Ph.D. thesis

Examination of the effects of estrogen deficiency, obesity, and voluntary exercise in animal models

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List of abbreviations

ALT: alanine aminotransferase

AST: aspartate aminotransferase

CO: carbon monoxide

CR: calorie restriction

CTRL: standard chow

CVD: cardiovascular disease

FFA: free fatty acid

HO-1, HO-2, HO-3: heme oxygenase-1, heme oxygenase-2, heme oxygenase-3

HT: high-triglyceride diet

IL-6: interleukin-6

LV: left ventricle

MAPK: mitogen activated protein kinase

MPO: myeloperoxidase

OGTT: oral glucose tolerance test

OVX: ovariectomy surgery

POVX: pharmacological ovariectomy

SnPP: tin-protoporphyrin IX

SO: sham operation

TNF-α: tumor necrosis factor-alpha

Introduction

In premenopausal women the cardiovascular morbidity and mortality is lower than in age-matched men, however, the risk of cardiovascular diseases (CVD) are almost similar in men and women at the onset of menopause. The risks related to postmenopause, which are mainly due to the interruption of estrogen, increase the incidence of CVDs by the regulation of lipid profile and glucose metabolism. As a result of estrogen insufficiency, glucose tolerance and insulin sensitivity decrease, body composition and fat accumulation alter, thus these changes result in visceral adiposity in postmenopausal women. The excess production of free fatty acids (FFA) coupled with enhanced lipolysis induce muscle and hepatic insulin resistance through abnormal modulation of the glucose production and glucose uptake. Additionally, the chronic exposure to FFA inhibits insulin secretion from pancreas and leads to β-cell apoptosis. Increased visceral fat is also associated with the increased production of adipocyte-derived cytokine and leptin. A series of metabolic complications including dyslipidemia, hyperglycaemia, hyperinsulinaemia and the elevated leptin concentration promote the development of nonalcoholic fatty liver disease. In addition to metabolic disorders, estrogen withdrawal may also be associated with damaged antioxidant state and endothelial dysfunction. Heme oxygenase (HO) enzymes are responsible for the breakdown of heme to yield free ferrous iron, equimolar amount of carbon monoxide (CO), and biliverdin, which is ultimately converted to bilirubin by biliverdin reductase. There are three known isoforms of HO. HO-2 and HO-3 are constitutively expressed, while the inducible isoform is the HO-1. It has been shown that both HO-1 and HO-2 isoforms play an important role in cardioprotection. Among the products of HO isoforms, bilirubin is the most potent scavenger of reactive oxygen species and CO exerts antiapoptotic and anti-inflammatory effects via mitogen activated protein kinase (MAPK) pathway. Moreover, CO influences the soluble guanylyl cyclase and cyclic guanosine monophosphate pathways, which serve to regulate both blood pressure and vascular contractility. While the termination of hormonal protective effects with the impairment of metabolic and the antioxidant mechanisms increases the risk state of postmenopausal women in itself, sedentary lifestyle, poor diet, and anomalies associated with aging further deteriorate the life expectancy of women.

Considering the length of postmenopausal women's lifetime, the reduction of risk factors accompanying the menopause has a major public health importance. Both pharmacological and non-pharmacological treatment strategies are used to manage and mitigate the postmenopausal complications. Lifestyle changes involving regular physical exercise and/or

an efficient dietary intervention with the improvement of a number of risk factors, may ameliorate the risk profile.

Aims

In addition to the complications accompanying the menopause, new life-threatening risk factors also have an important role in life expectancy of postmenopausal women.

1. In the first experiment, our aim was to clarify the effects of both age-related natural estrogen deficiency and experimental menopause on the inflammatory parameters, the activity and expression of HO enzymes, and the ischemic susceptibility of heart.

2. In our second experiment we set out to examine the metabolic effects of lifestyle changes (nutrition and physical exercise) in reproductive and ovariectomized rats.

There were two aspects of our investigation:

- How can estrogen deficiency and/or nutritional habits (control, high-triglyceride diet, and calorie restriction) modify the metabolic parameters?

- How can the metabolic parameters of reproductive and ovariectomized rats change due to non-pharmacological treatment strategies, including regular voluntary exercise, calorie restriction or combination of both?

Materials and methods

Effects of natural estrogen deficiency and experimental menopause

Experimental groups of animals

In our first study, experimental estrogen deficiency was induced pharmacologically (POVX, 750 μg/kg triptorelin *i.m.* every 4th week) or surgically with bilateral ovariectomy surgery (OVX). To investigate the effects of age-related natural menopause, 24-month-old, ovary intact aged rats were used. In control group the female rats were sham operated (SO). After four-week resting period, we measured the concentrations of pro-inflammatory tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), the activity of myeloperoxidase (MPO) and HO enzymes and the expression of HO-1 and HO-2 isoforms in cardiac left ventricle (LV). Moreover, the effect of HO activity blockade (by 24-hour and 1-hour pretreatment with tin-protoporphyrin IX, SnPP) on the epinephrine and phentolamine-induced electrocardiogram ST segment changes was examined *in vivo*.

Measurement of cardiac LV TNF-α and IL-6 concentrations

The concentrations of TNF-α and IL-6 of cardiac LV were determined by quantitative enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's directions where the values were expressed as pg/mg protein.

Cardiac LV MPO activity

MPO activity was detected by O-dianisidine dihydrochloride and hydrogen peroxide. It was measured spectrophotometrically and the values were expressed as mU/mg protein.

Cardiac LV HO activity

HO activity was assessed by measuring bilirubin formation spectrophotometrically and was defined as the amount of bilirubin (nmol) produced per hour per mg protein.

Cardiac LV HO-1 and HO-2 expression

The expression of HO-1 and HO-2 isoforms was determined by Western blot analysis. HO-1 and HO-2 were expressed as % (100 % being the maximal expression).

Protein determination

To determine the protein content we used Bradford microassay.

Experimental angina provoked by epinephrine plus phentolamine (ST depression)

The standard limb lead III of the electrocardiogram was recorded by HAEMOSYS system. The change in the ST segment was measured and used as an index of angina severity. In the epinephrine plus phentolamine model, a single dose of epinephrine (10.0 μg/kg) and 30 s later the -adrenoceptor antagonist phentolamine (15.0 mg/kg) were administered into the tail vein of the rat. The difference in the amplitude of the ST segment after and before the administration of the angina-provoking agents was calculated and expressed as the depression of the ST segment in mV. To investigate the effects of HO enzyme activity inhibition on ST segment changes tin-protoporphyrin IX (SnPP, 30.0 μmol/kg, s.c., pH 7.4) was administrated 24 h and 1 h before treatment.

Statistical analysis

Data are reported as means ± S.E.M. and statistical comparisons were performed by ANOVA. *P* values less than 0.05 were considered significant.

Examination of metabolic effects lifestyle changes (nutrition and physical exercise) in reproductive and ovariectomized (OVX) rats

Animals and experimental design

Female Wistar rats were underwent sham operation (SO) or bilateral ovariectomy surgery (OVX) in order to induce estrogen-deficient conditions. After a four-week resting period, the OVX and SO groups were randomized into new groups, based on the type of diet and

training. Rats were fed standard chow (CTRL subgroup), a high-triglyceride diet with 40 % fat content (HT subgroup) or 50 % calorie restricted food (CR subgroup). Calorie restriction meant a 50% reduction of the daily standard chow consumption. The running animals were placed into cages fitted with a running-wheel and allowed free access to the wheel for 24 h per day for 12 weeks. The exercising protocol, defined as a voluntary wheel-running model, which was selected in an effort to isolate the effects of exercising from the additional stress associated with forced exercise protocols. At the end of the twelve-week experimental period, body weight gain, glucose sensitivity, and levels of serum insulin, plasma triglyceride, leptin and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured.

Measurement of body weight

Each rat was labelled and weighed at the start and at the end of twelve-week experimental period.

Oral glucose tolerance test (OGTT)

The blood glucose level was detected at the end of twelve-week treatment period. After a 12 h fasting period tail blood was taken before the application of glucose by oral gavage (0.1 g/bw) and 30, 60, and 120 min afterwards. Glucose values were analysed via Accu Chek Active strips and expressed as mmol/l. Blood samples were collected for insulin detection at the same time as blood glucose measurements.

Determination of insulin, triglyceride, leptin, AST and ALT

At the end of twelve-week period, serum levels of insulin and plasma levels of triglyceride, leptin, AST, and ALT were determined by ELISA according to the manufacturer's instructions. Serum levels of insulin were expressed as mIU/l, plasma leptin as ng/l, triglyceride as mmol/l, AST and ALT concentrations as U/l.

Statistical analysis

Data are reported as means ± S.E.M. and statistical comparisons were performed by ANOVA. *P* values less than 0.05 were considered significant.

Results

Effects of natural estrogen deficiency and experimental menopause

The levels of the pro-inflammatory cytokines TNF-α and IL-6 as well as the activity of MPO were significantly elevated during aging and after ovariectomy in both the POVX and OVX groups in comparison with the SO control animals. Experimental menopause and age-related natural estrogen withdrawal were accompanied by a significantly lower HO activity and reduced cardiac expression of HO-1 and HO-2 proteins. The administration of phentolamine caused a significant ST segment depression 30 sec following epinephrine administration in the POVX, OVX, and aged rats. In the SO control females, the ST segment depression did not evolve. Pretreatment with the HO inhibitor SnPP resulted ST depression in SO animals, and the ischemia susceptibility of heart augmented it in the POVX, OVX and aged rats.

Examination of metabolic effects of lifestyle changes (nutrition and physical exercise) in reproductive and ovariectomized (OVX) rats

At the start of the training and feeding period the OVX rats exhibited the highest body weight. Twelve weeks of the treatment period further increased the weight of the OVX females, whereas the CR alone or in combination with physical exercise resulted weight reduction. At the end of twelve-week treatment period, serum insulin measurements revealed that the 50 % CR diet significantly decreased the fasting insulin level, while HT resulted an elevation in case of SO rats. We found that OVX augmented the fasting insulin values of each dietary subgroup as compared with the SO CTRL group. Changes in insulin sensitivity induced by OGTT show that the strongest reduction was observed in both SO and OVX animals which participated in the CR and physical exercise. Similarly to the insulin values, exercise training and CR diet caused a significant improvement in glucose sensitivity of SO and OVX rats. In case of plasma triglyceride, leptin, AST and ALT concentrations, estrogen deprivation and HT diet increased these values, whereas combined effects of CR and twelve weeks of exercise was the most effective in the reduction of pathological values.

Summary

- Our results clearly show that reduction in HO enzyme activity and the expression of HO-1 and HO-2 isoforms attenuated the anti-inflammatory and antioxidant effects of biliverdin/bilirubin and CO byproducts, which contributed to increased levels of inflammatory parameters and augmented cardiac ischemia susceptibility.

- Menopause by itself caused a negative impact on metabolic parameters, which was exacerbated by HT diet and the lack of physical exercise. Obesity is a pathological condition and is characterized by low grade inflammation, low insulin sensitivity, altered glucose homeostasis and lipid profile, which play a role in the development and progression of CVDs.

- In our work we established that regular physical exercise combined with a 50 % dietary intervention can be a potential non-pharmacological treatment in menopause. The incidence and progression of metabolic disorders and the caused complications can be slowed down and stopped by a balanced diet and regular exercise and consequently may improve the life expectancy of postmenopausal women.

Publications directly related to the PhD thesis

Renáta Szabó MTMT code: 10038382

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