INTRODUCTION

The macrophages are considered as an important component of innate immunity and the source of reactive oxygen species (ROS) and oxidative stress at the site of inflammation (1, 2). Oxidative burst in activated phagocytes is known to protect the body against invading microorganisms and these cells are considered as one of the first lines of organism defense system (3). The excessive ROS formation has been proposed to trigger the inflammatory signals responsible for the cell death due to the oxidative damage of cell macromolecules (4, 5). The ROS were postulated to evoke the inflammatory response via activation of transcription factors including nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) signaling, both involved in the regulation of expression of pro-inflammatory genes (6). The activation of NF-κB by the co-incubation of RAW 264.7 macrophages with lipopolysaccharide (LPS) has been shown to accelerate the expression of pro-inflammatory factors such as superoxide dismutase 1 (SOD-1), superoxide dismutase 2 (SOD-2), glutathione peroxidase (GSH-Px), catalase 9 (Cat-9), nuclear factor (erythroid-derived 2)-like 2-related factor (Nrf2) and heme oxygenase-1 (HO-1) were evaluated. We found that irisin applied in a concentration of 50 nM significantly attenuated the production of harmful H_2O_2 and this effect appears to be mediated by a significant increase in the expression of key enzymes involved with antioxidative stress pathways including SOD, GSH-Px and Cat-9 predominantly observed after stimulation of these cells with LPS. We conclude that 1) irisin exhibits a potent antioxidant and anti-inflammatory activities in non-stimulated and LPS-stimulated isolated murine macrophages in vitro, and 2) this protective and antioxidative activity of irisin in vitro might be considered as an important component of protective action of this peptide in vivo, especially under condition of exercise.

Irisin is a recently discovered myokine reported as protective protein released from exercising skeletal muscles. Although myokines were recently reported to possess the antioxidizing properties, the impact of irisin on the functions of macrophages with respect to its anti-inflammatory potential has not been fully elucidated. Here, we determined the ability of irisin to interact with reactive oxygen species (ROS) in RAW 264.7 murine macrophages. The macrophages were pre-incubated with irisin (0 – 50 nM), some of which had undergone additional co-incubation with bacterial lipopolysaccharide (LPS) (100 ng/ml). Cell viability, the reactive oxygen species scavenging potential as well as the mRNA and protein expression of key oxidative stress factors such as superoxide dismutase 1 (SOD-1), superoxide dismutase 2 (SOD-2), glutathione peroxidase (GSH-Px), catalase 9 (Cat-9), nuclear factor (erythroid-derived 2)-like 2-related factor (Nrf2) and heme oxygenase-1 (HO-1) were evaluated. We found that irisin applied in a concentration of 50 nM significantly attenuated the production of harmful H_2O_2 and this effect appears to be mediated by a significant increase in the expression of key enzymes involved with antioxidative stress pathways including SOD, GSH-Px and Cat-9 predominantly observed after stimulation of these cells with LPS. We conclude that 1) irisin exhibits a potent antioxidant and anti-inflammatory activities in non-stimulated and LPS-stimulated isolated murine macrophages in vitro, and 2) this protective and antioxidative activity of irisin in vitro might be considered as an important component of protective action of this peptide in vivo, especially under condition of exercise.

Key words: macrophages, irisin, fibronectin type III domain containing 5, superoxide dismutase 1, superoxide dismutase 2, heme oxygenase-1, reactive oxygen species, nuclear factor (erythroid-derived 2)-like 2-related factor, inflammation, obesity

INTRODUCTION

The macrophages are considered as an important component of innate immunity and the source of reactive oxygen species (ROS) and oxidative stress at the site of inflammation (1, 2). Oxidative burst in activated phagocytes is known to protect the body against invading microorganisms and these cells are considered as one of the first lines of organism defense system (3). The excessive ROS formation has been proposed to trigger the inflammatory signals responsible for the cell death due to the oxidative damage of cell macromolecules (4, 5). The ROS were postulated to evoke the inflammatory response via activation of transcription factors including nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) signaling, both involved in the regulation of expression of pro-inflammatory genes (6). The activation of NF-κB by the co-incubation of RAW 264.7 macrophages with lipopolysaccharide (LPS) has been shown to accelerate the expression of pro-inflammatory factors such as macrophage inflammatory protein 2 (MIP-2) and interleukin-2 (IL-2) (7). Such a ROS-dependent neutralization of pathogens by macrophages constitutes a natural element of the immune defense system (8), while chronic inflammation accompanied by oxidative stress has been implicated in the pathogenesis of chronic disorders including chronic lung disease, the chronic renal failure and diabetes (9-12). The adipose tissue exposed to prolonged oxidative stress has shown an elevated level of glucose (13) and diabetes-induced by a high-fat diet (14). Inflammation processes are controlled not only by immune-derived molecules but, as shown in recent studies, also by adipokines and myokines (15). Interleukin-6 (IL-6), a myokine released by skeletal muscles, impairs the pro-inflammatory activity by decreasing the production of tumor necrosis factor alpha (TNF-α) (16). Adiponectin has also been shown to influence the level of TNF-α, as demonstrated in adiponectin-knockout mice (17). Moreover, a recent study has demonstrated that adiponectin could alleviate oxidative stress in mice fed a high-fat diet (18). Among adipomyokines, irisin is a soluble protein secreted by cleavage of a type 1 membrane protein named fibronectin type III domain containing 5 (FNDC5) as a result of e.g. physical exercise (19-21). The skeletal muscle secretion of irisin depends on post-transcriptional co-activator PGC1α (22), however, irisin can be also released from white...
adipose tissue, and this process seems to be PGC1α-independent (20). Irisin is considered to act as beneficial for metabolic health, mainly, because of its ability to induce browning of white adipose tissue and increase energy expenditure (20, 21).

The high blood irisin level improves glucose tolerance of mice with insulin resistance fed a high-fat diet (22, 23) as well as severity of intestinal damage in mice with colitis (24) or acute lung inflammation (25). Moreover, in some chronic diseases such as diabetes (26) or chronic renal failure (27), the plasma level of irisin is decreased when compared to healthy individuals, thus, an important role of this myokine in the maintenance of human being health status has been proposed.

Irisin can play a regulatory role in an immune system activity. A recent study has shown that irisin decreased the expression of IL-6, TNF-α, and MIP1α and MIP1β inflammatory markers in LPS-activated macrophages. These irisin-related alterations of inflammatory cytokines accompany the change of macrophage polarization from M1 to M2 (28). Our previous results revealed that irisin affects the macrophage inflammatory response by enhancing phagocytosis and reducing respiratory burst (29). The irisin-induced anti-inflammatory activity is mediated by downregulation of TLR4/MyD88 pathways and results in a reduction of pro-inflammatory release such as TNF-α, IL-1α, IL-6 or MCP-1 (30). The present study was designed to expand the knowledge of the effects of irisin on inflammatory environment generated by macrophages, with focus to the insight into the mechanism of irisin exhibiting an anti-inflammatory and antioxidizing activities in vitro conditions.

MATERIALS AND METHODS

Cell line and reagents

A murine macrophage RAW 264.7 cell line was obtained from the European Type Culture Collection (Sigma). DMEM medium, fetal bovine serum (FBS) and antibiotics were sourced from Sigma Aldrich. An ROS-Glo™ H2O2 Cell-Based Assay medium, fetal bovine serum (FBS) and antibiotics were supplied by Thermo Fisher Scientific (Rockford, IL, USA). Horse anti-mouse (7076S) HRP-conjugated IgG was purchased from Cell Signaling Technology (Danvers, MA, USA). Human recombinant irisin (fibronectin type III domain, FND5C, 11451) was ordered from the Cayman Chemical Company (Ann Arbor, MI, USA). Lipopolysaccharide (LPS, Escherichia coli, serotype O111:B4, L2630) and a Total Protein Kit (Micro Lowry, Peterson’s Modification (TP0300)) were supplied by Bio-Rad (Hercules, CA, USA). WesternBright Sirius Western Blotting HRP Substrate was ordered from Advansta (Menlo Park, CA, USA).

Cell culturing and cell lysate preparation

The cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a CO2 humidified incubator (Lab-Line Instruments). Regular testing confirmed that the cells were free from mycoplasma contamination. Cells were treated with irisin in a concentration range between 0 – 50 nM for 2 or 24 h and then part of the cells were stimulated overnight with LPS (100 ng/ml). Cells were harvested upon 80% confluency by scraping. For Western blotting analysis, the cells were lysed using a Nuclear and Cytoplasmic Extraction Reagent Kit containing protease inhibitors according the manufacturer’s instructions. The lysates were clarified by centrifugation (1500 rpm) for 10 min at 4°C. Protein concentration was determined in cell extracts using a Total Protein Kit (Micro Lowry, Peterson’s Modification (TP0300)) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) was purchased from Cell Signaling Technology (Danvers, MA, USA).

Real-time PCR for quantitative assessment of gene expression

Quantitative analysis of superoxide dismutase 1 (SOD-1) and superoxide dismutase 2 (SOD-2), as well as hemoxygenase 1 (HO-1), glutathione peroxidase (GSH-Px), nuclear factor (erythroid-derived 2)-like 2-related factor (Nrf2) and catalase 9 (Cat-9) gene expression, was performed using SybrGreen in Real time PCR. RNA was prepared using an RNaseasy Protet Kit (Micro Lowry, Peterson’s Modification) containing protease inhibitors according the manufacturer’s instructions. The lysates were stored at –60°C until use.

Table 1. Primer sequences.

<table>
<thead>
<tr>
<th>gene name</th>
<th>primer forward (5’-3’)</th>
<th>primer reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD-1</td>
<td>CACGTCTTACATGATGCTGGG</td>
<td>CTGCTTCCTTCAGCAGTCACA</td>
</tr>
<tr>
<td>SOD-2</td>
<td>GCTGTCTGGGAGTTCAAAGG</td>
<td>CCCAGTGCAATTGCTGCA</td>
</tr>
<tr>
<td>HO-1</td>
<td>CACGCAATATACCCGGCTACCT</td>
<td>CCAGGTGGTTACCCAGGAGA</td>
</tr>
<tr>
<td>GSH-Px</td>
<td>ACAGTCCACCGTATGCTCTTC</td>
<td>CTCCTCTTCTTGCACATTTCTC</td>
</tr>
<tr>
<td>Nrf2</td>
<td>AACAGAAGGCGCCTAAAGGCA</td>
<td>TGAGCTGCACTAGGAGAC</td>
</tr>
<tr>
<td>Cat-9</td>
<td>AACACATGTCACTCAGTGCGGA</td>
<td>GGGATGTTCTTCACACAGGGTCC</td>
</tr>
<tr>
<td>Actb</td>
<td>CCCATCTATGAGGTTACGC</td>
<td>TTAATGTCACGCAGATTTC</td>
</tr>
</tbody>
</table>

SOD-1, superoxide dismutase 1; SOD-2, superoxide dismutase 2; HO-1, hemoxygenase 1; GSH-Px, glutathione peroxidase; Nrf2, nuclear factor (erythroid-derived 2)-like 2-related factor; Cat-9, catalase 9; Actb, β-actin.
Cell-based assays

Cells were seeded into 96-well plates at a density of 100,000 cells per well in 100 µl DMEM and treated with irisin for 2 or 24 hours. The next day, the following cell-based assays were performed. The levels of ROS were measured using the ROS-Glo™ H2O2 Cell-Based Assay according to a protocol supplied by the manufacturer. Briefly, 20 µl of H2O2 substrate solution was added to 80 µl of culture medium and incubated in standard conditions (37°C, 5% CO2 incubator) for 6 hours. Then, ROS-Glo™ Detection Solution was added and after 20-min incubation at RT the luminescent reaction was measured in an Infinite M200 microplate reader (Tecan, Switzerland) at a value of integration time (IT) of 250 ms. The ratio of reduced to oxidized glutathione (GSH/GSSG) was calculated based on the measurement of both glutathione forms using the GSH/GSSG-Glo™ Assay according to the manufacturer’s instructions. Each experimental variant was duplicated on multiwell plates to assess both GSH and GSSG levels. All procedure steps were performed at RT. After removal of culture medium, the cells were lysed for 5 min using Total and Oxidized Glutathione Reagents (50 µl per well). Then, the same volume of Luciferin Generation Reagent was added and the plates were incubated for 30 min. Finally, after the reaction with Luciferin Detection Reagent (100 µl per well, 15 min) luminescence was read in a Tecan Infinite M200 microplate reader at a 250 ms IT value and the ratio of GSH/GSSG was counted. Cell viability, cytotoxicity and apoptosis were evaluated using the ApoTox-Glo™ Triplex Assay according to the manufacturer’s protocol. Briefly, 5 µl of Viability/Cytotoxicity Reagent containing GF-AFC Substrate and bis-AAF-R110 Substrate was added to the wells and the plate was incubated for 30 min at 37°C. Fluorescence was measured using an excitation wavelength of 400 nm and emission wavelength of 505 nm in an Infinite 200 PRO microplate reader (Tecan, Switzerland) to determine cell viability. Cell cytotoxicity was evaluated by fluorescence measurements at 485 nm (excitation) and 520 nm (emission). Then to assess apoptosis, 25 µl of Caspase-Glo® 3/7 Reagent was added to the wells and the plate was incubated for 30 min at RT. Finally, the intensity of luminescence was measured in an Infinite M200 microplate reader (Tecan, Switzerland) at an IT value of 250 ms.

Statistical analysis

Data were tested for normality of distribution, and differences between groups were determined using ANOVA with Tukey post hoc analysis. All data were expressed as means and medians with standard error box ± SD with the level of statistical significance (p) set at 0.05.

RESULTS

Irisin is known to modulate pro-inflammatory action of macrophages (29, 30) as well as adipocyte (31) activities. In the present study, the impact of irisin was analyzed with respect to regulation of oxidative stress on both, the mRNA and protein levels as well as in cell-base tests that allow for direct cell activity assessment.

Irisin improves reactive oxygen species neutralization by quiescent macrophages

To assess whether irisin affects the mechanisms of ROS neutralization by macrophages, we performed an analysis of both quiescent and LPS-stimulated cells using a ROS-Glo test, which allows for the evaluation of H2O2 neutralization by the tested cells. As shown in Fig. 1, the status of macrophage activation has an impact on their capacity for ROS neutralization. The overnight LPS-stimulated macrophages showed significantly more efficient H2O2 neutralization (P < 0.001) than quiescent cells, and the level of ROS was similar regardless of the irisin concentration after the incubation period applied in this study. An apparent effect of irisin on ROS neutralization was observed also in non-stimulated macrophages. There was a statistically significant decrease in ROS content indicating the attenuation of ROS activation by this peptide, which was dependent on irisin concentration in

![Fig 1. The effect of irisin (IR) on the capacity of macrophages to scavenge ROS as determined by the cell-based ROS-Glo test. The RAW 264.7 macrophages pretreated with IR (0 – 50 nM) for 2 hours, were co-incubated overnight with or without vehicle (saline) or LPS (100 ng/ml). The results are expressed as a mean percentage vs. control group (IR0) presented as a 100% with 25 – 75 box ± SD of four to five independent experiments. Statistical significance (P < 0.05) were determined with ANOVA with Tukey post hoc analysis.](image-url)
conditional medium (P < 0.001). These results are particularly important, and are corroborative with our previous observations indicating that resting macrophages inhibited ROS when they have been pre-incubated with irisin (29). This ROS inhibitory action of irisin reported elsewhere (29) can explain, at least in part, the more efficient scavenge of ROS by irisin observed under LPS-stimulatory conditions.

To assess whether the inhibitory effect of irisin on ROS generation in macrophages is related to the prolongation of cell viability by irisin, we evaluated irisin impact on macrophage viability, cytotoxicity and caspases 3/7 activity using an ApoTox-Glo test. As shown in Fig. 2, after the termination of incubation period, the cell viability was significantly lower in LPS-stimulated macrophages than in quiescent cells, but irisin failed to significantly affect other parameters tested, i.e. cytotoxicity and caspases 3/7 activity quantified by the ApoTox-Glo test.

In macrophages co-incubated with irisin both, the total glutathione (tGSH) and its oxidized form (oGSH) tended to increase, however, these increases failed to reach statistical significance (Fig. 3). We have noticed only a tendency toward an increase in the concentration of both forms of glutathione in cells co-incubated with irisin and this tendency was more pronounced in oGSH than tGSH concentrations in cells treated with graded concentrations of irisin.

Irisin affects the gene expression of anti-oxidant enzymes superoxide dismutase 1 and 2, glutathione peroxidase and Cat-9

We evaluated whether the observed attenuation of ROS by irisin determined by ROS-Glo test, is linked with a change in the expression of key antioxidant enzymes such as superoxide dismutase 1 and 2 (SOD-1; SOD-2), glutathione peroxidase (GSH-Px) and catalase (Cat-9). As shown in Fig. 4, SOD-1 mRNA expression was significantly reduced after LPS stimulation (P < 0.001). Moreover, irisin treatment (IR5) resulted in a significant increase in SOD-1 mRNA expression in resting macrophages (P < 0.001), as well as when LPS stimulation preceded 2-hour incubation with irisin (P < 0.02). Longer irisin pre-incubation (24 h) failed to influence SOD-1 mRNA expression in stimulated macrophages.

As shown in Fig. 4B, irisin failed to significantly alter SOD-2 mRNA expression in resting macrophages but its stimulatory effect can be seen in LPS-activated macrophages because LPS activation significantly enhanced SOD-2 expression (P < 0.05). Moreover, a significant increase in SOD-2 mRNA expression was observed when LPS stimulation was preceded by 2-hour pre-incubation with irisin (P < 0.02). In the case of 24-hour pre-incubation with irisin, the opposite effect was observed because the SOD-2 mRNA was significantly lower in irisin-treated cells than in control cells (P < 0.05).

As shown in Fig. 4C, LPS stimulation significantly reduced the GSH-Px mRNA expression in control cells (IR0; P < 0.005), but this expression was altered by irisin treatment. The mRNA expression of GSH-Px was significantly lower in irisin-treated quiescent macrophages than in control cells (P < 0.05). Moreover, long term pre-incubation with irisin (24 h) intensified GSH-Px mRNA expression observed after LPS stimulation (P < 0.05). Shorter irisin pre-incubation (24 h) remained without a significant effect on GSH-Px mRNA expression in stimulated macrophages.

The analysis of the expression of CAT-9 mRNA (Fig. 4D), an enzyme responsible for the degradation of H₂O₂ to H₂O and O₂⁻, revealed a significant decrease in CAT-9 mRNA after LPS-stimulation in IR0 cells. Moreover, the increase in CAT-9 mRNA was observed when LPS-stimulated macrophages were co-incubated with irisin for 2 hours for 24 hours prior to the activation with LPS (P < 0.01). Irisin insignificantly affected the CAT-9 mRNA after shorter (2 hours) co-incubation with the macrophage cell line.

Fig. 2. Irisin impact on macrophages viability, cytotoxicity and caspase 3/7 activity assessed by the ApoTox-Glo test. The macrophage RAW 264.7 cell line pretreated with irisin (0 – 50 nM) for 2 hours, were overnight co-incubated with and without vehicle (saline) or LPS (100 ng/ml); (A) macrophage cell viability, (B) cytotoxicity level, and (C) caspase 3/7 activity. All results are expressed as the mean percentage vs. control group (IR0) presented as 100% with 25 – 75 box ± SD of four to five independent experiments. Statistical significances (P < 0.05) were determined with ANOVA with Tukey post hoc analysis.
Fig. 3. The effects of irisin (IR) applied in gradually increased concentrations (0 – 50 nM) on the total and oxidized glutathione status measured using the GSH/GSSG-Glo™ assay. The macrophages RAW 264.7 pretreated with irisin (0 – 50 nM) for 2 hours, were overnight cultured with and without vehicle (saline) and LPS (100 ng/ml); (A) total glutathione, (B) oxidized form of glutathione. The results are expressed as a mean percentage vs. control group (IR0) presented as 100% with 25 – 75 box ± SD of four to five independent experiments. Statistical significances (P < 0.05) were determined with ANOVA with Tukey post hoc analysis.

Fig. 4. Irisin effects on the gene expression of crucial anti-oxidant enzymes. Macrophages RAW 264.7, pretreated with irisin (0 – 5 and 25 – 50 nM) for 2 or 24 hours, were cultured with or without LPS (100 ng/ml) for an additional 6 h: (A) superoxide dismutase 1 (SOD-1) expression, (B) superoxide dismutase 2 (SOD-2) expression, (C) glutathione peroxidase (GHS-Px) expression, and (D) catalase 9 (Cat-9) expression. The results are expressed as a mean percentage vs. control group (IR0) presented as 100% with 25 – 75 box ± SD of four to five independent experiments. Statistical significances (P < 0.05) were determined with ANOVA with Tukey post hoc analysis.
Irisin affects Nrf2 and heme oxygenase-1 (HO-1) expression

The expression of Nrf2 and HO-1 was measured at both mRNA as well as protein levels. As presented in Fig. 5A, both the duration time of incubation with irisin and the status of cell activation have influenced the action of this peptide on macrophages. We observed the opposite effects of irisin on stimulated and non-stimulated macrophages. In quiescent cells pre-incubated with a high irisin concentration (50 nM), the significant reduction of the mRNA for Nrf2 was observed (Fig. 5A), while when irisin preceded LPS stimulation, the alterations in Nrf2 mRNA level were also observed. Analysis of nuclear and cytoplasmic proteins indicated that irisin treatment significantly improved the Nrf2 translocation to the nucleus in quiescent IR50: P < 0.02 as well as in LPS-stimulated macrophages which had been pre-incubated with irisin for 2 hours (Fig. 5B).

In the case of HO-1 expression, short-term pre-incubation with irisin (2 h) prior to LPS stimulation resulted in a significant increase in HO-1 mRNA expression (P < 0.02). The longer 24-hour pre-incubation with irisin resulted in a significant increase in expression only for application of the highest dose of irisin (50 nM; P < 0.02). In non-stimulated macrophages, the HO-1 expression was significantly increased (P < 0.05) after the co-incubation with irisin, starting from the dose of 25 mg/ml of this peptide (Fig. 5C). The upward trend in HO-1 expression upon irisin treatment was observed with a significant increase after IR25 pre-incubation. The intensification of HO-1 protein expression was confirmed by the Western Blot technique (Fig. 5D). The HO-1 protein level was significantly higher in both stimulated and unstimulated cells pre-incubated with the higher irisin concentrations (50 nM) compared to the respective control cells (0 nM).

**DISCUSSION**

Our present study demonstrated the efficiency of irisin to accelerate antioxidant mechanisms in macrophage cells. In the present study, we evaluated the effect of irisin on macrophages maintained at two conditions, namely the quiescent and LPS-
activated cells. Our major goal was to examine the role of irisin to affect the ROS in non-stimulated and LPS-stimulated macrophages to mimic the action of this peptide that might be timely associated with the course of inflammation. The proper functioning and efficiency of mechanisms that allow for maintaining a balance in the production and elimination of ROS is particularly substantial, because the imbalance can lead to damage of nucleic acids, proteins as well as cellular damage, and could serve as the pathogenic background for the development of some diseases (9). Moreover, the elevated ROS levels are observed in pathogenesis of some human being disorders such as cancer, neurodegeneration or Crohn’s disease (32). Therefore, the new agents of peptide structure with anti-oxidant as well an anti-inflammatory potential are particularly important.

Irisin is a molecule secreted from skeletal muscle in response to physical exercise. In recent years, several studies have highlighted the ability of irisin to promote a variety of biological activities. Irisin was originally identified as a myokine that induces browning of white adipocytes and subsequently non-shivering thermogenesis (22). Irisin has been shown to increase the total body energy expenditure, promote the weight loss, improve glucose tolerance, alleviate insulin resistance and suppress inflammatory processes (9, 30, 33). In our previous study, we have reported that irisin reduces the severity of the oxygen respiratory burst generated by macrophages, while diminishing the ROS production by quiescent cells (29). In recent years, the indirect impact of irisin on ROS status has become a subject of numerous studies. Increased plasma irisin level has been positively correlated with improvement of oxidative status and reduction of inflammatory oxidative stress in obese individuals (34) or pregnant women with gestational diabetes (35). Moreover, it has been demonstrated that irisin can protect the heart against ischemia/reperfusion injury (36) as well as improved the endothelial functions in DM2 (37), cultured endothelial cells (38) and arteriosclerosis (39), where the authors have also indicated this myokine indirect antioxidant potential (40).

In our current research, we have shown that the reduction in intensity of oxygen burst, or more efficient neutralization of resulting products ROS, may be associated with irisin’s influencing the antioxidative mechanisms responsible for the scavenging activity of harmful radicals. In the concentrations used in this study, irisin showed no cytotoxic effect and failed to affect the cell viability. However, the significant influence of this myokine on the expression of key enzymes related to the neutralization of ROS has been observed. These data seem to provide an explanation for the previously observed reduction in the level of ROS produced by resting macrophages pre-incubated with irisin (29). Analyzing the results presented in this study, the major attention should be paid to irisin exhibited the elimination of ROS in quiescent cells. Bearing this in mind, irisin prevented the fall in the expression of key antioxidant enzymes possibly due to a weaker stimulation of cells by ROS concentrations below the threshold observed in control cells. This might be due to cell adaptation more favorable conditions created in cells pretreated with irisin but not to a weakening of antioxidative mechanisms. A different effect of irisin has been observed in LPS-stimulated cells, where a massive respiratory burst occurred along with the enhanced ROS production. As we presented previously (29), the pre-incubation with irisin significantly reduced the severity of the respiratory burst. Our current work shed a little more insight into this irisin-induced protective mechanism against oxidative stress because we have shown that the ROS elimination in mediated by the activation of the Nrf2/HO-1 pathway and consequently, by an increase in the expression of key antioxidant enzymes.

Nrf2 is a transcription factor responsible for regulating the expression of critical antioxidant enzymes, and has exerted a cytoprotective activity in the cell (41). Due to the close relationship between oxidative stress and inflammation, Nrf2 also plays an important role in the regulation of inflammatory processes (42). Our research has shown a significant increase in nuclear levels of Nrf2 after pre-incubation with irisin suggesting that this myokine can directly increase the expression of key antioxidative enzymes. Indeed, we have observed an increase in both SOD-1, SOD-2, GSH-Px and Cat-9 levels, depending on the degree of activation of the cells and the time duration of irisin pre-incubation. Free radicals such as activated oxygen ($O_2^•_r$) are metabolized to $H_2O$ in the sequence of sequentially converting to $H_2O_2$ by dismutase enzymes (SOD 1 and SOD 2) and then to $H_2O$ by glutathione peroxidase or to $H_2O_2$ and $O_2$ by catalases (35). Our findings support the notion that irisin can protect the cell from ROS-induced serious cellular oxidative damage by the activation of antioxidative mechanisms. We assume that this mechanism may be strongly associated with expression and translocation of Nrf2 and activation of subordinate genes encoding for antioxidant enzymes. Moreover, an increase in HO-1 expression known to exert both antioxidant and anti-inflammatory effects (35) observed in the present study extends, at least in part, the further downstream mechanism involving the interaction of HO-1 and Nrf2 in antioxidative action mediated by irisin.

Considering the above, the current study also provides new insights into the potential anti-inflammatory mechanisms of irisin’s action. As previously reported, irisin inhibited pro-inflammatory activation of macrophages (30) by the TLR4/NF-$κB$ signaling pathway via the potent attenuation of release of proinflammatory cytokines such as TNF-$α$, IL-1β, IL-6 or MCP-1 by macrophages. In isolated adipocytes, irisin markedly reduced the level of TNF-$α$, IL-6 and MCP-1 (31). Furthermore, irisin effectively alleviated endothelial dysfunction caused by the oxidative/nitrosative stress and this protective activity of irisin was dependent upon the inhibition of PDK4/NADPH oxidase and NF-$κB$/NO pathways and the decrease in formation of peroxynitrite (37). Interestingly, irisin known to be associated with physical activity which has been reported to have in general, a favorable impact on mental functions, exhibited no correlation with depressiveness, anxiety and perceived stress as well as symptoms of eating disorder in obese woman (43). This indicates that irisin might be not involved in psychoendocrine pathways of the regulation of depression or other mental functions such as anxiety and perceived stress (43).

To best our knowledge, our present study is the first one which suggests that irisin acts directly on the target cells linked with inflammatory conditions such as macrophages and efficiently activates the protective mechanisms leading to the ROS scavenging. This conclusion comes out from our observations that irisin altered the expression of key antioxidant enzymes and activated the Nrf2/HO-1 signaling pathways. This ROS attenuation by irisin can be similar to other reported ROS scavengers such as quercetin because administration of quercetin in association with moderate exercise training not only attenuated the diabetic conditions but also restored sciatic nerves injuries by controlling hyperglycemia to down-regulate the generation of ROS as well as the elevation of antioxidant enzymes (44).

In conclusion, our study has indicated that irisin possesses an important antioxidizing and anti-inflammatory properties and may protect cells from the damage induced by ROS under inflammatory conditions by activating of the Nrf2/HO-1 pathway. Our present data are corroborative with a previous observation (30) that irisin released by working out skeletal muscle inhibited the pro-inflammatory activation by alterations in the downstream pathway of TLR4/MyD88. However, we are fully aware that further experimental and clinical studies in vitro
are needed to definitively verify the potential mechanism(s) of the antioxidative action of irisin released by skeletal muscle during voluntary and endurance exercise.

**Author contributions:** A.I. Mazur-Bialy conceived, designed and coordinated the experiments; A.I. Mazur-Bialy, K. Kożłowska and E. Pochec performed the experiments; A.I. Mazur-Bialy, E. Pochec, J. Bilski and T. Brzozowski analyzed the data; A.I. Mazur-Bialy performed statistical analysis and prepare figures; A.I. Mazur-Bialy and E. Pochec contributed to the purchase of reagents/materials/analysis tools; all the Authors prepared figures; A.I. Mazur-Bialy and E. Pochec contributed to the data; A.I. Mazur-Bialy performed statistical analysis and coordinated the experiments; A.I. Mazur-Bialy, K. Kozłowska and E. Pochec contributed to the purchase of reagents/materials/analysis tools; all the Authors have read and written the revised paper.

**Acknowledgements:** This study was financially supported by the research project no. K/ZDS/006358 (Agneszka I. Mazur-Bialy) and by the grant no. UMO-2013/09/B/NZ4/01566 from National Centre of Science in Poland (Tomasz Brzozowski).

**Conflict of interests:** None declared.

**REFERENCES**


Received: December 15, 2017
Accepted: February 26, 2018

Author’s address: Dr. Agnieszka Irena Mazur-Bialy, Department of Ergonomics and Exercise Physiology, Faculty of Health Science, Jagiellonian University Medical College, 20 Grzegorzecka Street, 31-531 Cracow, Poland.
E-mail: agnieszka.mazur@uj.edu.pl