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 meanopause 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 menopausa 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 menopausa, might play and important role in the 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 menopausa. However, in contrast to the expectations, well-known, extensive, randomized chlinical 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 menopausa. 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 restenosos 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 menopausa. 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 menopausa, 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_2 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 acticity of enzymes was blocked by tin-protoporphyrin IX (SnPP: 30.0 μg/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 anesthesy 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 expresed 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 α-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 ± S.E.M. The results of Western-blot experiments are demonstrated in representative photos. Statictical 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 ± 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 menopausa 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 enzimes, as well as enhancing the release of other vasodilatators, 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 enzimes 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 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-proloferative agent – can be uptaken within a short time into coronary vessels in a concentration that is sufficient for its anti-proloferative 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-proloferative 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 menopausa.

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.

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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

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