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Possible Participation of EDRF-NO in the Hormonal Regulation of Bone Blood Flow in Rats

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Summary

An increase in bone blood flow (BBF) was observed in rats after castration whereas a decrease in BBF occurred after oestradiol or testosterone. The possible participation of prostaglandins in these changes was demonstrated. The present results show that the endothelium-derived relaxing factor, i. e. nitric oxide (EDRF-NO), might play a role in these hormonal actions on BBF. Until now, almost nothing is known about the possible action of NO on bone circulation. Methylene blue (MB) as a substance blocking EDRF-NO was administered to sham-operated or oophorectomized (OOX) female rats. We determined local blood flow (^{85}Sr-microsphere uptake), cardiac output, blood pressure, heart rate, density of the tibia and ash weight, as well as 24-h incorporation of ^{45}Ca and ^{3}H-proline into the tibia. The administration of MB (0.5 % in the food for 4 weeks) significantly lowered both ^{85}Sr-microsphere uptake and blood flow values in the tibia and distal femur of sham-operated and OOX rats. MB lowered cardiac output and blood pressure to the same extent, indicating no change in the vascular resistance. After the administration of MB (0.1 % in the food), ^{85}Sr-microsphere uptake decreased significantly in the tibia of OOX females while no significant change was found in soft tissues. Bone density and ash weight were significantly lower in OOX rats and in sham-operated rats after MB treatment. Finally, the 24-h incorporation of both ^{45}Ca and ^{3}H-proline decreased significantly in OOX females after MB administration (0.04 % in the food). It can be concluded that 1) MB lowers BBF, suggesting the participation of EDRF-NO in BBF regulation, 2) MB does not influence or may even suppress cardiac output and blood pressure in high dosage, 3) MB lowers 24-hour incorporation of ^{45}Ca and ^{3}H-proline into the tibia of OOX rats, which is in agreement with the circulatory effect, 4) MB lowers bone density and ash weight of the tibia in non-castrated female rats. The effects of MB observed in our experiments partially differ from those of arginine-derived blocking agents. This requires further elucidation.

Key words
Bone blood flow – Endothelium-derived relaxing factor – Nitric oxide – Rat

Introduction

An increase in bone blood flow (BBF) was observed after castration whereas BBF decrease occurred after oestradiol or testosterone treatment in both female and male rats (Schoutens *et al.* 1984, Kapitola *et al.* 1991, Kapitola *et al.* 1995). Thus oestradiol and testosterone seem to have inhibitory effects on BBF. The question arose as to the mechanism of the hormonal actions observed. The experiments with acetylsalicylic acid indicated a participation of vasodilatory prostaglandins, most probably PGE_2, as a mediator of BBF changes occurring after deficiency or administration of estrogen (Kapitola *et al.* 1994).

At present, however, the most interesting factor influencing the blood circulation seems to be endothelium-derived relaxing factor (EDRF), now known as nitric oxide (NO). Its extensive effects on circulation, including local blood flow in different organs and tissues, are already well known (for review see e. g. Lowenstein *et al.* 1994). However, the influence of NO on bone blood flow was studied only exceptionally (Davis and Wood 1992).

We therefore started to investigate the possible participation of EDRF-NO in the hormonal

actions on BBF, the incorporation of ^{45}Ca and ^{3}H-proline and bone mineral content in female rats. Methylene blue (MB) was used as an agent blocking the production and action of EDRF-NO. Our working hypothesis was that the inhibition of BBF increase by simultaneous blocking of EDRF-NO with MB would indicate the possible participation of EDRF-NO in oophorectomy-induced BBF changes. We present here the results of three experiments which seem to support this hypothesis.

Material and Methods

Three experiments were performed on 133 female Wistar rats aged 70 days (Institute of Pharmacy, Prague, Czech Republic) fed a standard laboratory diet (Bergman, Jesenice, Czech Republic) and drinking water *ad libitum*. All experiments were carried out in the following groups: *group 1:* controls (sham operation), *group 2:* methylene blue, *group 3:* oophorectomy (OOX), *group 4:* OOX + methylene blue. Oophorectomy was performed by the dorsal approach four weeks before the actual experiment. Methylene blue (Sigma, USA) in a concentration of 0.5 % (exp. A), 0.1 % (exp. B) or 0.04 % (exp. C) was added to the food for four weeks preceding the examination.

The local blood flow and cardiac output was determined by means of microspheres labelled with radioactive strontium ^{85}Sr (Rudolph and Heymann 1967, Kapitola *et al.* 1987). The rats were anaesthetized with Thiopental (Research Institute of Antibiotics and Biotransformations, Roztoky, Czech Republic) and were given an i. v. injection of 200 IU heparin in 0.2 ml. A catheter was introduced into the right femoral artery and connected to a Type 304 peristaltic pump (Zalimp, Poland), which acted as an artificial organ for the determination of cardiac output. Another catheter, which was connected to LMP 160 pressure transducer with LDP 186 blood pressure recorder and LKM 210 cardiomonitor (Tesla, Czech Republic), was introduced *via* the right carotid artery into the left heart ventricle under control of the pressure curve. Through this tubing we injected a dose of approximately 18.5 kBq, i.e. 0.5 μCi ^{85}Sr-microspheres (diameter 15 μm, NEN, USA) and immediately rinsed the catheter with saline. Each rat was decapitated after about one minute. The following organs and tissue samples were removed: left tibia, an approximately 7 mm segment of the distal end of the left femur, diaphysis of the left femur and calvaria, both kidneys, one lobe of the liver, left gastrocnemius muscle and a sample of the skin from the ventral part of the body. The samples were weighed on a PRLT TW2 torsion balance (Tecniprot, Poland) and their radioactivity was measured together with ^{85}Sr-microsphere standards using Gamaautomat NA 3601 (Tesla, Czech Republic). The uptake of ^{85}Sr-microspheres was expressed as the percentage of the dose per 1 g of tissue; this value was used in the present paper as a relative indicator of local circulatory changes, independent of simultaneous changes in cardiac output. The local blood flow and cardiac output were computed according to generally employed formulas (Kapitola *et al.* 1987).

The 24-h incorporation of ^{45}Ca and ^{3}H-proline, as an indication of mineralization and of the formation of the organic matrix, was determined after Globus *et al.* (1986). 7.4 kBq, i.e. 0.2 μCi ^{45}Ca, in the form of $CaCl_2$ (Polatom, Poland) and 185 kBq i.e. 5 μCi ^{3}H-proline (Amersham, England) per 100 g body weight were injected i. p. in a single dose. The rats were sacrificed on the next day, their left tibia was dissected out, cleaned and dissolved in concentrated HCl. Some of the diluted material was measured in a liquid scintillator (Insta-Gel Packard, USA) on a 1219 Rackbeta Liquid Scintillation Counter (LKB, Finland). The results are given as the number of dpm per mg osseous tissue.

Bone density was computed on the basis of the principle of Archimedes, after weighing the tibia on a torsion balance under water and in the air. Ash weight was determined after incinerating the bone for about 18 h in a muffle furnace at 800 °C by weighing the ash on a 2004 MP balance (Sartorius, Germany). The results are given in mg of ash per ml bone volume.

Statistics

To assess the significance of group differences we used the one-way analysis of variance. The appropriateness of the ANOVA model was checked by investigating whether the data within each group appeared to be normally distributed and whether the population variance was the same in each group. Standardized skewness and kurtosis was used for testing the assumption of normality and the Bartlett test was employed to test the equality of variances. The non-parametric Kruskal-Wallis test was used for the parameters where one of these assumptions was not fulfilled. As a multiple comparison test we used the Scheffe or LSD test.

Results

Experiment A – effect of 0.5 % MB in the food on blood flow in the tibia and distal femur (Table 1). Four weeks after OOX, ^{85}Sr-microsphere uptake was significantly increased in the tibia; ^{85}Sr-microsphere uptake and blood flow in the distal femur were not significantly altered. MB administration markedly lowered both microsphere uptake and blood flow in the tibia and distal femur of both OOX and sham-operated rats. It also significantly lowered cardiac output, blood pressure and heart rate. Bone density and ash weight decreased after OOX, but they did not change after MB administration.

Table 1
Influence of methylene blue administration (0.5 % in the food for 4 weeks) on local blood flow in tibia and distal femur, on mineralization of tibia and on general haemodynamics in female sham-operated or oophorectomized (OOX) rats. Experiment A.

Group	1 Controls	2 OOX	3 Methylene blue	4 OOX + Methylene blue
Number of rats	11	12	11	12
Body weight (g)	234±3	252±2[a]	193±5a	215±5[ab]
Cardiac output (ml/min)	50.0±4.2	49.2±4.7	33.5±3.6[a]	35.1±3.2[ab]
(ml/min per 100 g)	21.4±1.8	19.5±1.8	17.2±1.8[a]	16.2±1.3[a]
Heart rate (beats per min)	424±12	413±9	330±9[a]	337±9[ab]
Mean arterial pressure (mm Hg)	114±5	115±2	91±2	89±5
Tibia				
^{85}Sr-microsphere (%/g)	0.46±0.03	0.54±0.04[a]	0.33±0.02[a]	0.41±0.03[b]
Blood flow (ml/min per g)	0.20±0.02	0.21±0.02	0.11±0.01[a]	0.13±0.01[ab]
Distal femur				
^{85}Sr-microsphere (%/g)	0.72±0.06	0.86±0.08	0.56±0.04[a]	0.66±0.06[b]
Blood flow (ml/min per g)	0.32±0.03	0.34±0.05	0.19±0.02[a]	0.21±0.03[ab]
Density of tibia	1.56±0.01	1.51±0.01[a]	1.56±0.01	1.51±0.01[a]
Ash weight (mg/ml)	0.65±0.01	0.59±0.01[a]	0.65±0.01	0.60±0.01[a]

Means ± S.E.M., significantly different ($p<0.05$): a – from controls, b – from OOX rats

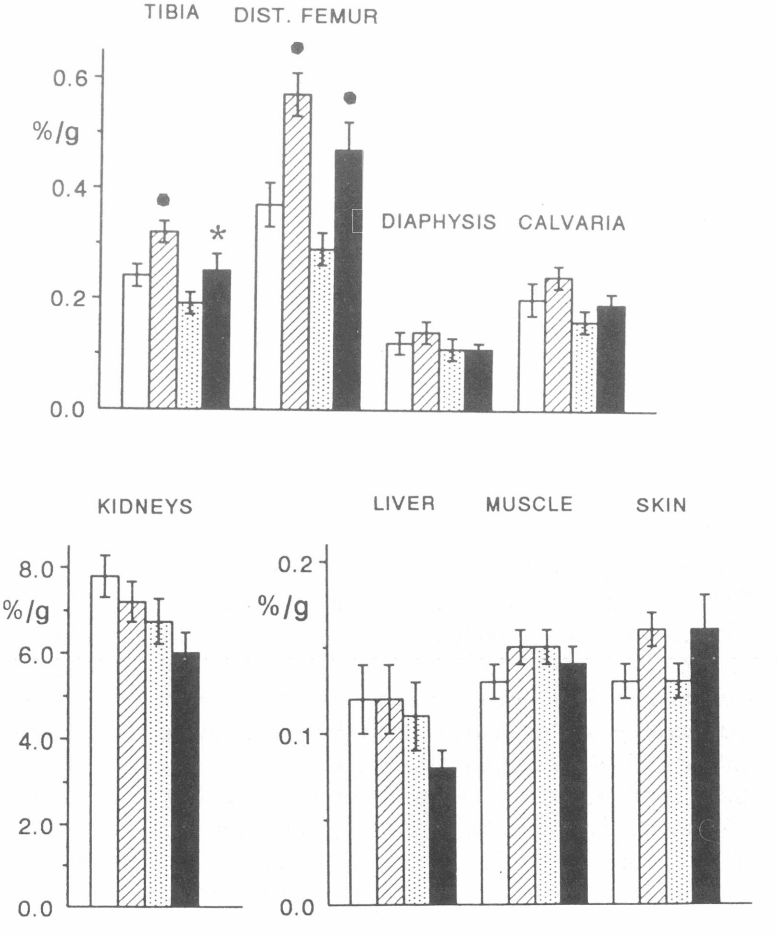

Fig. 1. *^{85}Sr-microsphere uptake as a relative indicator of local blood flow changes induced by methylene blue administration (0.1 % in the food for four weeks) in female rats. Open columns – intact controls, hatched columns – oophorectomized rats, dotted columns – methylene blue-treated intact rats, full columns – methylene blue-treated oophorectomized rats. Statistically different ($p<0.05$): full dots from controls, asterisks from oophorectomized rats.*

Table 2
Influence of methylene blue administration (0.1 % in the food for 4 weeks) on local blood flow (in ml/min per g) through bones and some other tissues in female sham-operated or oophorectomized (OOX) rats. Experiment B.

Group	1 Controls	2 OOX	3 Methylene blue	4 OOX + Methylene blue
Tibia	0.13±0.01	0.16±0.01[a]	0.10±0.01	0.14±0.02
Distal femur	0.21±0.02	0.28±0.02	0.15±0.02	0.26±0.04
Diaphysis of femur	0.07±0.01	0.07±0.01	0.06±0.01	0.06±0.01
Calvaria	0.20±0.03	0.24±0.02	0.16±0.02	0.19±0.02
Kidneys	4.24±0.31	3.44±0.18	3.45±0.28	3.33±0.30
Liver	0.07±0.01	0.06±0.01	0.05±0.01	0.04±0.01
Gastrocnemius muscle	0.07±0.01	0.07±0.005	0.08±0.01	0.08±0.01
Skin	0.07±0.01	0.08±0.01	0.06±0.005	0.08±0.01

Means ± S.E.M., significantly different ($p<0.05$): a – from controls

Table 3
Effect of methylene blue administration (0.1 % in the food for 4 weeks) on general haemodynamics and mineralization of the tibia in female sham-operated and oophorectomized (OOX) rats. Experiment B.

Group	1 Controls	2 OOX	3 Methylene blue	4 OOX + Methylene blue
Number of rats	9	11	10	10
Body weight (g)	207±3	250±3[a]	198±4	234±5[ab]
Cardiac output (ml/min)	57.4±4.1	61.2±3.7	51.5±2.9	65.0±3.9
Cardiac output (ml/min per 100 g)	27.6±1.9	24.4±1.3	26.0±1.5	27.6±1.2
Heart rate (beats per min)	453±9	433±7	416±8[a]	434±10
Mean arterial pressure (mm Hg)	130±3	129±4	119±4[a]	122±4[ab]
Density of tibia	1.57±0.01	1.53±0.004[a]	1.55±0.01[a]	1.50±0.01[a]
Ash weight (tibia) (mg/ml)	0.70±0.01	0.62±0.005[a]	0.65±0.01[a]	0.61±0.01[a]

Means ± S.E.M., significantly different ($p<0.05$): a – from controls, b – from OOX

Experiment B – effect 0.1 % of MB in food on the blood flow through bone and soft tissues. The ^{85}Sr-microsphere uptake is depicted in Figure 1, local blood flow values are given in Table 2 and the rest of the results is shown in Table 3. OOX raised the ^{85}Sr-microsphere uptake significantly in the tibia and distal femur; no significant changes were found in the diaphysis of the femur and the calvaria. After MB administration, ^{85}Sr-microsphere uptake decreased significantly in the tibia of OOX females but no other differences in microsphere uptake and in bone blood flow were encountered. In soft tissue samples, no significant differences in the ^{85}Sr-microsphere uptake and blood flow were observed. The heart rate was significantly lower after MB, but cardiac output and blood pressure remained unaltered. Bone density and ash weight were lower not only in OOX rats, but also after the administration of MB in sham-operated animals.

Experiment C – effect of 0.04 % MB in the food on 24-h incorporation of ^{45}Ca and ^{3}H-proline (Table 4). The incorporation of both indicators was increased in OOX rats and decreased in OOX+MB rats as compared with the OOX group.

Table 4
Influence of methylene blue administration (0.04 % in the food for 4 weeks) on the 24-h incorporation of ^{45}Ca and ^{3}H-proline into the tibia of female sham-operated or oophorectomized (OOX) rats. Experiment C.

Group	1 Controls	2 OOX	3 Methylene blue	4 OOX + Methylene blue
Number of rats	10	18	13	12
Body weight (g)	241 ± 4	309 ± 10	229 ± 2[a]	262 ± 6[ab]
24-h incorporation (dpm per mg)				
^{45}Ca	1.89 ± 0.05	2.22 ± 0.09[a]	1.75 ± 0.08	1.86 ± 0.09[b]
^{3}H-proline	1.30 ± 0.05	1.85 ± 0.07[a]	1.27 ± 0.05	1.61 ± 0.06[ab]

Means ± S.E.M., significantly different ($p < 0.05$): a – from controls, b – from OOX

Discussion

EDRF-NO, activates soluble guanylyl cyclase, causing the formation of cyclic guanosine monophosphate (cGMP) and vascular relaxation. Methylene blue is known as an agent non-specifically blocking this system: it interferes with soluble guanylyl cyclase activated by NO, reduces the level of cGMP and inhibits endothelium-dependent vascular relaxation (Wang *et al.* 1995). Moreover, there is also evidence that MB acts as a direct inhibitor of NO synthase (Mayer *et al.* 1993).

EDRF-NO system is active in many tissues and plays an important role in many physiological processes. In our laboratory, the inhibition of oestradiol-induced adenohypophysial growth and of oestradiol-induced stimulation of bone mass by methylene blue was described (Schreiber *et al.* 1993, Broulík and Schreiber 1994).

Our present interest concerned the possible participation of EDRF-NO system in hormonal regulation of bone blood flow. The results obtained are generally in agreement with the hypothesis formulated in the Introduction, namely that MB administration inhibited, at least partially, BBF increase in OOX female rats and inconsistently also decreased the BBF in sham-operated animals. Similarly, MB influenced the 24-h incorporation of ^{45}Ca and ^{3}H-proline into the bone. Thus, EDRF-NO acts as a mediator in BBF changes induced by estrogen deficiency.

There are, however, local differences in the circulatory changes induced by OOX and MB administration because ^{85}Sr-microsphere uptake was significantly modified in the tibia and distal femur but not in the diaphysis and calvaria (Experiment B).

The reliability of the local circulatory changes is supported by alteration in the 24-h incorporation of ^{45}Ca and ^{3}H-proline into the tibia, the reactions were similar and the differences were significant even with the low dose of MB.

The results of whole body circulatory indicators were rather unexpected. With lower MB dosage (0.1 %) there was no change in cardiac output and blood pressure, while a marked decrease in cardiac output and blood pressure occurred after high dosage (0.5 %) of MB. This indicates that no evident changes in peripheral vascular resistance have taken place in both cases. No change or even a decrease in blood pressure after MB administration is rather difficult to explain. However, Wang *et al.* (1995) did not also observe any rise in blood pressure of rats after MB and Loeb and Longnecker (1992) described even a decreased blood pressure in rats after MB treatment.

On the other hand, blocking substances derived from arginine (e.g. L-NAME, L-NMMA), which inhibit vasodilatation by blocking the synthesis of NO, usually raise vascular resistance and blood pressure. The exact explanation of this discrepancy is still not known (Wang *et al.* 1995).

As far as the main topic is concerned, i.e. bone blood flow, our preliminary results suggest the possible participation of the EDRF-NO system in BBF changes induced by a deficiency of estrogen and possibly also in the basal BBF. Our results are, thus, in agreement with the conclusions of Davis and Wood (1992) that both prostaglandins and EDRF-NO may participate in the regulation of bone circulation.

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