INTRODUCTION

The prevalence of obesity has reached pandemic proportions and is a major risk for several metabolic and cardiovascular diseases. It is believed that this phenomenon is related to an increased energy intake and reduction of physical activity, leading to diminished insulin sensitivity, the reduced skeletal muscle mass and an increase of visceral adipose tissue and epididymal fat (1-4). Chronic low-grade inflammation, accompanied by proinflammatory macrophages infiltration in white adipose tissue (WAT), has been closely correlated with obesity and its comorbidities (5-8). Adipose tissue, though historically considered as inert energy storage, has been now recognized as an endocrine organ due to secretion of an array of signaling molecules called adipokines (9, 10). Adipokines are also involved in the regulation of inflammatory responses and the dysregulation of adipokines secretion might lead to the development of an excess of adiposity and the decrease of insulin sensitivity (9-12). On the other hand, it is generally accepted that regular, moderate physical activity is inversely correlated with systemic low-grade inflammation supporting the notion that protective effects of exercise in patients with chronic diseases could be attributed to the exercise-induced an anti-inflammatory action (13, 14).

These protective effects of exercise could be due to the secretion of muscle-derived peptides, so-called 'myokines' released by contracting skeletal muscles, and exerting a direct anti-inflammatory effects, and/or specific effects on adipose tissue (3, 15, 16). Of particular interest is this myokine ability to modify adipose tissue metabolism and its contribution to a cross-talk of signals from skeletal muscle and adipose tissue. It was suggested that skeletal muscle myokines could be of therapeutic value in chronic diseases in which the exercise has been demonstrated to exhibit a beneficial effect like obesity, type 2 diabetes, hypertension and others (3, 15). The particularly promising therapeutic target seems the pathways activated by irisin, the myokine identified by Bostrom et al. (17). Irisin is a peptide released into the circulation by cleavage of fibronectin type III domain containing protein 5 (FNDC5), a membrane-bound protein in skeletal muscle and released in response to exercise or muscle shivering, to cause browning and to regulate the thermogenesis within white adipose tissue (17-19).
In skeletal muscle FNDC5 expression strictly depends upon an increased level of PGC-1 peroxisome proliferator-activated receptor gamma (PPARY) which is known to regulates genes in response to nutritional and physiological cues especially to a physical exercise (20).

It is of the interest that the WAT is not only a target for irisin action, but also it can be a source of FNDC5 which is upregulated after exercise (21-24). Recent studies questioned the existence of irisin in humans (25) but Jedrychowski et al. (26) using quantitative mass spectrometry confirmed the presence of human irisin and its release by exercise.

Since irisin exerts a beneficial protective effect in several tissues, it is believed that irisin possesses also anti-inflammatory properties, however anti-inflammatory activities of this peptide are relatively little studied. In some studies anti-inflammatory action of exercise was associated with increase of plasma irisin and suggesting that this peptide might be implicated in this action (27-30). In recent study irisin was shown to decrease the expression of inflammatory markers by peritoneal macrophages and stimulate macrophage shift from M1 to M2 types (31). Irisin was also reported to suppress proinflammatory activation of macrophages (32, 33) as well as NFkB activation in malignant breast epithelial cells (34).

While irisin metabolic effect on white adipose tissue has been well studied and some anti-inflammatory actions have been shown in other tissues, to our best knowledge this is the first attempt to characterize the anti-inflammatory effects of irisin in adipocytes, the main non immunocompetent components of adipose tissue, which plays a crucial role in pro-inflammatory activation of macrophages in the obesity state.

**MATERIALS AND METHODS**

**Chemicals and materials**

DMEM medium, antibiotics (streptomycin and penicillin), fetal bovine serum (FBS), and accutase were from PAA (Pasching, Austria). Lipopolysaccharide (LPS), calf serum, 2',7'-dichlorodihydrofluorescein diacetate (DCFDA), β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO). Annexin V kit was purchased from BD Biosciences Pharmingen (San Diego, CA, USA). RNeasy Plus Mini Kit (74134) for RNA isolation was obtained from Qiagen (Hilden, Germany). High Capacity RNA-to-cDNA Kit (4387406) for reverse transcription and TaqMan Gene Expression Master Mix (4369026) for real time PCR were provided by Applied Biosystems (ThermoFisher; Foster City, CA, USA). Phospho-NFkB p65 and total NFkB p65 and secondary antibodies were from Cell Signaling Technology (Beverly, MA, USA). ELISA kits for TNF-α, IL-6 and leptin were supplied by IBL International (Hamburg, Germany). ELISA kits for mouse MCP-1 was purchased from R&D BioVendor.

**Cell culture**

The study was conducted in the Mycoplasma-free mouse preadipocytes 3T3 L1 cell line differentiated to adipocytes. Cell line was kindly provided by Professor Alicja Jozkowicz from the Department of Medical Biology, Jagiellonian University. For the differentiation assessment cells were grown until confluence in DMEM medium supplemented with 1%
antibiotic solution and 10% calf serum. After additional 2 days of culture the growth medium was replaced with differentiation medium for next 2 days (DMEM medium with 10% fetal bovine serum, 0.5 mM isobutylmethylxanthine - IBMX, 0.25 µM dexamethasone). After that period of time the cells were incubated in a DMEM medium containing 10% FBS and 1 µg/ml insulin until the end of 3-week differentiation process. Oil-Red-O staining was used for evaluation of adipocyte differentiation. Then adipocytes were co-cultured with graded concentrations of irisin (0, 10, 50 or 100 nM) for 3 weeks labeled as IR0, IR10, IR50 and IR100 groups, respectively. In order to induce a state of mild inflammation at day 21 cells were stimulated with LPS (100 ng/ml; Escherichia coli, serotype 0111: B4) or vehicle (medium). During incubation 3T3 L1 cells were cultured under standard condition (37°C, 5% CO₂). Fresh cells were used for cytometric assessment and RNA isolation. Supernatants and cell pellets were frozen (–60°C) for future quantification of protein levels.

Cell activity and viability

Overall cell viability and activity was measured using a commercial CellTiter kit (Promega). Moreover, the percent of apoptotic and necrotic cells were quantified cytometrically using an Annexin V-PE Apoptosis Detection Kit I (BD Pharmingen). Both tests were performed according to the manufacturer's instruction using a spectrophotometer (Expert Plus, ASYS/Hitech; for CellTiter), or a FACScan cytometer (BD Biosciences; for apoptosis test).

Real-time PCR analysis of gene expression

Total RNA was purified from the 3T3 L1 adipocytes cultured in four separate repetitions for each variant of the experiment. RNeasy Plus Mini Kit with elimination of genomic DNA was used to RNA extraction. The RNA concentration and quality was assessed using NanoDrop 2000 spectrophotometer (Thermo Scientific). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit. Real-time PCR gene expression analysis for 7 genes (TNF-α, IL-6, adiponectin, leptin, PPARγ, NFκB, MCP-1) was performed using Applied Biosystems StepOne system and Taq Man primers. Glucose-6-phosphate dehydrogenase (GAPDH) was used for normalizing the amounts of cDNA. Gene expression changes in the presence of three different doses of irisin were calculated based on 2^ΔΔCt algorithm (35).

Western blotting assay

To the analysis of total pool of NFκB and its phosphorylated form as well as PPARγ proteins were extracted from adipocyte pellets according to the manufacturer's protocol. The protein concentration was quantified using the BCA protein assay kit. Then the samples were subjected to the SDS-PAGE gels (10 –
15%), and after electrophoretic separation were transferred onto the PVDF membrane. The nonspecific binding sites were blocked with 5% dried milk in TBSS for 1 h (37°C) and the membranes were incubated overnight with primary antibodies (4°C; 1:1000), and then with secondary antibodies conjugated with horseradish peroxidase (HRP; 1:5000) for 2 hours at room temperature. After reaction with DAB, membranes were imaged and the relative protein expression was normalized to β-actin expression used as the internal reference.

Macrophage migration

To assess the effect of irisin on a release of chemotactic factors by activated adipocytes, a 48-well microchemotaxis chamber (Neuro Probe) and RAW 264.7 macrophage cell line were used. The test was performed according to the manufacturer's protocol. Briefly, the lower wells of chamber were filled with the supernatants collected from 3T3 L1 adipocytes (cultured in the presence of various irisin concentrations: 0; 10; 50 or 100 nM) 24 hours after LPS stimulation, control supernatants, fMLP as a positive control, or DMEM medium as a negative control. The lower wells of chamber were covered with polycarbonate membrane (3 µm pore size; Neuro Probe). The upper wells of chamber were filled with 50 µl of RAW 264.7 macrophages suspension (2 mln/ml) and incubated for 45 min in standard culture condition. After that, the membrane was washed in PBS and stained with Diff-Quick solution (Medion Diagnostic). The macrophages that had migrated to the lower side of membrane were counted in four microscopic fields in each well. The results are expressed as the mean numbers of migratory macrophages per well.

Determination of cytokine/chemokine release

After 24-hours incubation with or without LPS the supernatants were collected and the levels of cytokine were quantified using commercial Elisa Kit. The concentration level of TNF-α, IL-6, MCP-1, adiponectin and leptin was determined according to the manufacturer's instructions and analyzed using Expert Plus spectrophotometer (ASYS/Hitech, Austria).

Statistical analysis

Data were tested for normality of the distribution, and differences among groups were determined using the Duncan's new multiple range test 3.1. All data were expressed as means ± standard deviation (S.D.) with the level of statistical significance (P) set at 0.05.

RESULTS

The influence of irisin on the inflammatory activity of adipocytes was evaluated in LPS-activated 3T3 L1 cells cultured in the presence of three different concentrations of irisin (10 nM, 50 nM and 100 nM) in relation to the fat cells not-treated by

![Fig. 3. The expression of mRNA and secreted protein levels for adiponectin (A) and leptin (B). Adipocytes were differentiated from 3T3 L1 cell line as described in the Section of Materials and Methods and then cultured for 3 weeks in the presence of 10 nM (IR10), 50 nM (IR50) or 100 nM (IR100) irisin. After LPS stimulation, adipocytes were harvested to evaluate the amount of adiponectin and leptin mRNA transcripts (6 h, real time PCR) and both adipokines concentration was measured in cell culture medium (24 h, ELISA). Values are means ± S.D. of 4 - 5 independent experiments. Asterisk (*, protein) and cross (+, mRNA) indicate a significant change in mRNA and protein concentration as compared with non-treated (IR0) and irisin (IR10) -treated cells at P< 0.05 by Duncan's test. CTR, control (non-activated adipocytes); IR, irisin; LPS, lipopolysaccharide.](image-url)
Irisin (IR0). As the control to adipocyte activation, the 3T3 L1 cells were treated with medium instead of LPS.

The effects of irisin on adipocyte viability and activity

To assess whether irisin affects the overall activity of the adipocytes as well as their viability first we performed the study using a CellTiter test. As shown in Fig. 1A, the incubation of adipocytes with irisin induced an intensification of general cell activity in a dose dependent manner. The adipocyte activity was significantly elevated in both IR50 and IR100 groups (P > 0.05) as compared to IR0 control cells. This effect was seen in both LPS-stimulated and non-stimulated adipocytes. Furthermore, to assess whether the increased activity is related to cell viability, we performed an examination of irisin impact on apoptosis and necrosis induction. As shown in Fig. 1B, the highest irisin concentration (IR100) efficiently inhibited LPS-induced apoptosis resulting in an increase of lipid droplets accumulation in these adipocytes (P > 0.05).

Irisin reduces expression and secretion of pro-inflammatory cytokines

The expression and secretion of two pro-inflammatory cytokines (TNF-α and IL-6) was assessed in irisin-treated with two adipocytes compared to irisin-free culture of adipocytes by the real time PCR and cytokine protein secretion to the culture medium using enzyme-linked immunosorbent assay (ELISA).

The significant reduction of TNF-α and IL-6 mRNA expression was observed in 3T3 L1 cells treated by the highest concentrations of irisin (50 nM and 100 nM) in comparison to adipocytes treated with vehicle (saline). The concentration of TNF-α and IL-6 quantified in supernatants were also significantly reduced when adipocytes were growing in the presence of 50 nM and 100 nM concentrations of irisin (P < 0.05) (Fig. 2A and 2B).

Effects of irisin on adiponectin and leptin mRNA expression and protein secretion

Figs. 3A and 3B shows the expression of two major adipokines: adiponectin and leptin in adipocytes cultured for 3 weeks in the presence of the three different doses of irisin. The activation of adipocytes by LPS significantly reduced adiponectin mRNA and protein expression compared to CTR but irisin significantly enhanced these expressions nearly to the level observed in vehicle - control adipocytes (Fig. 3A). In the case of leptin expression, we have observed an opposite effect because the activation of adipocytes by LPS significantly increased mRNA and protein for leptin but irisin administration markedly decreased its expression both, at mRNA as well as protein levels resulting comparable value in the presence of 100 nM irisin as in control (CTR) LPS-untreated cells (Fig. 3B).

PPARγ synthesis is elevated in irisin-treated adipocytes

The co-culture of adipocytes with irisin in a presence of LPS dose-dependently increased the PPARγ mRNA and protein expression. The statistically significant differences in expression of PPARγ mRNA and protein were observed when irisin was applied in concentration of 50 nM and 100 nM (Fig. 4A). Fig. 4B shows the representative Western blot of PPARγ protein expression in response to graded concentrations of irisin in the presence of LPS. As shown in Fig. 4B the expression of PPARγ protein was increased in a concentration-dependent manner in adipocytes co-incubated with irisin.

Fig. 4. The PPARγ mRNA and protein expression (A) and representative Western blot of PPAR protein expression (B) in irisin-treated adipocytes exposed to LPS (100 ng/ml). Adipocytes were differentiated from 3T3 L1 cell line as described in the Section of Materials and Methods and then co-cultured in the presence of 10 nM (IR10), 50 nM (IR50) or 100 nM (IR100) irisin for 3 weeks. After LPS stimulation, adipocytes were harvested to evaluate the amount of mRNA transcript (6 h, real time PCR) or the protein level (24 h, Western blotting). Values are means ± S.D. of 5 independent experiments. Asterisk (*, protein) and cross (+, mRNA) indicate a significant change in mRNA and protein concentration as compared with non-treated (IR0) and irisin-treated cells at P < 0.05 by Duncan’s test. CTR, control (non-activated adipocytes). IR, irisin; LPS, lipopolysaccharide, PPARγ; peroxisome proliferator-activated receptor gamma.
**DISCUSSION**

In this study, we demonstrated that irisin exerts the marked anti-inflammatory activity in the lipopolysaccharide-activated adipocytes.

Regular physical activity has a significant role in the modulation of immune and anti-inflammatory functions presumably due to myokines released from contracting skeletal muscle (3). Nowadays, irisin is considered as myokine of particular interest, released in response to exercise and muscle shivering. Irisin exerts its action on white adipose cells causing their transformation into so called ‘bright’ cells - white fat cells with a phenotype similar to that of brown fat cells. Moreover, irisin has been implicated in process of induction of thermogenesis and increased energy expenditure (18). Huh et al. (36) demonstrated that, irisin induced UCP1 and consequently increased adipocyte energy expenditure, the expression of metabolic enzymes and metabolite intermediates, caused an inhibition of lipid accumulation in human adipocytes. They also observed that irisin and FNDC5 reduced preadipocyte differentiation, which can be an additional mechanism in decreasing adiposity (36). Irisin has been shown to reduce high-fat-diet induced insulin resistance through its action on WAT and increased energy expenditure (37, 38). Liu et al. (39) observed that T2D patients had lower plasma irisin level compared to non-diabetic controls. The possible therapeutic role has been suggested for irisin in human metabolic disease, obesity and other disorders to which the exercise seems to be beneficial (40, 41).

The results of recent study (42) have suggested that obesity represents a low-grade chronic inflammation in WAT manifested by hypertrophied adipocytes and increased number of adipose tissue macrophages (ATM). This inflammation state is characterized by the activation of pro-inflammatory signaling pathways and the increased pro-inflammatory cytokine production (42). Thus, adipocytes are considered since now as members of the immune system, because they were shown to...
produce and release of several pro-inflammatory cytokines and chemokines such as IL-6, TNF-α, and MCP-1 in addition to adipokines (43, 44). In obesity, the inflammatory molecules may originate not only from adipocytes but also from infiltrated macrophages. This proinflammatory mediators can exhibit local effect on adipose tissue and also systemic effects on other organs as well as they contribute to the insulin resistance. Macrophages’ infiltration in white adipose tissue is associated with obesity causing a phenotypic switch in these cells from an anti-inflammatory M2 to a pro-inflammatory M1 state (45).

Our results show that irisin reduced expression of pro-inflammatory cytokines, TNF-α and IL-6 in LPS-activated adipocytes and this effect depend upon concentration of this peptide. The similar effect was observed in LPS-activated peritoneal macrophages (32, 33) because of irisin not only decreased the expression of TNF-α and IL-6 but also stimulated macrophage polarization from M1 into M2 response (31).

In line with these inhibitory effects of irisin on pro-inflammatory cytokines, this peptide has been shown to exert an opposite effect on adiponectin and leptin expression in LPS-treated adipocytes. While LPS-activation leads to reduced adiponectin and increased leptin expression in adipocytes, irisin was shown in our study to reverse this effect resulting in an increase in adiponectin and an apparent fall in leptin expression. Leptin, one of the first proteins shown to be secreted from adipose tissue, exhibits pro-inflammatory properties and the concentration of this adipokine is increased in obese subjects (46). Adiponectin is believed to act as the main anti-inflammatory mediator produced in and released by adipose tissue. In contrast to leptin, the expression and plasma levels of adiponectin, the anti-inflammatory adipokine, are down-regulated in obesity (46).

Recently, TNF-α has been shown to reciprocally increase the expression of TNF-α and IL-6 and inhibited the expression of adiponectin in adipocytes, these effects mediated by the activation of the cellular transcription factor NFκB (47). It is interesting to note, that in our present study, irisin exerted a suppressive effect on expression of NFκB in LPS-activated adipocytes. This observation strongly supports the notion that irisin exerts an anti-inflammatory action, potentially counteracting inflammatory cytokines including TNF-α induced activation of NFκB pathway. Our findings are consistent with previous studies showing that irisin suppressed NFκB activation in macrophages (33) and in malignant breast epithelial cells (34).

Our present study provides an evidence that MCP-1 expression was downregulated in irisin-treated adipocytes followed by the reduced macrophage migration in the presence of irisin. Adipocytes were shown to release MCP-1, Fig. 6. The mRNA and protein expression of MCP-1 in adipocytes exposed to LPS (100 ng/ml) (A) and the macrophage migration ability (B) are modified by irisin action. Adipocytes were differentiated from 3T3 L1 cell line as described in the section Materials and Methods and then co-cultured in the presence of 10 nM (IR10), 50 nM (IR50) or 100 nM (IR100) irisin for 3 weeks. After LPS stimulation, adipocytes were harvested to evaluate the amount of mRNA transcript (6 h, real time PCR) or cytokine concentration was measured in cell culture medium (24 h, ELISA). Macrophages RAW 264.7 migration to conditioned medium collected from adipocytes cultured in the presence of various concentrations of irisin for 24 hours. Normal medium was used as a negative control (CTR-), fMLP was used as a positive control (CTR+). Values are means ± S.D. of 4 – 5 independent experiments. Asterisk (*, protein and migration) and cross (+, mRNA) indicate a significant change as compared with non-treated (IR0) and irisin-treated cells (IR10) at P < 0.05 by Duncan’s test. CTR, control (non-activated adipocytes); IR, irisin; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein 1.
chemoattractant molecule which is implicated in macrophage infiltration of WAT (48). Previous studies revealed that when overexpressing of MCP-1, the WAT leads to an accumulation of macrophages in adipose tissue, an event associated with a development of metabolic disorders (6, 49, 50). The MCP-1 protein levels were significantly higher in the obese mice than those in the lean controls and the concentration of MCP-1 in these mice far exceeded that determined in mesenteric adipose tissue of lean mice (51). This reduction in MCP-1 expression in adipocytes co-incubated with irisin observed in our study can significantly contribute to the anti-inflammatory action of irisin and supports the protective activity of this peptide released from skeletal muscle during exercise especially in obese individuals. This local anti-inflammatory activity of irisin is in keeping with recent observation that this peptide is not associated with depressiveness, anxiety and perceived stress in female obese patients ruling out the assumption of irisin being involved in mental functions and psychoendocrine pathways (52).

In conclusion, we confirmed the anti-inflammatory activity of irisin in adipocytes. We demonstrated for the first time that irisin can directly attenuate the inflammation process in cultured adipocytes, in addition to inhibition of migratory activity of macrophages the action to this myozine that has been shown before.

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