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Altered cholesterol and calcium homeostasis observed in monocytes during the pathomechanism of obesity

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The Examination takes place at 1st Dept. of Medicine, Medical and Health Science Center, University of Debrecen, 2012 November 12, 11:00.

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INTRODUCTION

According to our present knowledge, obesity is one of the milestones in the development of metabolic syndrome. Some of the metabolic abnormalities, which are characteristics of obesity, are detectable in the early stage of the disease and its signs occur in patients with moderate overweight as well. Because of this, numerous studies examined the increased cardiovascular risk of overweight patients. The central role of obesity in the development of cardiovascular diseases is partially due to the adipokines originated from adipocytes. Leptin is able to increase the intensity of mevalonate pathway, which can lead to increased cholesterol biosynthesis. After binding to its receptor, leptin connects to the PLC–Ins(1,4,5)P3–Ca^{2+} signal pathway. The JAK/STAT, PI3K, MAPK/ERK and PKC signaling pathways also take part in the leptin induced signal transduction. However, the increased intensity of mevalonate pathway and cholesterol synthesis is not the sole consequence of the elevated leptin concentration. The membrane composition changes in parallel with the alterations of cholesterol level, which has an influence on the functions of the membrane channels. In neutrophils, decreased calcium influx was shown as a result of the effects of free cholesterol. In adipocytes the damaged ATP- and calmodulin-dependent H^+/Ca^{2+}antiport resulted in increased intracellular [Ca^{2+}], which led to a continuously increased intracellular [Ca^{2+}] level. This condition promotes increased lipogenesis and decreased lipolysis. These processes lead to the significant growth of adipose tissue indicating the role of intracellular [Ca^{2+}] in the pathomechanism of obesity. On the other hand, the formation of free radicals damages the ion transport through the membrane by directly damaging the Na^+/K^+-ATPase, the Na^+/Ca^{2+} exchanger protein and the ATP-dependent H^+/Ca^{2+}antiport proteins. In obesity the overexpression of leptin stimulates the NADPH oxidase by its long type receptors and induces superoxide production in numerous cells. The antioxidant activity of statins is known, and in patients with hypercholesterolemia it can inhibit the NADPH

oxidase by increased adiponektin production. The inhibition of NADPH oxidase results in decreased superoxide anion formation.

The previous experiments of our research group were carried out on monocytes of patients with hypercholesterolemia. These experiments highlight the importance of cPKC among the regulator factors of cholesterol biosynthesis and point out the difference of calcium homeostasis between the monocytes separated from the blood of healthy controls and hypercholesterolemic patients. According to our hypothesis, leptin is one of the factors that are responsible for the elevation of Ca^{2+} level in the monocytes of overweight patients. Confirming our hypothesis, we examined the effects of leptin on human monocytes, and we investigated the leptin-induced biphasic effect on cholesterol synthesis and the connection between the formation of superoxide anion and the intracellular free Ca^{2+} after adding leptin to the monocytes of subjects with normal weight and overweight. Furthermore, we examined the origin of Ca^{2+} signals in both groups of monocytes. Finally, to clarify the role of superoxide anion formation in intracellular free Ca^{2+} imbalance, we added fluvastatin to the cell suspension inhibiting NADPH oxidase activity.

Calcium

In the human body approximately 1% of calcium is solved in the intra- and extracellular fluid. The ionized calcium plays significant role in most physiological processes, therefore its concentration is strictly regulated. It is 1.16-1.18 mmol/L in the extracellular matrix and in the cell it is less than hundredth of this value. Parathyroid hormone (PTH), vitamin D and calcitonin play major roles in the maintenance of the physiological concentration.

The calcium, as a second messenger molecule, is important in the intracellular signal transduction pathways.

Molecules and inhibitors participating in the calcium signal transduction

Phospholipase C and neomycin

The phosphoinositide-specific phospholipase C (PLC) isoenzymes cleave the polar head of inositol phospholipids. They induce the formation of inositol 1,4,5- trisphosphate (Ins(1,4,5)P3) and diacylglycerol (DAG) through cell surface receptors by the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) located in the lipid bilayer. The PLC-β isoform is activated through G proteins, while the PLC-γ is activated via tyrosine kinase receptors. After phosphorilation, the PLC isoenzymes translocate into the membrane where they produce Ins(1,4,5)P3 and DAG from their substrates. Ins(1,4,5)P3 is a second messenger molecule participating in the mobilization of Ca^{2+} and DAG is the activator of proteinkinase C (PKC).

Neomycin is able to inhibit phospholipase C by binding to inositol phospholipids. It binds to phosphatidylinositol 4,5- bisphosphate with high affinity and PLC becomes unable to produce Ins(1,4,5)P3 and DAG.

Proteinkinase C

Proteinkinase C (PKC) isoenzyme family includes serine/threonine kinases. The enzyme family is divided into three isoforms: classical, novel and atypical. The members of the first group can be activated by both calcium and DAG; the novel PKCs are only sensitive to DAG, whereas the atypical PKCs are not sensitive to either of them. The PLC induces the hydrolyzation of PIP2, which leads to DAG formation and increasing intracellular calcium concentration. These second messenger molecules trigger the translocation of the cytosolic inactive classical PKC molecules to the membrane, which is followed by their conformational change. The active PKC, bound to the phosphatidylserine of the membrane, phosphorilates the serine and threonine side chains with a wide substrate-specificity.

Inositol trisphosphate (Ins(1,4,5)P3)

Ins(1,4,5)P3, the formation of which is induced by PLC, plays a regulatory role in the maintenance of intracellular calcium homeostasis. Its receptors are located on the surface of calcium stores in the cell. The binding of Ins(1,4,5)P3 to the receptors on the surface of the ER results the rapid depletion of the intracellular calcium stores. The decreased calcium concentration in the store is replaced by the Ca^{2+} influx through the membrane. This so-called "store-operated" Ca^{2+} current (SOC) controlled by the stores has an influence on numerous cellular responses, including enzyme activation, gene expression, secretion, cell division and cell death.

Gi protein and pertussis toxin

Gi proteins belong to the guanine nucleotide regulator protein (G protein) family. G proteins have a critical role in the regulation of signal transduction systems. These pathways include the adenylyl-cyklase/cAMP system and the receptor mediated activation of PLC, phospholipase A2, numerous hormone and neurotransmitter-regulated ion channels.

The pertussis toxin (PTX) is an ADP-ribosylation molecule consisting of five subunits. It irreversibly ADP-ribosylates the cysteine residuum of the α subunit of the Gi/o protein family prohibiting the inhibition of adenylate-cyclase (AC). This suggests that the inhibition of G protein coupled receptor (GPCR) triggers the formation of cAMP. The Epac (exchange protein directly activated by cAMP) molecule family binds the cAMP directly, which initiates calmodulin kinase II (CaMKII)-dependent signal transduction pathways, including Ca^{2+} release from endoplasmic reticulum.

Intracellular calcium translocation and thapsigargin

Thapsigargin (TG) is a sesquiterpene lactone, which is an effective and specific inhibitor of the intracellular Ca^{2+}ATPases, forming a so-called

"dead-end" complex with them, which inhibits the further catalysis. It leads to the depletion of intracellular Ca^{2+} stores and increased cytosolic Ca^{2+} level.

The discovery of TG and its utilization in research significantly facilitated the investigation of calcium-related cellular processes, including the mechanism of Ca^{2+} translocation to the cytosol.

Membrane channels and verapamil

Verapamil is a fenilalkilamin, which is the inhibitor of voltage-dependent Ca^{2+} channels. Presumably, it inhibits by binding to the pore of the ion channel in a voltage-independent manner and blocking it. Therefore, it does not have an influence on the normal gating processes, because it does not affect the changes of the membrane potential of the ion channel.

Phosphatidylinositol-3-kinase (PI3K) and wortmannin

PI3Ks are heterodimer complexes, which consist of the tightly connected regulator/adapter and catalytic subunits. They take part in numerous cell functions phosphorylating the 3-hydroxyl group of phosphatidylinositol. The PI3K is one of the contact points of leptin and insulin signal transduction. Its main substrate is the phosphatidylinositol 4,5-bisphosphate (PIP2), and converts it to phosphatidylinositol 1,4,5-trisphosphate (PIP3). The PI3K takes part in the regulation of numerous biological processes due to the high number of possibilities for its activation and its crossing points with other signal transduction pathways.

Wortmannin (WMN), a widely used fungal toxin, is able to inhibit PI3K. WMN binds covalently to the catalytic subunit of the enzyme and inhibits the binding of ATP and PIP2.

NADPH oxidase (Nox)

NADPH oxidases are multicomponent enzymes using electrons originating from NADPH to generate superoxide anion. Due to enzyme catalysis the produced O_2^- is transformed to a wide range of reactive oxygen species (ROS) important in immune response.

In resting monocytes the Nox system is inactive: their components occur in the cytosol and the membrane separately. Upon activation, the cytosolic components translocate to the membrane where the active enzyme is built and able to catalyze O_2^- production.

Previous studies demonstrated that the lipid oxidizing ability of monocytes is affected by calcium influx and intracellular calcium mobilization. Changes in intracellular [Ca^{2+}] influences other important signaling pathways such as Ca^{2+}-dependent PKC and cytosolic phospholipase A2 (cPLA2) pathways. Increasing intracellular [Ca^{2+}] is able to regulate the activity of Nox in monocytes and macrophages through the generation of arachidonic acid by cPLA. The effect of cPLA and PKCs on Nox is not independent of each other. The function of cPLA is also regulated by the phosphorylation of PKCs. PKCs are able to activate the members of the MAPK family, the role of which is demonstrated in Nox derived, ROS induced signaling pathways. The relationship between ROS and MAPK is strong: MAPK directly influences the ROS production of Nox. Increase in ROS levels may trigger positive feedback resulting in high cellular ROS concentration.

The relationship of Ca^{2+} and Nox is bilateral. Not solely increased intracellular [Ca^{2+}] affects Nox activity but the opposite also occurs: Nox derived ROS activates ryanodin receptors, main activators of intracellular Ca^{2+} mobilization.

Cholesterol synthesis; the role and pleiotropic effects of statins

3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA-reductase) enzyme catalyzes the most important, rate-limiting step of cholesterol synthesis. The widely used statins inhibit *de novo* cholesterol synthesis by targeting this enzyme.

Statins also possess cholesterol independent, so called pleiotropic effects. The mevalonate pathway is responsible for the production of numerous non-steroid isoprenoids which isoprenylate essential intracellular proteins. These proteins include small GTPases e.g. Ras and Rho GTPase families the ability of which is to connect intracellular signaling processes and extracellular stimuli.

The ability of statins to reduce isoprenoid synthesis exerts multiple pleiotropic effects through intracellular signaling mechanisms. These pleiotropic effects include the reversal of endothelial function loss, reduced inflammation and smooth muscle cell proliferation and immunomodulatory role.

Regulatory molecules in cholesterol synthesis and their inhibitors

MAPK and PD98059

Among the leptin induced signaling pathways can be found the canonical MAPK/ERK1/2 pathway and the non-canonicalp38 MAP kinase pathway. Leptin affects the regulation of cholesterol synthesis through p38 MAPK- (SREBP)-1c. The A canonical MAPK-cascade, which is activated in the Ras-ERK-MAPK pathway, reaches the nucleus and by the regulation of gene expression modulates many biological processes. p38 MAPK/MSK1 mediates the intracellular cholesterol dependent transcriptional activity of

NFκB. The NFκB activating ability of p38 MAPK pathway turned out to be inhibitable by fluvastatin. The MAPK cascade plays a major role in cholesterol homeostasis and inflammatory processes.

A MAPK proteins, ERK1 and ERK2, is activated by the phosphorylation of MEK. PD98059 is a flavonoid molecule, which specifically inhibits MEK. It is widely used for the elucidation of the roles of MAPK cascade members in biological processes.

SCAP and 25-HC

The maintenance of normal cholesterol level is carried out by the transcriptional regulation of genes responsible for cholesterol synthesis and uptake. One of the central components of this regulatory mechanism is the sterol regulatory element binding protein (SREBP) family. SREBPs are synthesized in the ER and activated by the SREBP cleavage activating protein (SCAP).SCAP has dual role: it acts as a sterol sensor and is responsible for SREBP transport. SCAP carries SREBP-t to the Golgi where the activation takes place. Following the activation of SREBP it enters the nucleus and regulates gene expression related to cholesterol synthesis and uptake.

Inhibition of the transcriptional regulation of cholesterol synthesis may be carried out by 25-hydroxicholesterol (25-HC) that supports the formation of SCAP-Insig protein complex.

AIMS

In previous experiments our research group studied the signaling mechanisms of granulocytes and monocytes. In monocytes, the PKC activation dependence of endogenous cholesterol synthesis has been shown after *in vitro* LDL administration. Fluvastatin treatment beneficially affected

the Ang-II induced O_2^- generation and alterations in the signaling mechanisms of neutrophils separated from the blood of hypercholesterolemic patients. *In vitro* leptin treatment increased the cholesterol synthesis of monocytes, which was higher in OW monocytes. The leptin-induced increase in cholesterol synthesis was augmented by fluvastatin and 25-HC. In neutrophils of hypercholesterolemic patients the PTX-sensitive Ins(1,4,5)P3 signaling damaged and calcium signals decreased. Furthermore, the Ca^{2+} signals originated from the extracellular medium and not from the intracellular pools as it is seen in healthy cells. FMLP and κ-elastin treatment triggered reduced Ca^{2+} signals in monocytes of elderly subjects and the return of the induced Ca^{2+} levels to the baseline levels was lagging.

Based on the results of our research group I aimed at carrying out the following experiments:

- we planned to study the effect of leptin on the monocytes of overweight, but healthy men;
- multiple leptin concentrations were used for the elucidation of the hypothesized differences in the regulation of cholesterol synthesis. To examine leptin signaling in more detail we inhibited SCAP and MAPK;
- we hypothesized the role of mPKC in the regulation of leptin induced cholesterol synthesis and we measured mPKC levels in our monocytes;
- calcium levels were measured and the calcium related signaling was mapped by the aid of inhibitors used against the members of signaling processes in connection with calcium mobilization and transport;
- we examined Nox activity because of the effect of O_2^- on membrane channels and the effect of intracellular [Ca^{2+}] on Nox;
- we aimed at the reduction of O_2^- production by fluvastatin and expected the normalization of calcium signaling and membrane transport.

MATERIALS AND METHODS

Patients

The study was performed on 16 lean (control) and 18 overweight healthy male subjects. Enrolling only male volunteers enabled us to exclude interfering sex differences such as hormonal factors or difference in lipid profiles.

Determination of laboratory parameters

The venous blood samples (10–15 mL) were taken at intervals of 4–5 days from 6 to 8 patients and 3 to 4 control volunteers for each set of experiments. The interassay coefficient did not exceed 15%. Determination of cholesterol and triglyceride levels from sera was carried out enzimatically, using colorimetric tests (GPO-PAP, Modular P-800 Analyzer, Roche/Hitachi), while HDL-cholesterol and LDL-cholesterol were measured with homogenous, enzymatic, colorimetric assay (Roche HDL-C plus 3rd generation and Roche LDL-C plus 2nd generation, respectively). Apolipoprotein determination was carried out by immuno-turbidimetric assay (Roche Tina-Quant APO A (Version 2), Tina-Quant APO B (Version 2), Roche Ltd). Concentration of leptin in the sera was measured by competitive enzyme immunoassay (Cayman Chemical Company, Ann Arbor, Michigan, USA).

Isolation of monocytes

Monocytes were isolated from venous blood, according to the methods of Boyum (1968). The mononuclear cell suspensions were placed in Nunc petri

dishes (90 mm diameter), pretreated with fetal calf serum, and further separated by the method of Kumagai et al. (1979). The final cell suspension was 93–96% pure for monocytes and the proportion of viable cells was 90–95%.

Experimental conditions

Monocytes were suspended in Hepes-buffered HBSS. For stimulation of monocytes leptin (Sigma) was administered at a final concentration of 1–500 ng/mL doses. Different kinds of inhibitors were applied on the basis of previous studies as follows: 5 µM neomycin (Sigma) for 60 min, 100 ng/mL pertussis toxin (PTX, Sigma) for 120 min, 1.0µM thapsigargin (Sigma) for 60 min, for inhibition of Ca^{2+}-influx cells were plated in medium V (10 µM verapamil(Sigma) + 3 mmol/L EGTA in Ca^{2+}-free medium) for 60 min, 1.0µM H-7 (Sigma) for 60 min, 20 nM wortmannin (WMN, Sigma) for 30 min, 50µM PD98059 (Sigma) for 30 min, 5µM fluvastatin (Merck) for 60 min, and for inhibition of sterol regulatory element-binding protein cleavage-activating protein (SCAP) the 25 µM 25-hydroxycholesterol (Sigma) complexed with methyl-β-cyclodextrin according to the method of Adams et al. (2004)for 120 min. All groups of monocytes 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 to enable the above-mentioned lengths of incubation prior to leptin stimulation. Experiments on monocytes were carried out within 6 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 the studied parameters. Cells were incubated in a humidified CO_2 incubator at 37 ◦C (CO_2 5%, air 95%, humidity 95%).

Determination of membrane-bound proteinkinase C

The method was carried out as described by Bell et al. (1986), modified by Gopalakrishna et al. (1986). At the 0–45th min after leptin stimulation the monocyte suspensions (10^5 cells/mL) were rapidly centrifuged at 4 ◦C. The pellet was resuspended in Hepes-buffered ice-cold HBSS containing EDTA, 0.5 mmol/L EGTA, phenyl-methyl-sulfonylfluoride (Sigma), and leupeptin (Sigma). Cells were disrupted ultrasonically (Branson Sonifier 450) and centrifuged at 100,000×g for 45 min at 4 ◦C (Beckman L5-65B). The pellets were then solubilized with Chaps (Sigma) 1% Nonident P-40 (Sigma). The pellets were rehomogenized and centrifuged again as above. The PKC activities of the membrane fractions were determined by measuring the ^{32}P incorporation from 100 to 200 cpm/mg [^{32}P]-ATP (Institute of Radiochemical Research, Budapest) into 100 µg/mL histone III-S (Sigma) in the presence of 10 mmol/L $MgCl_2$, 1.5 mmol/L $CaCl_2$, 96µg/mLl-phosphatidyl-l-serine (Sigma), 6.5 µg/mL 1-oleoyl-2-acetyl-glycerol (Sigma), 50 µmol/L adenosine 5-triphosphate Na2 (ATP, Sigma), and 100–200 cpm/mg [^{32}P]ATP. The reaction was terminated after 10 min by adding ice-cold trichloroacetic acid and bovine serum albumin as carrier. The precipitate was then filtered through a 0.45 µm Millipore HA filter and washed in 5× 2 mL ice-cold trichloroacetic acid. Radioactivity was determined with a Packard 2200 CA liquid scintillation counter, using a toluol cocktail to dissolve the filter. PKC activity was expressed as incorporated ^{32}P (pmol/min/mg protein).

Measurement of endogenous cholesterol synthesis

The assay was performed as described by McNamara et al. (1985). Monocytes (10^6 cells) in 1.0 mL HBSS containing 2.5 nmol/L[2-14C]acetate with 1.8 GBq/mmol specific activity (Isotope Institute of the Hungarian Academy of Sciences) were incubated with 1–500 ng/mL leptin and also with the above-mentioned inhibiting drugs. The reaction was terminated with 0.5 mL of 1.0 mol/LKOH at the end of the 4-h long incubation, and the samples

were saponified for 90 min at 70 ◦C. As an internal reference, standard [1,2-3H]cholesterol (1480 GBq/mmol) was used. The unsaponified lipids were extracted with hexane. The extracts were placed on aluminium oxide columns, and the steroid fraction was eluted with a 1:1 mixture of acetone/diethyl ether. After drying eluates their radioactivity was measured, and values were expressed in pmol of synthesized cholesterol/h/10^7 monocytes.

Superoxide anion generation

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

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. The appropriate concentrations of inhibitor drugs were added to the cell containing mixture 30–120 min prior to the end of myo-[3H] inositol preincubation. Following vigorous washing cell-bound radioactivity was determined. The inhibitors were readministered to the monocytes, and the cells were stimulated with 50 ng/mL leptin. After 20 s, the reaction was terminated. Ins(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.

Determination of intracellular calcium concentration

Monocytes were incubated in 1 mL volume containing 5 x 10^6 cells + 20 µL Indo 1/AM (Calbiochem) for 30 min at 37 ^oC 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 determination of intracellular [Ca^{2+}] was carried out at 405 and 485 nm in a spectrofluorimeter (Hitachi, F-4500), under constant stirring at 37 ^{o}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 intracellular [Ca^{2+}] 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.

Statistical analysis

PKC activity and intracellular [Ca^{2+}] values were expressed in AUC. To evaluate the degree of inhibitions, AUC values were calculated which were characterized by descriptive statistics. They were analyzed by one way ANOVA and Newman–Keuls test. Changes were considered statistically significant at $p \leq 0.05$.

RESULTS

In the first set of experiments the effect of leptin on cholesterol synthesis was examined by using different leptin concentrations.

The basal level of cholesterol synthesis in resting monocytes was higher in the OW than in the control lean group. In control monocytes 10–100 ng/mL leptin-stimulated cholesterol synthesis whereas higher, 250–500 ng/mL leptin concentrations suppressed it. In contrast, 10–500 ng/mL leptin concentrations lead to remarkable enhancement of endogenous cholesterol

synthesis in monocytes of OW patients. Furthermore, we studied the downstream regulation of 50 and 500 ng/mL leptin-induced endogenous cholesterol synthesis in both monocyte groups. Leptin in 50 ng/mL concentration increased the cholesterol synthesis in both monocyte types; however, the reaction was more pronounced in OW cells. Enhanced cholesterol synthesis was inhibited in both of these monocyte groups by the PI3K inhibitor WMN, the MAPK inhibitor PD98059, the HMGCoA reductase inhibitor fluvastatin and the SCAP inhibitor 25-HC.

In control cells leptin-induced cholesterol synthesis was also decreased by the PLC inhibitor neomycin and the intracellular Ca^{2+} pool suppressor thapsigargin. In contrast, in OW monocytes the verapamil containing medium V was able to inhibit leptin-induced cholesterol synthesis.

Furthermore, we studied the signaling pathways in both monocyte groups after stimulation with 500 ng/mL leptin. The decrease in cholesterol synthesis in control monocytes under these circumstances was abolished by thapsigargin, H-7 and WMN pretreatment suggesting the role of Ca^{2+} signaling, cPKC and PI3K activation in the regulation of leptin induced cholesterol synthesis. However, in the OW monocyte group the inhibition of Ca^{2+} influx by Medium V, WMN, 25-HC and fluvastatin decreased the elevated cholesterol synthesis.

The PKC activity was measured in monocytes of lean and OW subjects for 45 min after stimulation with 50 and 500 ng/mL leptin. In the lean group we only found an early peak 5 min after stimulation with low leptin concentration whereas after treating the cells with 500 ng/mL leptin, we observed a second high peak 30–35 min after adding leptin to the culture medium. Leptin-induced PKC time curves in OW monocytes show that PKC activity in resting cells was sevenfold higher in OW than in control monocytes. Moreover 50 ng/mL leptin caused a time curve with two peaks in cell of OW subjects, whereas after 500 ng/mL leptin treatment we found only one large peak with a maximum at 30 min. When we calculated the areas under time curves (AUC), we found that in control lean groups the 50 and 500 ng/mL leptin-induced first peaks were nearly equal, however, the second large peak only appeared after addition of 500 ng/mL leptin.

In the OW groups the leptin-induced membrane bound PKC activation was more pronounced than in control cells, and the one large peak after stimulation with 500 ng/mL leptin was higher than the sum of two peaks after addition of 50 ng/mL leptin.

In both treatment groups of control lean monocytes the membrane-bound PKC activity peaks were inhibited by WMN preincubation, while the second peak after 500 ng/mL leptin treatment PKC was inhibited by both WMN and H-7. In OW monocytes the resistance of PKC activity to H-7 is independent of the applied leptin concentration, and PKC activity was inhibited only by the PI3K inhibiting WMN. Summarizing the above results, we conclude that in OW monocytes the lack of H-7 sensitive cPKC activation plays a potential role in the increased stimulation of cholesterol synthesis by leptin treatment.

In the next set of experiments the intracellular [Ca^{2+}] changes triggered by leptin in C and OW monocytes were compared. We found resting intracellular [Ca^{2+}] levels in OW monocytes to be higher than in control cells, whereas peaks of Ca^{2+}-time curves were lower and appeared later in OW cells. We demonstrated Ca^{2+} signals, i.e. intracellular [Ca^{2+}] in leptin-stimulated minus resting monocytes. Values in AUC were significantly higher in OW monocytes than in control cells, which maybe a consequence of the delayed return to the basal level despite the low peak. To elucidate the origin of Ca^{2+} signals, we preincubated monocytes with pertussis toxin (PTX), neomycin, thapsigargin (TG), wortmannin (WMN) and in medium V. According to these data, PTX had no effect on leptin-induced Ca^{2+} signals. Neomycin, TG and WMN were found to inhibit Ca^{2+} signals in control cells more than in OW monocytes. The most pronounced inhibition was exerted in OW monocytes by incubation in medium V, whereas in control cells Ca^{2+} signals were not influenced by absence of extracellular Ca^{2+}, suggesting that verapamil-sensitive Ca^{2+} channels are involved in the leptin-induced Ca^{2+} signal generation only in OW monocytes. In contrast, we found that Ca^{2+} translocation from thapsigargin sensitive intracellular pools is greater in control cells, whereas its role in OW monocytes is not as pronounced.

We attempted to clarify the role of superoxide anion generation in altered Ca^{2+} balance. First, the leptin-triggered superoxide anion generation

was determined in both groups of monocytes. Data showed leptin to cause more intensive and PTX resistant superoxide anion generation in OW monocytes than in control cells. Neomycin, TG and WMN inhibited NADPH oxidase activation significantly in control monocytes, whereas the superoxide generation remained unchanged in medium V. TG caused the most pronounced inhibition suggesting the significance of Ca^{2+} translocation from intracellular pools in leptin signaling. In contrast, superoxide anion generation in OW cells was significantly inhibited in medium V, whereas it was affected moderately by neomycin, TG and WMN. These results suggest that Ca^{2+} signal is an important component of leptin-induced NADPH oxidase activation, and consequently, these signal pathways are different in control and OW monocytes. Additionally, the pretreatment with fluvastatin resulted significant decrease in leptin-stimulated superoxide anion generation in both monocyte groups, however, the inhibition was more pronounced in OW monocytes.

In the next set of experiments, the difference between control and OW monocytes in leptin-induced inositol-1,4,5-trisphosphate (Ins(1,4,5)P3) signal was further elucidated. In control cells, after leptin treatment, Ins(1,4,5)P3 was found to be remarkably inhibited by neomycin and WMN. In OW monocytes leptin treatment resulted in smaller Ins(1,4,5)P3 level increase, however, the basal level in resting OW cells was higher than in control monocytes. The results also indicated that leptin-induced activation of PLC was caused by activation of a WMN-sensitive pathway but did not occur through the PTX sensitive Gi protein.TG inhibited Ca^{2+} signal only in control cells, whereas medium V had inhibiting effect only in OW monocytes. Fluvastatin treatment had no effect in control cells, whereas in OW monocytes it restored the impaired intracellular [Ca^{2+}] balance, i.e. it corrected the cytosolic free Ca^{2+} decrease. When fluvastatin pretreated OW monocytes were incubated in the presence of either TG or medium V. The leptin induced intracellular [Ca^{2+}] peak was inhibited only by TG. These results suggest that the HMG CoA reductase inhibiting fluvastatin was able to restore intracellular [Ca^{2+}] homeostasis in OW monocytes.

Finally, we observed positive correlation between the leptin

triggered superoxide generation and Ca^{2+} signals in OW monocytes, whereas there was no correlation in control cells. This suggests that superoxide generation is involved in impaired intracellular [Ca^{2+}] in OW monocytes.

DISCUSSION

We examined the effects leptin, key regulator of obesity, on monocytes isolated from the blood of normal weight and overweight healthy men. Apart from the role of leptin in the control of hunger and energy intake, it also acts as a major regulator of intracellular processes. The importance of this adipokine is underlined by the complexity of its related signaling mechanisms. Previously, we showed altered FMLP and angiotensin-II triggered signaling mechanisms in neutrophils of patients suffering from type II diabetes mellitus and hypercholesterolemia compared to healthy controls.

In the present experiments leptin influenced the cholesterol synthesis, calcium levels and Nox activity of OW and C monocytes in a different manner. We treated the monocytes with leptin in concentration gradient. In OW monocytes the rate of cholesterol synthesis increased with the increasing leptin levels while C monocytes reacted with decreased cholesterol synthesis for high leptin concentration. On the basis of these results, the hypothesized differences in the signaling mechanisms were further studied by 50 and 500 ng/mL leptin stimulation. Ca^{2+} signals of different origins, PI3K, MAPK and HMG-CoA play a role in this process. In connection with leptin signaling, inositol phospholipids are key regulators of numerous cellular processes. Leptin has a modulatory role in the cytokine production of monocytes regulating inflammatory processes. In the state of obesity, the adipose tissue creates a mild, long-lasting inflammation, which elevates the expression of SREBP and SCAP leading to increased cholesterol synthesis. Treating our monocytes with 25-HC and fluvastatin, we observed the inhibition of cholesterol production. This finding indicated that the SREBP2–SCAP axis might contribute to the metabolic alteration observed in

OW monocytes. The differences in the regulation of cholesterol synthesis between the two monocyte groups raised two questions: in control monocytes what is the mechanism responsible for the decrease in cholesterol synthesis after high concentration of leptin stimulation and why this mechanism cannot be observed in OW monocytes. The 500 ng/mL leptin triggered decreased cholesterol synthesis was stopped by the pretreatment with H-7 and WMN. Thus, the role of Ca^{2+}-dependent cPKC in the decrease of cholesterol synthesis after 500 ng/mL leptin treatment was underlined by the use of H-7. Additionally, SOCS3 is able to reduce the activity of long type leptin receptors and functioning as antagonists of STAT3 activation. Therefore, SOCS3 is considered as an important player in the development of leptin resistance. The role of PKC and PI3K signaling has been demonstrated in the increased expression of SOCS3 and ERK1/2 in activated macrophages. The function of PKC and SOCS family is not independent of each other, which supports the hypothesized role of PKC in leptin resistant cholesterol synthesis. Our research group has already observed the leptin signaling in monocytes, one element of which is the DAG induced PKC activation. Stimulation by 50 ng/mL leptin lead to increased cholesterol production is both C and OW monocytes. This increase was highly sensitive to WMN pretreatment, which indicates that PI3K and consequently the production of Ins(3,4,5)P3 play a role in increased cholesterol synthesis followed by treatment with moderate leptin dose. Furthermore, Ins(3,4,5)P3 elevates the activation of PKCζ autophosphorilation leading to increased NFκB expression.WMN sensitive PKC activity appeared following both moderate and high dose of leptin treatment. The second peak in C cells was shown to be H-7 sensitive. However, OW monocytes had H-7 resistant PKC synthesis. The cholesterol synthesis and PKC levels changed parallely: both monocyte groups showed WMN sensitive cholesterol synthesis, while only C monocytes were susceptible to H-7. According to our results PKCζ represents the link between PKC activity and cholesterol synthesis. PKCζ activates NFκB through the phosphorilation inhibitor-κB (I-κB), which acts as a transcriptional activator in the nucleus. The close relationship of NFκB and cholesterol production is further supported by the sensitivity of NFκB towards statins and increased cholesterol synthesis. Apart from PKC,

different regulation of PI3K, altered membrane composition and rigidity and cholesterol accumulation in the lipid rafts of OW monocytes may also contribute to the differences observed in the C and OW groups.

Additionally, in our experiments we found changes in the calcium homeostasis of OW monocytes. We detected significant elevation of intracellular calcium concentration in resting OW monocytes and the peak of Ca^{2+} signals after leptin stimulation were lower than in control cells. Additionally, return of intracellular [Ca^{2+}] to the baseline level was delayed. Overall, these indicate a significant imbalance of the Ca^{2+} homeostasis in OW monocytes. Based on our results we concluded that in OW monocytes leptin – among other cytokines – might be involved in the increase in intracellular [Ca^{2+}] because of the strong free radical generating ability of these cells. This disturbed intracellular [Ca^{2+}] homeostasis may be characterized by the failure of Ca^{2+} translocation from intracellular pools and slow Ca^{2+} extrusion from the cytosol.

Calcium imbalance in OW cells may be characterized by: (1) The failure of the canonical signal through the receptor – inositol-1,4,5-trisphosphate – intracellular Ca^{2+} pools – Ca^{2+} signal pathway. (2) A shift in the origin of Ca^{2+} signals to Ca^{2+} influx through the verapamil-sensitive Ca^{2+} channels. (3) The delayed return of intracellular [Ca^{2+}] to the baseline, as seen on Ca^{2+} time-curves after leptin stimulation.

When time-curves of Ca^{2+} signals were analyzed applying the AUC concept we found the total Ca^{2+} signals in OW monocytes markedly elevated compared to controls. The leptin-triggered enhancement of AUC was caused by the delayed normalization of Ca^{2+} signals in OW monocytes. The inhibiting effect of thapsigargin in all groups of monocytes may be attributed to the participation of wortmannin-sensitive phosphatidylinositol 3 (PI3)-kinase in the signaling of long type leptin. PI3 kinase is able to split phosphatidylinositol to diacyl-glycerol and Ins(3,4,5)P3 which activates phospholipase C enzyme inducing Ins(1,4,5)P3 generation. In our experiments leptin caused significant increase in the inositol-1,4,5-trisphosphate levels in both subject groups. These elevated levels of Ins(1,4,5)P3 in the monocytes separated from normal weight and overweight men were reduced by WMN through the inhibition of phosphoinositide 3-

kinases and neomycin due to the inhibition of phospholipase C. However pertussis toxin had no effect on Ins(1,4,5)P3 levels in either patient groups. Since Ins(1,4,5)P3 is an important element of the intracellular signaling resulting in calcium release from the endoplasmic reticulum these results inform us about the share of intracellular calcium release to the overall calcium concentration in the cell. The inhibiting drugs applied in the Ins(1,4,5)P3 determination suggested that phosphoinositide 3-kinases and phospholipase C participated in the production of Ins(1,4,5)P3 whereas the pertussis toxin inhibited G proteins had no effect in this process. These results further support our calcium measurements after the preincubation with these inhibitors and verify the significance of Ins(1,4,5)P3 in cellular calcium homeostasis.

The attenuated inhibiting effect of ZG on OW monocytes suggests that leptin-induced Ca^{2+} signal in OW cells is caused by Ca^{2+}-influx, however there is also evidence in the literature that PTX-resistant Ins(1,4,5)P3 takes part in Ca^{2+} signal, too. TG had inhibiting effect on superoxide anion generation both in control and in OW-monocytes indicating that Ca^{2+} signal-induced activation of NADPH oxidase depends partly on the intracellularly mobilized free Ca^{2+}. The significant decrease in leptin-induced Ins(1,4,5)P3 elevation with WMN preincubation confirms our assumption about the role of phosphatidylinositol 3-kinase in leptin-induced Ca^{2+} signal.

In monocytes, store depletion subsequently results in extracellular Ca^{2+} entering the cell to help refilling the stores. This rise in intracellular $[Ca^{2+}]$ is essential for the activation of NADPH oxidase, the enzyme responsible for the production of ROS. The relation between intracellular Ca^{2+} homeostasis and free radical generation in control and OW monocytes needs to be further evaluated.

Previously we published that angiotensin II and leptin increased superoxide anion and leukotriene generation in neutrophils of hypercholesterolemic patients compared to controls and free radical generation was partially inhibited by both *in vitro* and *in vivo* statin-administration. The calcium/calmodulin dependent ATPase located in the plasma membrane is the only system in non-excitable cells for calcium-extrusion. Therefore, it is the most significant enzymatic Ca^{2+} pump in the

control of intracellular calcium concentration. In the present study, we found a significant increase in the leptin generated superoxide anion production of OW monocytes. Decrease in superoxide anion generation of OW-monocytes, in medium V and after pretreatment with fluvastatin suggests not only altered signal processing, but also the mevalonate cycle-dependence of superoxide anion generation and the endogenous antioxidant activity of statins. Statins are inhibitors of the 3-hydroxy-3-methyl-glutarylcoenzymeA reductase, which is the key-enzyme in the mevalonate cycle. Moreover, statins have pleiotropic effects, inhibiting not only cholesterol synthesis but the members of the Rho superfamily, which are responsible for cell proliferation, tumor development, and activation of the Rac1 small GTPase. This latter protein plays role in the regulation of the NADPH oxidase activity in monocytes, therefore fluvastatin is able to decrease partially superoxide anion generation induced by Angiotensin II or leptin stimulation in vitro and in vivo. The mevalonate cycle-dependent (fluvastatin-inhibitable) portion of the leptin-induced superoxide anion generation is more pronounced in OW monocytes compared to control cells. This finding supports the hypothesis that endogenous antioxidant activity is not the sole characteristic of statins in the inhibition of ROS generation. Fluvastatin is able to eliminate the delayed restoration of intracellular [Ca^{2+}] in OW cells, possibly, by inhibiting the enhanced superoxide anion generation, therefore it causes significant decrease in Ca^{2+} signal. In fluvastatin-treated cells Ca^{2+} signal originated from the thapsigargin sensitive pools, the Ca^{2+} influx through the Ca^{2+} channels stopped, and the free Ca^{2+} disappearance from cytosol recovered. Furthermore, we found in OW monocytes significant and strong positive correlation between the leptin-induced superoxide anion generation and AUC-values of Ca^{2+} signals.

We can draw the conclusion that in OW monocytes a H-7 resistant, but WMN sensitive leptin signaling is present which may be the consequence of an atypical, Ca^{2+}-independent PKC activation. This PKC activation may be the culprit in the increased cholesterol synthesis in OW monocytes following leptin treatment. Our results suggest that increase in Ca^{2+} signals in OW-monocytes is caused partly by impaired Ca^{2+} efflux from the cells, as a

consequence of the leptin triggered superoxide anion induced injury of the membrane transport.

Finally, we must consider that Ca^{2+} imbalance plays a key role in many pathological symptoms of disorders e.g. obesity, insulin resistance, hypertension and atherosclerosis. Agouti protein, a gene product of weight control, influences Ca^{2+} influx and along with increased$[Ca^{2+}]_i$ levels inhibits lipolysis and promotes lipogenesis in human and murine adipocytes. Since humans express agouti mainly in adipose tissue, it may similarly exert paracrine effects on $[Ca^{2+}]_i$ and thereby stimulate de novo lipogenesis and promote obesity. Thus, Ca^{2+} signaling represents a target for therapeutic intervention in obesity. Statins, due to their ability of diminishing intracellular calcium concentration intracellular $[Ca^{2+}]$ levels through inhibition of free radical generation, and inhibiting cholesterol synthesis, play important role in the treatment of obesity-related diseases e.g. insulin resistance, hypercholesterolemia and atherosclerosis.

SUMMARY

Obesity, as a significant risk factor, takes major part in the development of numerous diseases including atherosclerosis and cancer. Leptin is an adipose tissue derived hormone, the level of which is increased in overweight. Leptin takes part in local inflammations and in the regulation of cholesterol biosynthesis in human monocytes.

In our studies we aimed at defining the concentration dependence of the effect of leptin in monocytes of overweight men (OW), and the downstream signaling of high and low leptin concentrations targeting the regulation of cholesterol synthesis. Additionally, Ca^{2+} signals, NADPH oxidase activation, and their hypothesized correlation in monocytes of lean and overweight men were investigated in our experiments. To reveal the signaling mechanisms in connection with calcium and cholesterol

triggered by leptin we stimulated OW and control monocytes with leptin in the presence or absence of different inhibitors.

Our results demonstrate that the biphasic effect of control monocytes depends on concentration, while OW monocytes only presented elevated cholesterol synthesis. The stimulation with 50 ng/mL leptin induced Ca^{2+} signal, activation of PI3K, MAPK and HMG CoA reductase. The suppression of cholesterol synthesis in the control monocytes stimulated with 500 ng/mL leptin was shown to be dependent on the Ca^{2+} signal, PI3K activation and the H-7 sensitive cPKC, whereas the cholesterol synthesis in OW monocytes was only increased by PI3K. Our results demonstrate that the OW monocytes had higher basal intracellular [Ca^{2+}] level than the control cells. On the other hand, in the OW monocytes the leptin-induced peak of the Ca^{2+} signal was lower with a delayed return to the basal level. Despite the lower Ca^{2+} peaks OW monocytes presented higher Ca^{2+} signals than control monocytes due to the delayed return to the baseline values. The utilization of different cellular signaling inhibitors revealed that the Ca^{2+} signals in control monocytes were originated from intracellular pools, while they were originated mostly by Ca^{2+}-influx from medium in the OW cells. Leptin induced superoxide anion generation showed significant positive correlation with Ca^{2+} signals in OW monocytes. The treatment with fluvastatin completely restored the intracellular [Ca^{2+}] homeostasis in OW monocytes. The beneficial effect of statins on elevated intracellular [Ca^{2+}] level might be the consequence of their pleiotropic effects (e.g. inhibiting free radical generation). Statins therefore might be useful for the treatment of obesity not only due to their ability to decrease cholesterol synthesis, but their anti-oxidative effect.

Our results demonstrate that damage of H-7 sensitive cPKC activation, PI3K mediated PKC activation and elevated Ca^{2+} levels as the consequence of damaged calcium channels characterize the leptin-signaling in OW monocytes.

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List of other publications

3. Sztanek, F., Seres, I., Harangi, M., Lőcsey, L., **Padra, J.T.**, jr. Paragh, G., Asztalos, L., Paragh, G.: Decreased human paraoxonase-1 (PON1) lactonase activity in hemodialyzed and renal transplanted patients. A novel cardiovascular biomarker in end-stage renal disease. *Nephrol. Dial. Transplant. Epub ahead of print (2012)* DOI: http://dx.doi.org/10.1093/ndt/gfr753 IF:3.396 (2011)

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