Functional polarization of macrophages of rats with progesterone-induced obesity treated with melanin from the Antarctic yeast Nadsoniella nigra

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Introduction

Obesity is the disease associated with the development of inflammation not only in adipose tissue but in the whole organism as well. In response to fat accumulation, adipocytes increase their size leading to secretion of proinflammatory cytokines. In obese patients the adipose tissue produces tumour necrosis factor α (TNF-α), interleukin-1, -6, -8, -10, chemokines MCP-1 and MIP-α, C-reactive protein (Kern et al., 2001). This leads to macrophage recruitment to the adipose tissue with subsequent secretion of cytokines and chemokines (Sell & Eckel, 2010, Hartford et al., 2011). The circulation levels of proinflammatory cytokines increase (Curat et al., 2006), reflecting the fact that low-grade inflammation in adipose tissue causes the systemic inflammation in distant organs and tissues (Buillo et al., 2003, Engeli et al., 2003).

The model of progesterone-induced obesity remains incompletely studied. Progesterone is a hormone involved in regulation of reproductive function. The increase of its production in the luteal phase of menstrual phases leads to an increase in food intake and even binge eating (or emotional eating), which may be accompanied by depression (Klump et al., 2013, Hildebrandt et al., 2015). Progesterone is included in menopausal hormone therapy (MHT) (Coquoz et al., 2018).

Progesterone derivatives used for contraception in adolescent girls (form 12 to 18 years) increase weight gain (Bonny et al., 2006). The treatment of women with progesterone in MHT also increases the food intake, and therefore fat accumulation in the organism (Bonny et al., 2014, Coquoz et al., 2019). Similar effects were observed in rats and mice, which led to the development of the animal model of progesterone-induced obesity (Kaur & Kulkarni, 2001, Chidrawar et al., 2011, Gundamaraju et al., 2012, Suneeatha et al., 2013). However, only sparse data obtained using such model are available, and these are insufficient for an understanding of progesterone-induced obesity mechanisms.

Macrophages play a pivotal role in the inflammation process in obesity, especially those infiltrating visceral fat tissue (Heilbron & Campbell, 2008). They not only secrete inflammatory molecules but also undergo polarization towards M1 type (Sell & Eckel, 2010). M1 type macrophages produce pro-inflammatory cytokines, nitrogen monoxide and reactive oxygen species (ROS) (Sica & Mantovani, 2012), thus exacerbating inflammation. Conversely, M2 type macrophages alleviate inflammation, producing anti-inflammatory cytokines and expressing arginase, which is the marker of M2 cells (Rath et al., 2014).

Although the role of visceral fat tissue macrophages in inflammation in obesity is studied the most, more research is needed to clarify the role of peritoneal macrophages. These cells are resident macrophages of the abdominal cavity, involved in the immune response to infection and inflammation by the production of inflammatory mediators, IL-12, MIP-1α, TNF-α, and RANTES in particular (Ghosn et al., 2010). The NO production by PLS-stimulated peritoneal macrophages was established, showing their polarization towards M1 type (Sica & Mantovani,
The isolation of peritoneal macrophages. After euthanasia, 10 mL of cold physiological solution was injected intraperitoneally and massaged. The abdominal fluid was aspirated by a syringe and then collected into sterile tubes that were centrifuged at 1,500 rpm for 10 min. The precipitate (cell suspension) was resuspended in 1 mL of RPMI medium which contained 10% FCS and 40 μg/mL gentamicin. Cell viability was determined using hemocytometer in 4% trypan blue exclusion technique (Skivka et al., 2013). Peritoneal macrophages were resuspended in the medium, as outlined above, and diluted to concentration 5 × 10⁶ cells/mL. The level of oxygen species was measured in isolated macrophages by NBT-test (nitro blue tetrazolium test) immediately. To determine the arginase activity and NO production macrophages were cultured for 18 hours at 37 °C.

**NO production assay.** The NO production by peritoneal macrophages was determined by the accumulation of stable degradation products—nitrates (Skivka et al., 2013). Cells were inoculated (2 × 10⁶ cells/mL) into quadruplicates in wells of a polystyrene flat-bottomed 96-well microplate and cultured in RPMI 1640 medium with addition of 10% fetal bovine serum and 1% gentamicin during 18 hours at 37 °C and 5% CO₂. The supernatant (cell medium) was collected and used for the determination of nitrates using Griess reagent (0.2% N-1-naphthyl ethylenediamine dihydrochloride mixed with an equal volume of 2% sulfanilamide dissolved in 10% phosphoric acid). 100 μL of Griess reagent was added to an equivalent volume of cell medium and incubated in quadruplicate in 96-well plate in room temperature for 30 min in the dark. The absorbance was read at 550 nm using an automated plate reader (Bio-Rad, USA). The nitrite level in the medium was calculated from a sodium nitrite (NaNO₂) standard curve and expressed as nmoles per 10⁶ cells.

**NBT assay.** Peritoneal macrophages were washed in Hank’s solution by centrifugation at 1,500 rpm for 10 min and then resuspended in the same solution to the concentration of 1 × 10⁶ cells/mL. The suspension of 100 mL of washed cells was inoculated to the wells of a polystyrene flat-bottomed 96-well microplate (Nest Biotech Co., Ltd, China) and incubated at 37 °C for 30 min to allow cells to adhere to the plate. The ROS production by peritoneal macrophages was estimated by NBT assay (Skivka et al., 2013). Then 100 μL of 0.1% nitro-blue tetrazolium chloride (NBT) (Sigma-Aldrich, USA) was added per well and incubated with 0.1% nitro blue tetrazolium (NBT) (Sigma-Aldrich, USA) at 37 °C for 15 min. To trigger the oxidative burst, zymosan A (from Saccharomyces cerevisiae, Sigma-Aldrich, USA) was added to the final concentration of 600 μg/mL.

The supernatant was removed from the wells and the reaction was stopped by adding the 100 μL of 2 M KOH. Insoluble formazan product was dissolved in 100 μL of 50% dimethyl sulphoxide (DMSO) (Sigma-Aldrich, USA). The absorbance was read at 630 nm by a plate reader (Bio-Rad, USA).

**Arginase activity assay.** Peritoneal macrophages were inoculated into wells of a flat-bottom microtiter plate (Nest Biotech Co., Ltd, China) at 2 × 10⁶ per well. Cells were cultured in RPMI 1640 supplemented with 10% Bovine fetal serum and 1% gentamicin and 5% CO₂ at 37 °C for 18 h. The next day nonadherent cells were separated from adherent cells by washing in 200 μL of Phosphate Buffer Solution (PBS). The cells were lysed by the addition of 100 μL of 0.1% Triton X-100.

Amount of 100 μL of 50 mM Tris-HCl, pH 7.4, 1.0 μM MnCl₂ was added to the suspension, all procedures were undertaken at 4 °C. Arginase activity in macrophages was activated by heating the mixture at 56 °C for 7 min. The reaction of L-arginine hydrolysis was initiated by the addition of 100 μL of arginine (0.5 M, pH 9.7). After that the mixture was incubated at 37 °C for 3 hours. The urea solutions of the following concentrations were used as standards: 7.5, 15, 30, 45, 60 μg per 100 μL of H₂O. The reaction was stopped by the addition of 800 μL of an acid mixture (H₃PO₄ : H₂SO₄ : H₂O = 1 : 3 : 7). The amounts of 900 μL of the same solution were added to the standards.

For colorimetric determination of urea, 40 μL of 6% o-iodosobiphenylphosphate (o-ISPP) (Sigma-Aldrich, USA) dissolved in 9% ethanol was added to all wells, including the standards (Skivka et al., 2013). Mixtures were incubated at 95 °C for 30 min and then at 4 °C for 30 min. The absorbance was measured at λ = 540 nm using an automated plate reader (Bio-Rad, USA). Arginase activity was calculated
using a standard calibration curve developed according to the standard concentrations of μg urea.

The data were expressed as units of arginase per 1 × 10⁶ cells: the amount of μg urea (μg) divided by 60 (molecular weight of urea) was multiplied by 50 (dilution factor) divided by the time (minutes) of incubation with arginine. One unit of arginase activity means the amount of enzyme needed to hydrolyze 1 μM arginine per minute.

Results are expressed as the average of the eight measures (x) and standard deviation (SD). Statistical analysis was performed using the Shapiro-Wilk test, the groups were compared using one-way ANOVA by homogeneity variance test (Levene’s test), followed by significant difference test (Bonferroni test). A P-value less than 0.05 was considered statistically significant.

**Results**

**Body weights.** To confirm the obesity development in animals in the first stage we determined body weights in all groups. The increase of body weight by 27% was observed in rats treated with progesterone for 28 days (Fig. 1). In the Pg withdrawal group of animals that had not been treated for a month, bodyweight did not return to its initial value and even did not decrease significantly: it exceeded the control level by 34% (Fig. 1). In animals that were treated with melanin for 28 days after 28-day progesterone treatment (Pg + Mel group), the body weight was 12% lower in comparison with the Pg group. Thus, melanin prevented further weight gain in animals with progesterone-induced obesity: in this case, it exceeded the control only by 9%. Notably, melanin did not affect the body weight in healthy animals (Mel group).

**Arginase activity.** The arginase activity was observed to decrease by 14% in rats treated with progesterone (Pg group), compared to control (Fig. 4). It means that the M2 polarization of macrophages is suppressed in this model of obesity. This data correlates with the data on increase of macrophages M1 polarization which is reflected in increase in NO production. However, in the Pg withdrawal group enzyme activity fell significantly and became lower than the control by 55%.

**ROS level.** The change of ROS production was not statistically reliable in the progesterone-treated rats in comparison to the healthy ones (Control group) (Fig. 3). Surprisingly, in the Pg withdrawal group the ROS level decreased 29% compared with control. At the same time, melanin introduced to animals 1 month after progesterone withdrawal (PG withdrawal group) decreased the ROS level by 18% compared with the Pg group. A similar trend was observed in the Control+Mel group: melanin administration to control animals decreased the ROS level by 10% in comparison with the Control group.
animals melanin decreased the enzyme activity in a less pronounced way and was 31% lower than the control.

Discussion

Our present study demonstrated on the animal model that in progesterone-induced obesity the inflammatory process developed in the abdominal cavity. We induced the obesity model by progesterone introduction to animals over 28 days. These data indicate that progesterone treatment causes obesity, which complements the data from mouse models of progesterone-induced obesity (Kaur & Kulkarni, 2001).

The weight gain in response to progesterone may be caused by an increase in food intake by animals (non-published results). Progesterone is a neuroactive steroid that, as was mentioned above, is produced in women during the menstrual phase and can cause a hyperphagic effect (Klump et al., 2013). The mechanism of progesterone-induced obesity differs from those for high-fat diet and is associated with the increase in food intake (emotional eating) (Klump et al., 2013).

Since, after progesterone withdrawal, the growth of body mass does not stop, but, on contrary, increases, we can assume that pathological processes which lead to obesity do not stop and aggravate the inflammation in the organism leading to disorders in metabolic pathways. In particular, changes in lipid metabolism may develop, leading to an increase of fat accumulation in adipocytes. This is consistent with the data about the stimulation of adipocyte growth in response to progesterone by indirectly enhancing the expression of the fatty acid synthase (Eberle et al., 2004).

Since Antarctic yeast melanin prevents progesterone-induced weight gain, it may have a beneficial effect when used for obesity prophylaxis. Probably, it causes such effect due to its antioxidant and anti-inflammatory properties as demonstrated in a number of studies (Falalyeyeva et al., 2009; Pernyakova et al., 2016; Belemets et al., 2017).

Studies in other obesity models have shown that local inflammation in adipose tissue causes systemic inflammation (Weisberg et al., 2003; Heilbronn & Campbell, 2008). The studies also revealed that not only fat tissue macrophages are implicated in inflammation in obesity; resident macrophages of other tissues are also involved in these events (Shaw et al., 2014). The mechanism of progesterone-induced obesity remains incompletely understood. It may be assumed that similarly to other types of obesity the fat accumulation in adipose tissue causes local inflammation which can further cause systemic inflammation. Therefore, to identify the inflammatory process in the organism, one should first assess the contributions of abdominal cavity macrophages which respond to changes in the fat tissue first.

Taking into account the data about inflammation obtained from other animal models of obesity (Sell & Eckel, 2010; Harford et al., 2011) and our results, we assume that in progesterone-induced obesity inflammation will possibly develop in the abdominal cavity. To confirm this assumption we evaluated the main markers of peritoneal macrophages polarization: pro-inflammatory M1 markers (NO production and ROS level) and anti-inflammatory M2 markers (arginase activity).

The study revealed an increase of NO level that could be an indication of polarization of macrophages towards pro-inflammatory M1 type, likely caused by inflammation in the adipose tissue. Our data coincide with the results which demonstrate that NO production intensifies in the fat tissue of humans (Hrabik et al., 2011) and animals (Gil-Ortega et al., 2010) with diet-induced obesity. Moreover, the increase of NO production was found not only in the fat tissue but also in leukocytes of children with inflammation associated with obesity (Hrabik et al., 2011).

At the next stage, we tested the arginase activity which is an important anti-inflammatory polarization marker. This enzyme is expressed together with pro-inflammatory cytokines in M2 macrophages (Rath et al., 2014) and is involved in the development of inflammation in response to viral infection and tumour growth (Narita et al., 2013; Takele et al., 2013).

The decrease of arginase activity in animals with progesterone-induced obesity is possibly associated with the decrease in peritoneal macrophages’ ability to polarize towards M2 anti-inflammatory type. Also, it is possible, that these cells can take no part in the immune response in obesity. This fact is indicated by the data obtained on the mouse model, in which the obesity M2 macrophages were not involved in the inflammatory status formation; in contrast, their number increased in lean mice (Lumeng et al., 2007).

Thus, the increase of NO production together with the decrease of arginase activity indicates the stimulation of their polarization towards pro-inflammatory phenotype and the suppression of polarization towards anti-inflammatory one respectively. Our findings suggest the development of inflammation in the abdominal cavity in progesterone-induced obesity.

Our suggestion is supported by the notion that excessive accumulation of visceral fat in abdominal obesity causes local inflammation in fat tissue followed by systemic inflammation (Bullo et al., 2003, Engelt et al., 2003). Therefore, the induction of inflammatory status in peritoneal macrophages in progesterone-induced obesity could be the outcome of inflammation in the abdominal cavity due to fat accumulation.

Also, in a model of progesterone-induced obesity we have revealed the link between the increases of animals’ body weight and the augmentation of polarization of abdominal cavity macrophages towards pro-inflammatory phenotype. A similar correlation between body mass, some metabolic parameters, and markers of inflammation has been demonstrated in obese patients with polycystic ovary syndrome (Durnas et al., 2017).

Our data regarding the implication of peritoneal macrophages in inflammation development in the organism are consistent with findings from other obesity models. The induction of inflammatory status in peritoneal macrophages in mice with high-fat-diet-induced obesity (Wu et al., 2013) and also the augmentation of M1 polarization along with the suppression of M2 polarization (Zhang et al., 2016) have been reported.

Oxidative stress is believed to be the integral part of inflammation in obesity not only in fat tissue (Trayhurn et al., 2008; Wood et al., 2009), but also in whole organism (Furukawa et al., 2004) with attendant production of reactive oxygen and nitrogen species (Nathan, 2008). Several reports suggest ROS accumulation not only in fat tissue but also in the serum of people and animals with obesity (Fernández-Sánchez et al., 2011; Marsiglia et al., 2014). In this context, we have estimated by NBT assay the ROS level in peritoneal macrophages of rats with progesterone-induced obesity. In spite of evidence of the occurrence of oxidative stress in fat tissue in obesity (Furukawa et al., 2004), we have not revealed the oxygen-dependent metabolism stimulation in macrophages of progesterone-treated rats. We surmise that with progesterone-induced obesity the augmentation of macrophages polarization towards the M1 pro-inflammatory phenotype is implemented through the NO production in contrast to ROS production. We suggest that these effects could reflect the immune response to inflammation in fat tissue.

Notably, the long-term consequences (Pg withdrawal group) include the decrease of all markers of M1 and M2 polarization: both NO and ROS level as well as arginase activity respectively. All markers became lower not only comparative to the progesterone group but also relative to healthy animals. It likely mirrors the suppression of functional capacity of peritoneal macrophages that is not restored and but rather aggravated in the inflammatory process upon obesity development. This suggestion is in agreement with the fact that in the progesterone withdrawal group the body-weight continues to rise after the progesterone withdrawal.

Our findings suggest that with progesterone-induced obesity the polarization of the macrophages towards pro-inflammatory type increased, while the polarization towards anti-inflammatory type is suppressed. Our data are consistent with the results indicating that inflammatory response in obesity is accompanied by an increase of M1 polarization and alleviation of M2 polarization of both macrophages in adipose tissue (Lumeng et al., 2007; Fujisaka et al., 2009) and peritoneal macrophages (Zhang et al., 2016). The revealed changes of these cells functioning could be interpreted as the response to the violation of the peritoneal cavity microenvironment, the production of inflammatory molecules (cytokines), produced in adipose tissue, in particular. This agrees with the evidence that systemic inflammation in obesity is the outcome of local inflammation accompanied by the decrease in the number of M2 macrophages in adipose tissue (Heilbronn & Campbell, 2008). Perhaps, 1 month after progesterone withdrawal, the consequences of the inflammatory process in fat tissue are not mitigated, resulting in the depletion of the functional ability of macrophages. The present study also aimed to examine the effect of Antarctic yeast melanin on the above-noted markers of macrophages po-
lizarion. Melanin has attracted particular attention due to its antioxidiant and anti-inflammatory properties. We have demonstrated that melanin not only prevents weight gain but also suppresses the polarization of peritoneal macrophages. It causes a decrease in M1 polarization markers (NO and ROS production) in obese animals. Notably, the effect of melanin in healthy animals was similar but less pronounced.

Arguably, melanin reduces the inflammation in the abdomen caused by body weight increase. This effect could be associated with melanin antioxidiant properties, meaning its ability to scavenging hydroxyl radicals and superoxide anions.

We suppose that in cases of obesity melanin prevents activation of abdominal cavity macrophages in response to low-grade inflammation in fat tissue and it, therefore, could have anti-inflammatory properties. This is consistent with previous studies, where melanin was shown to reduce the level of pro-inflammatory cytokine IL-1β and restore the level of anti-inflammatory cytokines (IL-10, TGF-β) in the serum of rats with non-alcoholic liver disease induced by monosodium glutamate (Belemets, 2017).

As noted previously, the antioxidiant properties of melanin are linked to its anti-inflammatory, anti-tumour and gastroprotective effects, as demonstrated in several articles (Furukawa et al., 2009; Golyshkin et al., 2015; Permyakova et al., 2016; Belemets et al., 2017). Taking into account that melanin doesn’t elevate but reduces arginase activity, which was decreased in obese animals, it can be assumed that it cannot stimulate the ability of macrophages to polarize towards the pro-inflammatory phenotype that was suppressed in obesity. Probably, it causes an anti-inflammatory effect via the suppression of NO production. This effect requires further examination. Our assumption is consistent with the reported studies, that is, endogenous melanin level increases in adipose tissue of obese patients (Page et al., 2011). It is suggested that this pigment has antioxidiant and anti-inflammatory properties: it can scaveng free radicals, thus reducing the oxidative stress and alleviating inflammation.

Taken together, the data from the present study have shown that the beneficial effect of melanin on hormonal obesity is implemented through the suppression of inflammatory processes in an organism, in particular by the prevention of macrophages’ activation. On this basis, melanin may have the potential to improve inflammatory and metabolic conditions in obesity which develops due to progesterone use in menopausal therapy or contraception.

Conclusions

The results of the present study suggest that the 28-day treatment of rats with progesterone causes obesity, as evidenced by the 27% increase in body weight. This is accompanied by the polarization of peritoneal macrophages towards the M1 pro-inflammatory phenotype: the production of NO is increased in contrast to ROS generation, which doesn’t change. M2 polarization of macrophages towards the M2 anti-inflammatory phenotype is suppressed: arginase activity decreases. Thus, progesterone-induced weight gain due to visceral fat accumulation causes inflammatory status formation that affects peritoneal cavity macrophages.

One month after progesterone withdrawal, weight gain remains increased, and the consequences of the inflammatory process are aggravated leading to depletion of functional capacity of macrophages. Their ability to polarize is suppressed, as reflected in the decrease of the studied M1 and M2 markers: NO and ROS production and arginase activity, respectively. It can be assumed that the inflammatory process caused by obesity starts moving from fat tissue to other tissues and organs and those changes are irreversible. The findings are aligned with the data from other obesity models regarding the development of a systemic inflammatory process in response to local inflammation in fat tissue.

Melanin from Antarctic black yeast N. nigro prevents the aggravation of progesterone-induced obesity in rats, impeding the weight gain and exhibits anti-inflammatory properties via lowering of NO and ROS production. Further studies are needed to establish the possibility of using melanin to mitigate the consequences of obesity.

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