octapeptide-induced experimental pancreatitis but fails to improve histology.

Pancreas. 2007;35:249-55.

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Whittle BJ, Varga C, Berko A, Horvath K, Posa A, Riley JP, Lundeen KA, Fourie AM, Dunford PJ.

Attenuation of inflammation and cytokine production in rat colitis by a novel selective inhibitor of leukotriene A(4) hydrolase.

Br J Pharmacol. 2008;153:983-91.

*Impact factor: 3.825*

Horvath K, Varga C, Berko A, Posa A, Laszlo F, Whittle BJ.

The involvement of heme oxygenase-1 activity in the therapeutic actions of 5-aminosalicylic acid in rat colitis.

Eur J Pharmacol. 2008;58:315-23.

*Impact factor: 2.432*

Hemetsberger R, Posa A, Pavo N, Farhan S, Csonka Cs, Csont T, Ferdinandy P, Garamvölgyi G, Petrasi Zs, Petnehazy Ö, Varga C, Pavo I Jr, Laszlo F Jr, Huber K, Wojta J, Glogar D, M Gyöngyösi.

Die involvierung des Nitric Oxids im kardioprotektiven Effekt des „early ischaemic preconditioning“ in Ischämie/Reperfusion Model in Schweinen.

Journal für Kardiologie (In press)

*Impact factor: 0.0*

***Cumulative impact factor of the publications based on the dissertation:  
7,764***

***Cumulative impact factor of the publications related to the dissertation:  
13.458***

## Conference abstracts

### **LXVIIth Meeting of the Hungarian Society of Physiology (2003)**

#### **Poster**

Effects of raloxifene and estradiol treatment on vasoconstriction caused by vasopressin in ovariectomized rats

László FA, Varga C, **Pósa A**, Molnár A, László F.

### **XXth Congress of the Hungarian Society of Endocrinology and Metabolism (2004)**

#### **Poster**

Investigation into the cardiovascular interaction of raloxifene and arginine vasopressin in rat

**Pósa A**, Horváth K, Varga C, László F, László FA.

### **XIIth Congress of the Hungarian Society of Hypertonia (2004)**

#### **Poster**

Effect of raloxifene on the activity of nitric oxide synthase and heme-oxygenase, on the basal blood pressure, aorta contraction and perfusion pressure of the heart in experimental menopause

Priger P, Molnár A, Varga C, László F, Molnár Z, László FA, Horváth K, Berkó A, Kordás K, **Pósa A**.

### **VIth Congress of the Hungarian Society of Pharmacology and Clinical Pharmacology (2005)**

#### **Oral presentation**

Raloxifene decreases the enhanced vasoconstriction caused by vasopressin in experimental menopause *in vivo* and *ex vivo* models

**Pósa A**, Priger P, Molnár A, Varga C, Molnár Z, Horváth K, Berkó A, Kordás K, László F, László FA.

The interaction of constitutive nitric oxide synthase and heme-oxygenase enzymes in the maintenance of the integrity of the vascular endothelium in male and female rats

Molnár Z, Priger P, **Pósa A**, Horváth K, Kordás K, Varga C, László FA, László F.

The endogenous estrogen protects rat heart against ischemia by the up-regulation of

heme-oxygenase

Priger P, Molnár Z, **Pósa A**, Horváth K, Kordás K, Varga C, László FA, László F.

## **Congress of the Hungarian Society of Cardiology (2006)**

### **Oral presentation**

Role of the estrogen caused up-regulation of heme-oxygenase enzyme in the protective mechanism of cardiovascular system

**Pósa A**, Horváth K, Varga C, Kordás K, Egresits J, Nemcsik J, László F.

## **XXIth Congress of the Hungarian Society of Endocrinology and Metabolism (2006)**

### **Poster**

Alterations in the expression of heme-oxygenase isoenzyme in the cardiovascular system at different states of estrogen fulness

Pécsi I, **Pósa A**, Berkó A, Varga C, László FA, Kordás K, László F.

## **LXXth Congress of the Hungarian Society of Physiology (2006)**

### **Poster**

Decreasing of NFκB and inflammatory mediators by the inhibition of glycogene synthase-3β in the colon of rats

Varga C, Berkó A, Horváth K, **Pósa A**, Molnár A, Collin M, Thiemermann C and Whittle BJR.

### **Oral presentation**

Raloxifene protects the cardiovascular system by increasing heme-oxygenase enzyme synthesis in experimental menopause

**Pósa A**, Pécsi I, Molnár Z, Priger P, Berkó A, Varga C, László FA, László F.

Role of the interaction of nitric oxid synthase and heme-oxygenase enzymes in the sexual dimorphism of the integrity of vascular endothelium

László F, Pécsi I, Molnár Z, Priger P, **Pósa A**, Berkó A, Horváth K, Varga C, László F.

Alterations of heme-oxygenase isoenzyme in the cardiovascular system at different states of estrogen fullness

Pécsi I, **Pósa A**, Berkó A, Varga C, László FA, László F.

Role of 5-aminio-salicylic acid caused glutathione decrease on the expression of heme oxygenase-1 in experimental colitis model of rats

Horváth K, László F, BJR Whittle, **Pósa A**, Molnár A, Berkó A, Varga C.

## **48th Meeting of the Hungarian Gastroenterological Association (2006)**

### **Poster**

5-amino salicylic acid induced depletion of glutathione protects the colon against trinitrobenzene sulphonic acid injury through heme-oxygenase-1 enzyme expression

Horváth K, László F, BJR Whittle, **Pósa A**, Molnár A, Berkó A, Varga C.

### **Oral presentation**

Hyperlipidemia aggravates necrotizing pancreatitis via altered nitric oxide synthase in rat

Czakó I, Szabolcs A, Tiszlavicz L, Csont T, Berkó A, **Pósa A**, Varga C, Lonovics J.

Beneficial effect of zerumbone on cholecystokinin-octapeptide induced acute pancreatitis in rat

Szabolcs A, Tiszlavicz L, Kaszaki J, Varga C, **Pósa A**, Berkó A, Lonovics J, Takács T.

### **Digestive Disease Week (DDW), American Gastroenterological Association (AGA) (2006)**

#### **Poster**

Modulation of rat colonic TNF $\alpha$ , iNOS and acute colitis through down-regulation of NF $\kappa$ B by glycogen synthase kinase-3 $\beta$  inhibition.

BJR Whittle, Varga C, Berkó A, **Pósa A**, Molnar A, Collin M & Thiermann C.

### **12th Meeting of the European NeuroEndocrine Association (ENEA) (2006)**

#### **Poster**

Time and concentration-dependent interaction between glutathione and hem-oxygenase-1 enzyme: an *in vitro* and *in vivo* study.

Horváth K, László F, BJR Whittle, **Pósa A**, Molnar A, Berkó A, Varga C.

### **European Society of Cardiology (2007)**

#### **Poster**

Involvement of Nitric Oxide in the Cardioprotective Effect of Early Ischemic Preconditioning in the Reperfusion Phase in Pigs

Hemetsberger R, **Posa A**, Farhan S, Csonka Cs, Csont T, Ferdinandy P, Garamvölgyi R, Petراسi Zs, Petnehazy Ö, Varga C, Laszlo F jr, Huber K, Wojta J, Glogar D, Gyöngyösi M.