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Leptin triggers Ca^{2+} imbalance in monocytes of overweight subjects

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ABSTRACT

Obesity is a major risk factor in numerous diseases, in which elevated intracellular Ca^{2+} plays a major role in increased adiposity. We examined the difference between Ca^{2+} signals in monocytes of lean and overweight subjects and the relationship between leptin induced NADPH oxidase activation and intracellular calcium concentration $[Ca^{2+}]_i$ homeostasis. Our results are as follows: (1) The basal level of $[Ca^{2+}]_i$ in resting monocytes of overweight subjects (OW monocytes) was higher than that in control cells, whereas the leptin-induced peak of the Ca^{2+} signal was lower and the return to basal level was delayed. (2) Ca^{2+} signals were more pronounced in OW monocytes than in control cells. (3) Using different inhibitors of cellular signaling, we found that in control cells the Ca^{2+} signals originated from intracellular pools, whereas in OW cells they were generated predominantly by Ca^{2+}-influx from medium. Finally, we found correlation between leptin induced superoxide anion generation and Ca^{2+} signals. The disturbed $[Ca^{2+}]_i$ homeostasis in OW monocytes was fully restored in the presence of fluvastatin. Statins have pleiotropic effects involving the inhibition of free radical generation that may account for its beneficial effect on elevated $[Ca^{2+}]_i$ and consequently on the pathomechanism of obesity.

1. Introduction

Obesity is considered as a crucial risk factor of cardiovascular morbidity and mortality due to its association with hypertension, type 2 diabetes mellitus and hypercholesterolemia (Romero-Corral et al., 2006). Atherosclerosis typically develops taking decades in most of the cases. However, several metabolic abnormalities characteristic for obesity are already detectable in patients with moderate weight excess. Therefore several studies focused on the increased cardiovascular risk of overweight patients (Calle et al., 1999). The central role of obesity in the development of cardiovascular diseases is due partially to the adipocyte derived adipokines which participate in the pathogenesis of obesity-associated metabolic syndrome (Matsuzawa, 2005, 2006). In adipocytes, injured ATP- and calmodulin-dependent H^+/Ca^{2+} antiport results in increased intracellular calcium concentration; consequently, high level of $[Ca^{2+}]_i$ exists continuously that results in the enhancement of lipogenesis and decrease in lipolysis (Zemel, 2002, 2005). These processes lead to significant expansion of the adipose tissue suggesting the crucial role of $[Ca^{2+}]_i$ in the pathomechanism of obesity (Shi et al., 2001). On the other hand, the increase in free radical production can abrogate ion-transport through the cell membrane by directly damaging the Na^+/K^+-ATPase, Na^+/Ca^{2+} exchange and ATP-dependent H^+/Ca^{2+} antiport proteins (Kourie, 1998; Stark, 2005). Leptin overexpression in obesity stimulates NADPH oxidase through its long type receptors and causes superoxide generation in various cells (Fernández-Sánchez et al., 2011; Fortuño et al., 2010; Koh et al., 2008). In vitro experiments showed that statins have intrinsic antioxidant activity (Franzoni et al., 2003). Furthermore, it was hypothesized that fluvastatin, in hypercholesterolemic patients, may inhibit NADPH oxidase via adiponectin up-regulation (Carnevale et al., 2010). Inhibiton of NADPH oxidase results in reduced superoxide anion production.

We hypothesized that leptin is one of the factors accounting for the increased calcium levels in monocytes isolated from overweight subjects (OW monocytes). Supporting our hypothesis we studied the effects of leptin on human monocytes and elucidated the relationship between leptin-induced NADPH oxidase activation and intracellular free Ca^{2+} balance. In present in vitro studies we compared superoxide anion generation and Ca^{2+} signals following leptin administration in control and OW monocytes. Furthermore, we analyzed the origin of Ca^{2+} signal in both groups of monocytes. Finally, to clarify the role of superoxide anion generation in intracellular free Ca^{2+} imbalance, we administered fluvastatin to the cell suspension in order to inhibit NADPH oxidase activity.

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2. Patients and methods

2.1. Patients

The study was performed on 16 lean (control) and 18 overweight healthy male subjects. This study population was the same as in another set of experiments previously published by our research group (Balogh et al., 2011). Enrolling only male volunteers enabled us to exclude interfering sex differences such as hormonal factors or difference in lipid profiles. Demographic data and laboratory parameters of control and overweight volunteers can be found in Table 1. All protocols were in compliance with the ethical guidelines of the Medical and Health Science Centre of the University of Debrecen.

2.2. Blood sampling

The venous blood samples (10–15 mL) were taken at intervals of 4–5 days from 6–8 overweight to 3–4 lean control volunteers for each set of experiments. The interassay coefficient did not exceed 15%.

2.3. Isolation of monocytes

Monocytes were isolated from venous blood and further separated as described previously (Balogh et al., 2011). The cell suspension was 93–96% pure for monocytes (as verified by anti-CD14 marker) and the proportion of viable cells was 90–95% (assessed by trypan blue exclusion test).

2.4. In vitro experimental conditions

Monocytes were suspended in HEPES Buffered Hanks Balanced Salt Solution (HBSS). For stimulation of monocytes leptin (Sigma) was administered at a final concentration of 50 ng/mL. Different inhibitors were applied on the basis of previous studies as follows: for phospholipase C (PLC) inhibition 5 μM neomycin, for G_i protein inhibition 100 ng/mL Pertussis toxin (PTX), for the inhibition of intracellular Ca^{2+} translocation 15 μM thapsigargin, for inhibition of Ca^{2+}-influx cells were plated in medium V (10 μM verapamil + 3 mmol/L EGTA in Ca^{2+}-free medium), for phosphatidylinositol 3-kinase (PI3K) inhibition 20 nM wortmannin (WTM), and for inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase 5 μM fluvastatin (Merck) were used for 60, 120, 2, 60, 30 and 60 min, respectively. Inhibitors, except fluvastatin, were purchased from Sigma. All monocyte groups were incubated in HBSS for 120 min prior to leptin treatment as this was the longest inhibitor incubation time. Different inhibitors were administered at various time points for enabling the above mentioned lengths of incubation prior to leptin stimulation. Experiments on monocytes were carried out within 3 h of cell isolation. Leptin was added to the cell suspensions containing the inhibiting drugs after the appropriate preincubation. The time-course of leptin-stimulation was dependent on the type of studied parameters: at inositol-1,4,5-trisphosphate determination 20 s, at $[Ca^{2+}]_i$ measurement 6 min, and at superoxide anion 30 min. Cells were incubated in a humidified CO_2 incubator at 37 °C (CO_2 5%, air 95%, humidity 95%).

Table 1
Demographic and laboratory parameters of control lean, and overweight male subjects.

Parameters	Control	OW
n	16	18
Age (years)	40.5 ± 5.8	42.2 ± 5.5
BMI (kg/m^2)	22.1 ± 1.5	29.7 ± 4.8*
WHR	0.81 ± 0.11	1.32 ± 0.15*
Fasting blood sugar (mmol/L)	5.2 ± 0.5	5.5 ± 1.0
Systolic blood pressure	122.7 ± 15.3	125.5 ± 15.5
Diastolic blood pressure	76.4 ± 10.1	80.3 ± 12.5
Fasting insulin (mU/L)	20.8 ± 3.6	21.6 ± 4.0
Leptin (ng/mL)	12.6 ± 2.1	39.7 ± 8.6*
HbA1c (%)	4.5 ± 0.8	4.7 ± 0.9
Cholesterol (mmol/L)	4.1 ± 0.6	5.0 ± 0.8
Triglyceride (mmol/L)	1.64 ± 0.18	2.71 ± 0.35*
HDL-C (mmol/L)	1.55 ± 0.25	1.50 ± 0.25
LDL-C (mmol/L)	3.48 ± 0.65	4.91 ± 0.68*

Each value represents the mean ± SD. *Abbreviations:* OW = overweight; BMI = body mass index; WHR = waist-to-hip-ratio; $HbA1_C$ = glycosilated Hgb.
* Differences between the control and the overweight groups are significant ($p < 0.001$).

2.5. Superoxide anion generation

Superoxide anion production was measured after leptin-stimulation for 30 min using superoxide dismutase inhibitable reduction of ferricytochrome C (Sigma) (Cohen and Chovaniek, 1978). Results were expressed as nmol of produced O_2^- nmol//30 min/10^6 monocytes.

2.6. Determination of inositol-1,4,5-trisphosphate

Determination of inositol-1,4,5-trisphosphate [Ins(1,4,5)P3] was carried out according to the method of Patthy et al. (1990). The appropriate concentrations of inhibitor drugs were added to the cell containing mixture 30–120 min prior to the end of myo-[^{3}H] inositol preincubation. Following vigorous washing cell-bound radioactivity was determined. The inhibitors were re-administered to the monocytes, and the cells were stimulated with 50 ng/mL leptin. After 20 s, the reaction was terminated. (1,4,5)P3 were isolated by reverse-phase ion-pair chromatography, using Ins(1,4,5)P3 as internal standard (Amersham). The amount of produced Ins(1,4,5)P3 was expressed as a quotient of pmol Ins(1,4,5)P3/mg total protein.

2.7. Determination of intracellular calcium concentration

The $[Ca^{2+}]_i$ was determined as described earlier (Kosztáczky et al., 2007). Briefly, monocytes were incubated in 1 mL volume containing 5×10^6 cells + 20 μL Indo 1/AM (Calbiochem) for 30 min at 37 °C in a shaker. The cells were then washed vigorously and aliquots were resuspended in HBSS. Inhibitors were added to the cells at previously indicated times prior to leptin-stimulation. The applied inhibitors did not influence the capacity of Indo 1/AM to measure the cytosolic free Ca^{2+} content. The determination of $[Ca^{2+}]_i$ was carried out at 405 and 485 nm in a spectrofluorimeter (Hitachi, F-4500), under constant stirring at 37 °C. The final mixture consisting of 10^6 monocytes in 2.0 mL HBSS was placed into a cuvette and the cells were stimulated with leptin in 50 ng/mL final concentration at 0 min of the 6-min-long measurement. The $[Ca^{2+}]_i$ levels were calculated according to the given equation. Ca^{2+} signals were calculated as leptin-stimulated minus basal $[Ca^{2+}]_i$. Areas Under Curves (AUC) values were calculated from Ca^{2+} signals measured during the six-min time-curves. AUC values showed the total amount of calcium present over a 6 min long period which well described the different velocity of calcium influx and efflux in monocytes from normal and overweight subjects.

2.8. Statistical analysis

Statistical analyses were carried out by ANOVA followed by Bonferroni's T test for repeated measurements. To evaluate the degree of inhibitions, AUC values were calculated which were characterized by descriptive statistics. They were analyzed by one way