ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA
VOL. XXIII, N 2, 33 SECTIO DDD 2010

I.Ya.Horbachevsky Ternopil State Medical University, Department of Medical Biochemistry

MYKHAYLO KORDA, SVITLANA YAVORSKA, TETYANA YAROSHENKO, OKSANA OSTRIVKA, SVITLANA PIDRUCHNA, NATALIYA SUSLOVA, IRYNA KUZ'MAK

The role of leptin in obesity induced nitrooxidative stress and endothelial dysfunction – nanomedical approach

Rola leptyny w indukowanym otyłością stresie nitrooksydacyjnym i dysfunkcji śródbłonka – podejście nanomedyczne

INTRODUCTION

Obesity is one of the most serious risk factors for vascular disorders such as hypertension and coronary artery disease. Evidence has accumulated that adipose tissue derived hormone leptin may affect the vascular tone. We hypothesized that chronic hyperleptinemia accompanying obesity can modify the endothelial $NO/O_2^{-}/ONOO^{-}$ metabolism and cause endothelial dysfunction.

MATERIAL AND METHODS

Human umbilical vein endothelial cells (HUVECs) were incubated for 2 or 12 h with different concentrations of leptin or coincubated with 0.1 μmol/l of leptin and elevated concentrations of L-arginine (3 mmol/l). C57BL/6J mice were assigned to low-calorie (10 kcal fats) or high-calorie (60 kcal fats) diet for 105 days. Mice fed with high-calorie diets were divided into two groups: group 1, received regular water; group 2, received L-arginine (100 $mg*kg^{-1}*day^{-1}$) in their water. Following euthanasia, the thoracic aorta was excised from each animal and the small segments from aorta were cut into rings. The rings were opened and pinned on the bottom of organ chamber filled with HBSS buffer. A system of electrochemical nanosensors was used to measure *in situ* NO, O_2^{-} and $ONOO^{-}$ [3]. HUVEC culture cluster in a well or aorta strip in chamber were placed on the stage of an inverted microscope. Nanosensors were lowered near the surface of a single cell membrane with the aid of micromanipulator. The release of NO, O_2^{-}, and $ONOO^{-}$ from HUVEC or aortic endothelium was stimulated by eNOS agonist calcium ionophore (CaI). The changes in NO, O_2^{-}, and $ONOO^{-}$ concentration were measured with a computer-based potentiostat (detection limit 1 nmol/l).

Blood was taken from mice and leptin concentrations were measured using a radioimmunoassay kit (Linco, St. Louis, MO). eNOS expression was measured by Western blot analysis. L-arginine concentrations in HUVECs and aortic endothelium were measured using HPLC coupled with

fluorescence detection. The concentrations of cGMP were measured in thoracic aortas using an ELISA kit.

Multiple comparisons were evaluated using ANOVA followed by the Student's *t*-test.

RESULTS

Leptin did not directly stimulate NO, O_2^-, and $ONOO^-$ release from HUVEC. After CaI administration, a rapid release of NO, O_2^-, and $ONOO^-$ was observed from both control and leptin-treated cells. The incubation of cells for both 2 and 12 h with leptin resulted in a dose-dependent increase of the peak NO, O_2^-, and $ONOO^-$ generation (Fig. 1). It is interesting to report that the incubation of HUVEC with leptin for 2 h stimulated the NO production to a higher degree than treatment with leptin for 12 h. In contrast to NO, the CaI stimulated peak production of O_2^- and $ONOO^-$ in HUVECs incubated with leptin for 12 h was higher than in HUVEC treated with leptin for 2 h. We used the ratio of NO to $ONOO^-$ concentration ($R=[NO]/[ONOO^-]$). A high R value indicates strong eNOS coupling and high bioavailable NO and/or low nitroxidative stress (low level of $ONOO^-$). Intriguingly, the R value was 2.8 ± 0.1 in HUVEC incubated for 2 h with leptin when compared with control cells (2.0 ± 0.1) (Fig. 2). In contrast, HUVEC treated with the same leptin concentration for 12 h showed a decrease of the R value to 1.3 ± 0.1.

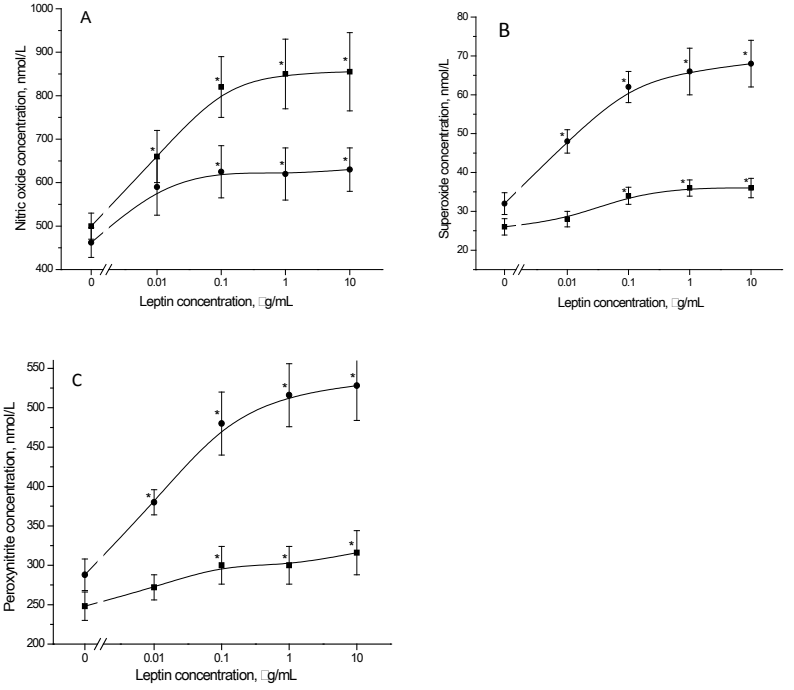

Fig. 1. The effect of leptin on CaI-stimulated peak of NO (A), O_2^- (B) and $ONOO^-$ (C) released from HUVEC. Cells were incubated with various concentrations of leptin for 2 (closed squares) and 12 (closed circles) hours *P < 0.05 vs control (without leptin), n=5 experiments

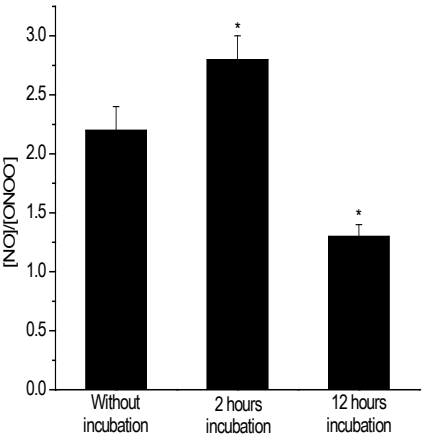

Fig. 2. The ratio of NO concentration to the concentration of ONOO- in HUVECs
*P < 0.05 vs control, n=5 experiments

As shown in Fig. 3, eNOS expression increased after cell incubation with leptin in a dose-dependent manner. HUVEC treated with leptin for 2 h showed a higher increase of eNOS expression compared with the cells incubated for 12 h. During incubation, leptin-treated HUVEC had significantly lower intracellular L-arginine concentration levels than untreated cells.

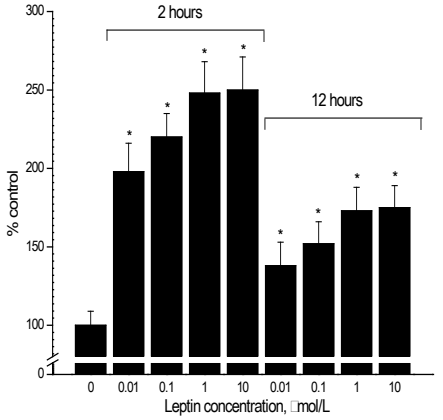

Fig. 3. eNOS expression in HUVEC incubated with varies concentrations of leptin for 2 and 12 hours *P < 0.05 vs control, N=5 experiments

After 105 days, the mean body weight of mice fed with high-calorie diets was about 1.4 times higher than that of the control group. The shape of curves reflecting the increase of leptin concentration in serum of mice fed with high- and low-calorie diets were very similar to the curves reflecting the

gain of body weight in corresponding animal groups. The increase in leptin concentration correlated with the increase in eNOS expression in obese mice. The cGMP level (aortic wall) correlated inversely with eNOS expression.

NO concentration released from the aortic endothelium of control mice was 420±12 nmol/l, about 1.7 times higher than from aortic endothelium of mice with obesity (250±11 nmol/l). Obese mice treated with L-arginine showed higher NO production (297±10 nmol/l) compared with untreated obese mice. In contrast, the CaI-stimulated peaks of O_2^- and $ONOO^-$ release were significantly elevated in obese mice in comparison with control. A supplementation with L-arginine resulted in the decrease of O_2^- and $ONOO^-$. The R value decreased about 64% in obese mice compared with control mice (Fig. 4). L-Arginine treatment increased the R value about 30% over the untreated obese mice.

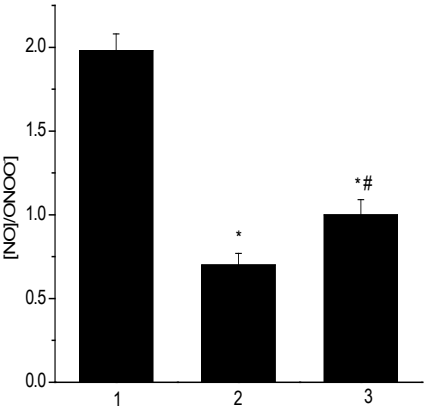

Fig. 4. The ratio of NO concentration to the concentration of ONOO- in aortic endothelium; (1) mice maintained on regular diet, (2) obese mice, (3) obese mice with supplementation of L-arginine *P<0.05 vs control, #P<0.05 vs obese mice, N=5 experiments

Obesity resulted in about a 30% decrease of L-arginine level in aortic endothelium of obese mice compared with control mice. L-Arginine supplementation caused a significant increase (30%) of L-arginine level in aortic endothelium of the obese mice.

DISCUSSION

Our results clearly demonstrated that leptin does not directly stimulate NO, O_2^-, or $ONOO^-$ release from the endothelium. However, the exposure of endothelial cells to leptin resulted in dose-dependent upregulation of eNOS expression and an enhanced NO production that lasted for about 2 h. The leptin-enhanced expression of eNOS in endothelial cells can be explained by a transcriptional and/or posttranscriptional mechanisms. A short-time exposure of HUVEC to leptin did not increase O_2^- and $ONOO^-$ levels significantly. This indicates that an acute exposure of endothelial cells to leptin may have beneficial effects on the cardiovascular system by increasing the potential for the generation of bioavailable NO. In contrast, long-term (12 h) exposure of endothelial cells to leptin

decreased CaI-stimulated bioavailable NO despite a twofold increase in eNOS expression in the endothelium. This apparent paradox can be explained by the enhanced generation of O_2^- and $ONOO^-$ after long-term exposure of the HUVEC to leptin. To better specify the relationship between leptin, eNOS expression, and obesity in animals, another portion of our experiments involved an inbred C57BL/6J strain of obese mice. These mice were not obese on a standard chow diet but became obese and developed hyperleptinemia when fed a high-fat diet. The present study provides direct evidence that an increased leptin level inversely correlates with NO bioavailability in the aortic endothelium and with cGMP in the aortic wall. Simultaneously, with the suppression of NO production in obese mice, we registered a considerable increase in O_2^- and $ONOO^-$ generation. The most probable mechanism for the NO release diminishment in this case, like in the experiments with cell culture, is the overproduction of O_2^-, resulting in a fast reaction with NO to form $ONOO^-$. This direct quantitative data are concordant with previous indirect studies that indicate the role of oxidative stress in obesity [1]. At the same time, the eNOS expression in aorta of obese animals was increased 8-fold compared with control. This apparent discrepancy can be clearly explained with the data presented in this study. One of the most probable mechanisms underlying the leptin-stimulated endothelial dysfunction may be the imbalance between eNOS expression and intracellular L-arginine and/or tetrahydrobiopterin level. According to our results, leptin significantly increases the amount of eNOS protein in HUVEC and endothelium of obese mice. Coincident with the increase in eNOS expression, we observed a tendency toward diminishment of the L-arginine concentration in leptin-incubated HUVEC and a significantly diminished L-arginine level in obese animals with hyperleptinemia. L-Arginine is required not only as a substrate for NO synthesis but also as a stabilizer of eNOS, preventing it from uncoupling. The relative L-arginine insufficiency results in the uncoupling of the oxidative and reductive domains of eNOS, which results in O_2^- generation [2]. The net effect of the reaction between NO and increased O_2^- caused diminishment of NO bioavailability. Furthermore, in our experiments, the obese mice treated with L-arginine showed significantly higher NO production and lower O_2^- and $ONOO^-$ release from the aortic endothelium compared with the untreated obese mice.

CONCLUSIONS

We provided direct evidence that in long-term exposure of endothelium to leptin, despite increased eNOS expression, bioavailable NO is reduced, whereas O_2^- and $ONOO^-$ levels are increased. Increased O_2^- and $ONOO^-$ production which contributes to high oxidative/nitroxidative stress in endothelial cells is most likely due to a discrepancy between enhanced eNOS expression and diminished intracellular L-arginine concentration in obesity.

REFERENCES

1. Fenster C.P, Weinsier R.L. et al.: Obesity, aerobic exercise, and vascular disease: the role of oxidant stress. Obes. Res., 10, 964, 2002.
2. Huk I., Nanobashvili J. et al.: L-Arginine treatment alters the kinetics of nitric oxide and superoxide release and reduces ischemia-reperfusion injury in skeletal muscle. Circulation, 96, 667, 1997.

3. Malinski T., Taha Z.: Nitric oxide release from a single cell measured *in situ* by a porphyrinic based microsensor. Nature, 358, 676, 1992.

SUMMARY

Hyperleptinemia accompanying obesity affects endothelial NO and is a serious factor for vascular disorders. NO, O_2^-, and $ONOO^-$ levels in HUVECs and aortic endothelium of obese mice were recorded using tandem electrochemical nanosensors. 12-h exposure of HUVECs to leptin increased eNOS expression and decreased the [NO]/[$ONOO^-$] ratio. In obese mice, a 2.5-fold increase in leptin concentration coincided with 100% increase in eNOS and about 30% decrease in intracellular L-arginine. This led to eNOS uncoupling, a reduction in bioavailable NO, and an elevated concentration of O_2^- and $ONOO^-$. L-Arginine supplementation reversed eNOS uncoupling and partially restored [NO]/[$ONOO^-$] balance in obese mice.

Key words: obesity, leptin, endothelial dysfunction, nitric oxide, superoxide, peroxynitrite

STRESZCZENIE

Hyperleptynemia towarzysząca cukrzycy wpływa na NO śródbłonka i jest ważnym czynnikiem występowania zaburzeń naczyniowych. W pracy oceniono poziomy NO, O_2^- oraz $ONOO^-$ w HUVECs i śródbłonku tętnic otyłych myszy przy zastosowaniu tandemowych nanosensorów elektrochemicznych. 12-godzinna ekspozycja HUVECs na lektynę zwiększała ekspresję eNOS i zmniejszała stosunek [NO]/[$ONOO^-$]. U otyłych myszy 2,5-krotnemu wzrostowi stężenia leptyny towarzyszył 100 % wzrost eNOS i około 30 % spadek zawartości wewnątrzkomórkowej L-argininy. Prowadziło to do rozpadu eNOS, redukcji biodostępnego NO i wzrostu stężenia O_2^- i ONOO. Suplementacja L-argininy odwracała rozpad eNOS i częściowo przywracała równowagę [NO]/[ONOO.

Słowa kluczowe: otyłość, leptyna, dysfunkcja śródbłonka, tlenek azotu, ponadtlenek, nadtlenek azotu